Stimulation of the gastrin-cholecystokinin<sub>B</sub> receptor promotes branching morphogenesis in gastric AGS cells

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Pagliocca, A., L. E. Wroblewski, F. J. Ashcroft, P. J. Noble, G. J. Dockray, and A. Varro. Stimulation of the gastrin-cholecystokinin<sub>B</sub> receptor promotes branching morphogenesis in gastric AGS cells. Am J Physiol Gastrointest Liver Physiol 283: G292–G299, 2002. First published March 28, 2002; 10.1152/ajpgi.00056.2002.—Epithelial organization is maintained by cell proliferation, migration, and differentiation. In the case of the gastric epithelium, at least some of these events are regulated by the hormone gastrin. In addition, gastric epithelial cells are organized into characteristic tubular structures (the gastric glands), but the cellular mechanisms regulating the organization of tubular structures (sometimes called branching morphogenesis) are uncertain. In the present study, we examined the role of the gastrin-cholecystokinin<sub>B</sub> receptor in promoting branching morphogenesis of gastric epithelial cells. When gastric cancer AGS-G<sub>4</sub> cells were cultured on plastic, gastrin and PMA stimulated cell adhesion, formation of lamellipodia, and extension of long processes in part by activation of protein kinase C (PKC) and phosphatidylinositol (PI)-3 kinase. Branching morphogenesis was not observed in these circumstances. However, when cells were cultured on artificial basement membrane, the same stimuli increased the formation of organized multicellular arrays, exhibiting branching morphogenesis. These effects were reversed by inhibitors of PKC but not of PI-3 kinase. We conclude that, in the presence of basement membrane, activation of PKC by gastrin stimulates branching morphogenesis.

Stomach; gastric glands; extracellular matrix; migration; epithelium

THE ORGANIZATION OF THE EPITHELIUM throughout the gut is maintained by cell proliferation followed by migration and differentiation. Proliferating cells in the gastric epithelium are located in the isthmus region of the glands (12). Cells that leave the cell cycle may migrate either toward the surface, adopting a mucus-secreting phenotype, or toward the base of the gland, where the main differentiated cell types are parietal, chief, and endocrine cells. The latter cell types arrange and maintain themselves in characteristic tubular ensembles that constitute the gastric glands. The assembly of tubular structures is thought to depend on cell-cell and cell-matrix interactions and is modulated and regulated by growth and morphogenetic factors. Loss of gastric glands and reduction in gland length are features of the premalignant condition of gastric atrophy (4). Cells may also be arranged in characteristic tubuliform like ribbons in some gastric cancers, so that the control of tubulogenesis may be of pathological as well as physiological relevance.

In kidney, mammary gland, and liver cells, hepatocyte growth factor (HGF) is known to promote the process of tubule formation, or branching morphogenesis (1, 11, 15, 21, 23, 27). The mechanisms by which gastric epithelial cells form tubuliformlike arrays are less well studied. It has, however, been reported that heparin (also known as neuregulin), which belongs to the epidermal growth factor (EGF) family (24), promotes formation of tubuliformlike structures via activation of erb-B2 and erb-B3 members of the EGF receptor group in a gastric cell line (3). Moreover, another EGF-like growth factor, transforming growth factor (TGF)-α, appears to stimulate branching morphogenesis in RGM-1 cells (derived from rat gastric mucous cells), probably via induction of cyclooxygenase-2 (28). In addition, the gastric trefoil factor TFF-2 has been reported to stimulate branching morphogenesis in MCF-7 cells (derived from human mammary carcinoma) (17). Interestingly, expression of the mucin MUC-1 is also linked to branching morphogenesis in mammary and kidney cell lines (9).

It is well recognized that gastrin influences the organization of the gastric epithelium, as well as acutely regulating acid secretion (33). Elevated plasma gastrin concentrations are associated with increased parietal cell mass (25) and hyperplasia of histamine-producing enterochromaffinlike (ECL) cells (2, 18, 19). In transgenic mice with hypergastrinemia, there are initially increased parietal cell numbers and increased gland length (16, 34), indicating that gastrin influences the organization of the gastric mucosa (20). In the present study, we used an assay of branching morphogenesis to study the response of gastric AGS cells to activation of the gastrin-CCK<sub>B</sub> receptor. The data indicate that gastrin stimulates branching morphogenesis when cells are cultured on basement membrane via activation of protein kinase C (PKC).

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MATERIALS AND METHODS

Cells and materials. The gastric cancer cell line AGS was obtained from the American Type Culture Collection. In contrast to previous reports (10), in our hands these cells did not express the gastrin-CCK\textsubscript{B} receptor as determined by Northern blot, binding of \textsuperscript{125}I-labeled heptadecapeptide gastrin (G-17), or responses to incubation with G-17 (up to 10 nM, 92 h). The cells were stably transfected with the gastrin-CCK\textsubscript{B} receptor as previously described (35). Cells were cultured in Ham’s F-12 medium supplemented with 10% fetal bovine serum (FBS) and 1% wt/vol penicillin/streptomycin (Life Technologies, Paisley, UK) as described (35). G-17 was obtained from Bachem (St. Helens, UK); the gastrin-CCK\textsubscript{B} receptor antagonist L-740093 was a gift from Merck, Sharpe & Dohme (Rathaway, NJ). Tetramethylrhodamine B isothiocyanate (TRITC)-conjugated phalloidin, phorbol 12-myristate 13-acetate (PMA), TGF-\alpha, lysophosphatidic acid (LPA; oleoyl), and actinomycin D were obtained from Sigma (Poole, UK). Pertussis toxin (PTX), PD-98059, PD-153035, AG-1478, LY-294002, wortmannin, and Ro-32-0432 were obtained from CN Biosciences (Beeston, UK); HGF was obtained from Genentech (San Francisco, CA). Artificial basement membrane was obtained from either Sigma or Becton Dickinson (Bedford, UK).

Morphological studies. Cells (10^4/well) were plated on six-well dishes and incubated for 2 days in full medium and then serum-free medium containing G-17 (30 pM–3 nM), PMA, or other drugs. Cells extending long processes in response to stimuli were scored after 6 h as a proportion of total cells by counting duplicate fields in each of triplicate wells. In addition, cells were cultured on coverslips and F-actin was stained using TRITC-conjugated phalloidin. Images were captured from a Zeiss Axiovert 25 microscope (Carl Zeiss, Welwyn Garden City, UK) using Intelicam software (Matox Electronic Systems).

Adhesion assays. Confluent AGS cells expressing the gastrin-CCK\textsubscript{B} receptor (AGS-G\textsubscript{R} cells) were recovered in trypsin-EDTA (2.5 \times 10^5/well, 24-well plates), and incubated with or without G-17 and drugs for 30 min at 37°C. Media and nonadherent cells were then removed, cells were washed three times with PBS, and adherent cells were stained with 0.02% crystal violet. Adherent cells were then washed and solubilized with 2 mM Na\textsubscript{2}HPO\textsubscript{4}-50% ethanol, and absorbance was measured at 550 nm by using a SpectraCount plate reader (Packard BioScience, Pangbourne, UK).

Branching morphogenesis assays. Cells (5 \times 10^4) were plated on 24-well plates that had been coated with artificial basement membrane according to the manufacturer’s instructions. Cells were cultured in serum-free Ham’s F-12 medium, and treatments were applied at the time of plating. In some experiments, branching morphogenesis was studied by video time-lapse microscopy. For this purpose, cells were plated on coverslips previously coated with artificial basement membrane and mounted on the heated stage of a Zeiss Axiovert 100 microscope in a humidified chamber. Images were captured at 5-min intervals with a Hamamatsu 480–80 charge-coupled device camera (Hamamatsu Photonics, Hamamatsu City, Japan) and AQM-2001 software (Kinetic Imaging, Liverpool, UK). In routine assays of branching morphogenesis, cells were examined after 3 h by using a Zeiss Axiovert 25 inverted microscope and were scored by using a scale from 1 to 5 based on the following descriptors: 1, single cells or amorphous groups (<5 cells); 2, linear arrays of cells, without branches; 3, linear and branching arrays of cells; 4, the presence of one or more complete rings of cells (together with linear and branching arrays); and 5, extensive networks of cellular assemblies forming multiple-ringed structures. Experiments were conducted in triplicate wells, and five fields from each well were scored.

Statistics. Data are presented as means \pm SE. Sample groups for multiple-treatment experiments were analyzed by using Kruskal-Wallis ANOVA followed by pairwise comparisons of treatments. Where two independent sample groups were compared, data analysis was performed by using a Mann-Whitney U-test. Differences were considered significant when \( P \leq 0.05 \).

RESULTS

G-17 and PMA induce morphological changes and scattering. Subconfluent AGS-G\textsubscript{R} cells grew in colonies with epithelial-like morphology when cultured on plastic dishes (Fig. 1A). There was no difference between the parental cell line (AGS) and cells expressing the gastrin-CCK\textsubscript{B} receptor (AGS-G\textsubscript{R}). Incubation of AGS-G\textsubscript{R} cells with G-17 induced cell scattering and the extension of long processes, which was maximal at ~6 h (Fig. 1B). Phalloidin staining of F-actin revealed predominantly cortical actin in unstimulated cells (Fig. 1C). In response to G-17, phalloidin staining revealed cell spreading and membrane ruffling within 30 min (Fig. 1D), and by 6 h there was elaborate remodeling of the actin cytoskeleton supporting lamellipodia and the extension of long processes (Fig. 1E). Similar responses were produced by PMA (100 nM; Fig. 1F).

The extension of processes and formation of lamellipodia in AGS-G\textsubscript{R} cells treated with G-17 were concentration dependent over the range 10–1,000 pM (Fig. 2A). The response to G-17 was completely inhibited by the gastrin-CCK\textsubscript{B} receptor antagonist L-740093 (100 nM; Fig. 2B), but the extension of processes in response to PMA was not inhibited by L-740093 (not shown). The responses to both G-17 and PMA were also reversed by the PRC inhibitor Ro-32-0432 (1 \mu M; Fig. 2B). It is known that gastrin acts in part to activate phosphatidylinositol (PI)-3 kinase (6, 30), and we found that inhibition of PI-3 kinase by LY-294002 (20 \mu M; Fig. 2B) or wortmannin (100 nM; not shown) partially inhibited the morphological transformation in response to gastrin and PMA, indicating that PI-3 kinase might be downstream of PKC.

Gastrin stimulates EGF receptor activation in AGS-G\textsubscript{R} cells, which in turn activates the mitogen-activated protein (MAP) kinase pathway (32). However, this pathway appears not to be involved in the morphological transformation of AGS-G\textsubscript{R} cells. Thus the EGF receptor ligand TGF-\alpha (100 ng/ml) did not produce the morphological transformation seen with gastrin and PMA (results not shown); moreover, inhibiting EGF receptor tyrosine kinase activity with PD-153035 or inhibiting activation of the MAP kinase (MEK) with PD-98059 had no effect on gastrin-stimulated extension of processes. Similarly, although AGS cells express the receptor for HGF, i.e., c-Met, application of HGF (100 ng/ml) had no effect on AGS cell morphology.
**G-17 and PMA increase cell adhesion.** The morphological changes in AGS-GR cells in response to G-17 were associated with increased cell adhesion. Thus in an adhesion assay, G-17 produced a significant fourfold increase in cell adhesion. This response was inhibited by the gastrin-CCKB receptor antagonist L-740093. Interestingly, G-17-stimulated adhesion was completely abolished by inhibition of PI-3 kinase by LY-294002 (20–50 μM; Fig. 3) and wortmannin (100 nM; not shown) and was reduced but not abolished by the

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**Fig. 1.** Reorganization of the actin cytoskeleton and extension of processes in AGS cells expressing the gastrin-CCKB receptor (AGS-GR cells). Phase-contrast microscopy of control AGS-GR cells (A) and cells treated with heptadecapeptide gastrin (G-17; B; 1 nM, 6 h) show scattering and extension of processes. Phalloidin staining of F-actin in control cells (C) and cells treated with G-17 (D; 1 nM, 30 min) show gastrin-stimulated cell spreading. Phalloidin staining of F-actin in G-17- (E; 1 nM, 6 h) and PMA (F; 100 nM, 6 h)-treated cells shows remodeling of the actin cytoskeleton, formation of lamellipodia, and extension of processes. Scale bars = 50 μm.

**Fig. 2.** The proportion of cells extending processes in response to G-17 is concentration dependent and mediated by protein kinase C (PKC) and phosphatidylinositol (PI)-3 kinase. A: proportion of cells extending processes in response to G-17 increases with concentration (means ± SE, 3 independent experiments). B: proportion of cells extending processes in response to G-17 (1 nM, 6 h) is abolished by the gastrin-CCKB receptor antagonist L-740093 (100 nM) and significantly inhibited by the PKC inhibitor Ro-32-0432 (1 μM) and the PI-3 kinase inhibitor LY-294002 (20 μM). The effects of PMA (100 nM 6 h) were also inhibited by Ro-32–0432 and LY-294002. Open bar, control; hatched bars, gastrin; wavy bars, PMA. Results are means ± SE; *P < 0.05 vs. G-17, †P < 0.05 vs. PMA (ANOVA).
Fig. 3. Gastrin stimulates cell adhesion, and this is mediated by PI-3 kinase. Adhesion of AGS-GR cells to plastic is increased by G-17 (1 nM, 30 min) and by PMA (100 nM). The effects of G-17 are inhibited by LY-294002 (1 μM) and L-740093 (100 nM). There is a small increase in adhesion in response to PMA (100 nM) that is inhibited by Ro-32–0432 and LY-294002. Results are means ± SE; *P < 0.05 vs. G-17, †P < 0.05 vs. PMA (ANOVA).

PKC inhibitor Ro-32–0432. PMA (100 nM) produced a small but significant increase in cell adhesion that was also inhibited by Ro-32–032 (1 μM), LY-294002 (20–50 μM), and wortmannin (1 μM; not shown) (Fig. 3).

Branching morphogenesis on artificial basement membrane. When cells were cultured on the surface of artificial basement membrane (5 × 10^4 cells/well, 24-well dishes) and in appropriate conditions (see below), they assembled into multicellular, linear, and branching complex structures, i.e., exhibited branching morphogenesis. Video time-lapse microscopy indicated a progression from initially dispersed populations of single cells, which through the extension of processes and migration led to the assembly of elaborate multicellular assemblies (Fig. 4). During this phase, the extension of processes appeared to provide a scaffold along which more complex structures formed. Tracking of individual cells by video time-lapse microscopy indicated that the assembly of these cellular complexes was dependent almost exclusively on migration and not on cell proliferation.

Assembly of multicellular complexes is stimulated by G-17, serum, PMA, and LPA. Branching morphogenesis of AGS-GR cells was stimulated by G-17 in concentrations from 30 to 1,000 pM when cells were cultured in serum-free medium on artificial basement membrane. It was possible to classify the degree of assembly into organized multicellular structures and so score the effects of different treatments (Fig. 5). Similar effects to those of gastrin were produced by addition to serum-free medium of FBS (Fig. 6). Moreover, PMA (100 nM) and LPA (50 μM) in serum-free medium also stimulated the formation of multicellular assemblies (Fig. 6). There was no effect of HGF and TGF-α (not shown) on
the branching morphogenesis exhibited by AGS-GR cells.

**G-17 stimulates branching morphogenesis via the gastrin-CCKB receptor.** We then examined the effect of gastrin on branching morphogenesis in AGS-GR cells treated with the gastrin-CCKB receptor antagonist L-740093. The formation of multicellular assemblies was inhibited by L-740093 (Fig. 7). To establish the specificity of this response, we showed that L-740093 had no effect on LPA, PMA, or serum-induced branching morphogenesis (Fig. 7). PTX (which inhibits signaling through Go11 and Gi2) had no effect on responses to either gastrin or LPA (data not shown), consistent with signaling through the PTX-insensitive Gq11 pathway.

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**Fig. 5.** Concentration-dependent stimulation of branching morphogenesis by G-17. Increasing concentrations of G-17 stimulated progressively more complex and elaborate assemblies of AGS-GR cells. These were scored by using descriptors provided in MATERIALS AND METHODS. AGS-GR cells (5 × 10⁴ cells/well in 24-well plates, 3 h) were cultured with various concentrations of G-17, and representative fields for each score are shown. A: mean data from 3 independent experiments (each of triplicate wells) showing graded responses to 30–300 pM G-17; *P < 0.01 vs. control (ANOVA). B: control; C–F: representative fields from gastrin-stimulated cells exhibiting each grade of branching morphogenesis. Scale bars = 500 μm.

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**Fig. 6.** Stimulation of branching morphogenesis by fetal bovine serum (FBS), lysophosphatidic acid (LPA), and PMA but not hepatocyte growth factor (HGF). FBS (5 and 10%), PMA (100 nM), and LPA (50 μM) stimulated branching morphogenesis of AGS-GR cells cultured on artificial basement membrane. HGF (100 ng/ml) had no effect. In each case, appropriate control data (open bars) and response to treatment (wavy bars) are shown. Results are means ± SE; *P < 0.005 by Mann-Whitney U-test or ANOVA, as appropriate; N.S.D, not significantly different.

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**Fig. 7.** Inhibition of branching morphogenesis in response to gastrin by L-740093. The branching morphogenesis stimulated by G-17 (300 pM) was inhibited by the gastrin-CCKB receptor antagonist L-740093 (100 nM). The latter had no significant effect on the responses to PMA (100 nM), LPA (50 μM), or 10% FBS. Open bars show the appropriate control observation for each treatment. Means ± SE; *P < 0.001 (ANOVA).
gastrin and tubulogenesis in AGS-GR cells, we studied the role of PKC in branching morphogenesis. The results presented here show that gastrin acts via PKC to stimulate remodeling of the actin cytoskeleton in AGS-GR cells, increased cell adhesion, and the extension of long processes. In the presence of basement membrane, the extension of processes appears to provide a scaffold for the arrangement of cells into complex multicellular assemblies consistent with branching morphogenesis. This type of response may contribute to the formation of gastric glandular cells into tubules.

In complex epithelia, proliferating and differentiated cells are frequently localized to morphologically distinct domains that are maintained by cell migration following exit from the cell cycle. In the case of the gastric epithelium, a common class of stem cell is localized in the isthmus region of the gland (13). Migration of cells toward the mucosal surface is associated with differentiation to mucus-secreting phenotypes, whereas cells migrating toward the base of the gland may become parietal, ECL, or chief cells (12). The mechanisms regulating the assembly of cells into gastric glands are poorly understood. Disruption of the processes controlling migration may, however, be a feature in gastric atrophy and in any case is likely to underlie tumor cell invasion. Moreover, the capacity for tubulogenesis may account for the organization of some gastric tumors in which cells assemble into columns or tubulolike structures. We suggest that stimulation of PKC by gastrin is one of the mechanisms that influence these processes.

Assays of branching morphogenesis similar to that used here have been widely employed to study the assembly into organized multicellular structures of endothelial cells (21) and MDCK cells (1, 15, 26). In both endothelial and MDCK cells, HGF strongly stimulates branching morphogenesis in the presence of extracellular matrix (1, 15, 26, 27). Although AGS-GR cells express the HGF receptor c-Met, activation of this receptor in these cells is not associated with branching morphogenesis. Moreover, the branching morphogenesis stimulated by HGF in MDCK cells is mediated by PI-3 kinase. In AGS-GR cells, inhibition of PI-3 kinase blocks cell adhesion and reduces gastrin-stimulated extension of processes by cells cultured on plastic, but, interestingly, this has no effect on branching morphogenesis when cells were cultured on artificial basement membrane. The signaling pathways responsible for the latter phenotype in AGS-GR cells therefore appear to be different from those in MDCK cells. Together, the data suggest that the extension of processes that characterizes the formation of organized multicellular structures on basement membrane appears to reflect the PKC-dependent, PI-3 kinase-independent pathway responsible for remodeling of the actin cytoskeleton. Further work will be needed to identify downstream targets of PKC involved in branching morphogenesis in AGS-GR cells.

Previous studies have shown that gastrin stimulates pathways involving PKC, activation of MAP kinase, and activation of PI-3 kinase (5, 6, 30, 31). For the most part, the activation of these pathways has been linked to control of proliferation and apoptosis. There have been few direct studies of the way that gastrin might regulate events leading to migration or the formation of complex assemblies of cells. We did not find evidence for an involvement of the MAP kinase pathway in these events. The present data do, however, suggest differences in the relative importance of PKC and PI-3 kinase in mediating the remodeling of the actin cytoskeleton and in control of the cell-cell interactions required for adhesion, the extension of processes, and branching morphogenesis. The data imply that PI-3 kinase might in some circumstances be activated by PKC in AGS-GR cells, but this is unlikely to account for all of the present data, and further work on the rela-

![Fig. 8. Inhibition of the effects of PMA and gastrin by Ro-32-0432.](image-url)
tionship between, and the activation of, the relevant signaling pathways is needed.

The gastrin precursor progastrin yields the amidated gastrin via intermediates with a COOH-terminal glycine residue (the Gly-gastrins) (7). The latter peptides have low affinity for gastrin-CKβ receptors. Interestingly, however, recent reports suggest that Gly-gastrins may regulate epithelial migration (8) and that these effects might be expected to influence epithelial organization, although their relationship to branching morphogenesis induced by stimulation of the gastrin-CKβ receptor remains unclear. Normally, the gastrin-CKβ receptor is expressed by parietal and ECL cells (7). It is, however, worth noting that in prolonged hypergastrinemia the receptor appears to be expressed by mucus neck cells (22), and after damage to the mucosa it is also expressed by surface epithelial cells (29). The role of this receptor in regulating branching morphogenesis may therefore include both the maintenance of normal epithelial organization and adaptive responses to damage.

Previous studies of the mechanisms of branching morphogenesis in other gastric cell lines have identified heregulin (3), cyclooxygenase-2 (28), TFF-2 (17), and MUC-1 (9) as potential regulatory agents. Gastrin is the first neuroendocrine peptide acting through G protein-coupled receptors to be shown to be capable of stimulating branching morphogenesis. Since observations in genetically modified mice suggest that gastrin also regulates gastric mucosal morphology in vivo (34), we suggest that gastrin may be one factor controlling the capacity of gastric epithelial cells to organize into glandular structures. Gastrin regulates the maturation of some gastric epithelial cells (7); it will now be interesting to determine the extent to which the stimulation of tubulogenesis by gastrin is linked to the differentiation of gastric epithelial cells. Either way, it is reasonable to assume that other growth and morphogenic factors are also involved in maintaining the organization of gastric glands, and elucidation of the interactions and relative importance of these is now required.

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