Gastrin Stimulates Ca\(^{2+}\) Mobilization and Clonal Growth in Small Cell Lung Cancer Cells

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ABSTRACT

Gastrin has been postulated to be a physiological growth factor, but compelling in vivo evidence of this has been difficult to obtain. In the present study we investigated whether small cell lung carcinoma cell lines could provide a useful model system to study the effects of gastrin on signal transduction and cell proliferation in vitro. We found that the addition of gastrin to small cell lung cancer cells loaded with the fluorescent Ca\(^{2+}\) indicator fura 2-tetraacetoxymethyl ester causes a rapid and transient increase in the intracellular concentration of Ca\(^{2+}\) ([Ca\(^{2+}\)]\(i\)) followed by homologous desensitization. The [Ca\(^{2+}\)]\(i\) response was especially prominent in the small cell lung carcinoma cell line H510. In this cell line, gastrin I, gastrin II, cholecystokinin residues 26–33 (CCK-8), and unsulfated CCK-8 increased [Ca\(^{2+}\)]\(i\), in a concentration-dependent fashion with half-maximum effects at 7, 2.5, 3, and 5 nM, respectively.

The Ca\(^{2+}\)-mobilizing effects of gastrin and CCK-8 were prevented by proglumide, benzotript, and the specific gastrin/CCK\(_B\) receptor antagonist L365260. Gastrin stimulated the clonal growth of H510 cells in semisolid (agarose-containing) medium, increasing both the number and the size of the colonies. Gastrin and CCK agonists were equally effective in promoting clonal growth. The broad-spectrum neuropeptide antagonists [D-Arg\(^{6}\), D-Phe\(^{7}\), Leu\(^{8}\)] substance P and [D-Arg\(^{6}\), D-Phe\(^{7}\), Trp\(^{9}\), Leu\(^{11}\)] substance P (D−11) markedly inhibited gastrin-stimulated Ca\(^{2+}\) mobilization and clonal growth. These results show that gastrin acts as a direct growth factor through gastrin/CCK\(_B\) receptors and demonstrate, for the first time, that these peptides can stimulate the proliferation of cells outside the gastrointestinal tract.

INTRODUCTION

The possibility that the gastrointestinal peptide gastrin could act as a hormonal growth factor has attracted considerable interest. A considerable body of evidence has demonstrated that the administration of gastrin induces growth-promoting effects in the digestive tract and exocrine pancreas (1). In particular, an increase in the circulating levels of gastrin has been related to hyperplasia of the gastric enterochromaffin-like cells (2). Furthermore, a decrease in circulating gastrin induced by antrectomy resulted in atrophy of the colonic mucosa in the rat, an effect reversed by the administration of pentagastrin (3). Gastrin also appears to be a growth-promoting hormone for malignant cells grown as xenografts in nude mice (4, 5). While these observations strongly suggest that gastrin acts as a growth factor, it is difficult to obtain unambiguous evidence for a direct growth-promoting effect of gastrin in vivo because the administration of this peptide could stimulate the release of other biologically active peptides or growth factors which could act as the proximal effectors of the action of gastrin.

Cultured cells have provided useful experimental systems for elucidating the extracellular factors that promote cell growth without the many complexities of whole animal experimentation. Nevertheless, compelling evidence that gastrin acts as a cellular growth factor or as an autocrine factor in tumors has been difficult to document in clonal cell populations. Indeed, studies using receptor antagonists and colon carcinoma cell lines resulted in controversial results (6–8). The lack of a convenient model system has impeded the elucidation of the possible action of gastrin as a direct growth factor in vitro.

Cell lines established from SCLC\(^{2}\) provide a useful model system to study the effects of hormonal peptide agonists and antagonists on early signaling events and on cell proliferation (9–13). The SCLC cell line H345 has been shown to express receptors for gastrin (14, 15), but the effect of this peptide on H345 growth was not determined. In the present study we report that gastrin, at nanomolar concentrations, stimulates the clonal growth of the SCLC cell line H510, identified as an excellent model system for studying the effects of gastrin in vitro. The results demonstrate that gastrin acts as a direct growth factor and show, for the first time, that this hormone can stimulate the proliferation of cells outside the gastrointestinal tract.

MATERIALS AND METHODS

Cell Culture. SCLC cell line H510 was purchased from the American Type Culture Collection. Stocks were maintained in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum (heat inactivated at 57°C for 1 h) in a humidified atmosphere of 10% CO\(_2\)/90% air at 37°C. They were passaged every 7 days. For experimental purposes, the cells were grown in HITESA which consists of RPMI 1640 supplemented with 10 nM hydrocortisone, 5 μg/ml insulin, 10 ng/ml transferrin, 10 nM estradiol, 30 nM selenium, and 0.25% bovine serum albumin (16).

Determination of Intracellular Ca\(^{2+}\) Concentration. Aliquots of 4–5 × 10\(^{4}\) SCLC cells cultured in HITESA for 3–5 days were washed and incubated for 2 h at 37°C in 10 ml fresh HITESA medium. Then 1 μM fura-2-tetraacetoxymethyl ester AME from a stock of 1 mM in dimethyl sulfoxide was added, and the cells were incubated for further 5 min. The cell suspension was centrifuged at 2000 rpm for 15 s, and the cells were resuspended in 2 ml of electrolyte solution [140 mM NaCl, 5 mM KCl, 0.9 mM MgCl\(_2\), 1.8 mM CaCl\(_2\), 25 mM glucose, 16 mM 4(-2-hydroxyethyl)-piperazineethanesulfonic acid, 16 mM Tris, and a mixture of amino acids at pH 7.2], transferred to a quartz cuvette, and stirred continuously at 37°C. Fluorescence was recorded continuously in a Perkin-Elmer LS5 luminescence spectrometer with an excitation wavelength of 336 nm and an emission wavelength of 510 nm. [Ca\(^{2+}\)]\(i\), was calculated as previously described (13).

Clonogenic Assay. Cultures of the SCLC cell line H510, 3–5 days postpassage in HITESA, were washed and resuspended in the same medium. Cells were then disaggregated into a single cell suspension by two passes through a 19-gauge needle and then through 20-μm nylon gauze. Viability was determined by Trypan blue exclusion on a standard hemocytometer. Approximately 10\(^4\) viable cells/ml, measured using a Coulter counter, were suspended in HITESA containing 0.3% agarose.

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2. The abbreviations used are: SCLC, small cell lung cancer; GRP, gastrin-releasing peptide; HITESA, 10 nM hydrocortisone, 5 μg/ml insulin, 10 μg/ml transferrin, 10 nM estradiol, 30 nM selenium, and 0.25% bovine serum albumin; AME, tetraacetoxymethyl ester; [Ca\(^{2+}\)]\(i\), intracellular concentration of Ca\(^{2+}\); CCK, cholecystokinin; CCK-8, cholecystokinin residues 26–33.
Then, 1 ml of this mixture was plated in five replicate 35-mm plastic dishes containing a 2-ml base layer of 0.5% agarose in HITESA that had hardened. The two layers contained neuropeptide at the same concentration. Cultures were incubated at 37°C in a humidified atmosphere at 10% CO2:90% air for 21 days and then stained with the vital stain nitroblue tetrazolium. Colonies of >120 μm diameter (16 cells) were counted under a microscope, and, using a ×4 lens, the image is relayed via a TV camera and analyzed on a Macintosh IIcx computer running the digital image processing and analysis program Image.

Materials. Gastrin 17-I (unsulfated), gastrin 17-II (sulfated on position 6 from the COOH terminus), CCK residues 26–33 sulfated on the 7th position from the COOH terminus (CCK-8), desulfated CCK-8 [des(SO3)CCK-8], and CCK 10–27 were purchased from Sigma Chemical Co. (St. Louis, MO); [d-Arg4, d-Phe5, d-Trp7,9, Leu11] substance P and [Arg8, d-Trp7,9, MePhe8] substance P (6–11) were purchased from Peninsula Laboratories (Belmont, CA); L364,718 and L365,260 were a kind gift from John Walsh (University of California, Los Angeles, CA); fura-2-AMΕ was from Calbiochem Corporation (La Jolla, CA); and agarose was from SeaKem (Rockland, ME). All of the other reagents were of the highest grade commercially available.

RESULTS

Gastrin Stimulates Ca2+ Mobilization. The addition of gastrin I or CCK-8 to either H69 or H345 SCLC lines loaded with the fluorescent Ca2+ indicator fura-2 AMΕ caused a rapid and transient increase in [Ca2+]. These findings (Fig. 1) are in agreement with other reports that demonstrated that gastrin induces Ca2+ mobilization in the SCLC cell line H345 (14, 15). However, the increase of [Ca2+], by gastrin or CCK-8 (∆[Ca2+]), 20–30 nm) was considerably smaller than that induced by other peptides including bradykinin (H69) or GRP (H345) (∆[Ca2+]), 80–100 and 85–120 nm, respectively.

The salient feature shown in Fig. 1 is that gastrin I and CCK-8 induced a prominent Ca2+ mobilization in the SCLC cell line H510. The magnitude of the [Ca2+]i response induced by gastrin I in this cell line (∆[Ca2+]i, 150 nm) was greater than the responses induced by other Ca2+-mobilizing neuropeptides including bradykinin, vasopressin, or galanin (∆[Ca2+]i, 100, 70, and 50 nm, respectively). Identical results were obtained when CCK-8 was added instead of gastrin I (Fig. 1). The increase in [Ca2+]i induced by gastrin I in H510 cells results from Ca2+ mobilization from internal stores, since it still occurred after the addition of ethylene glycol-bis(β-aminopropyl) ether)-N,N,N',N'-tetraacetic acid to chelate extracellular Ca2+, just prior to the addition of gastrin I (results not shown). In view of the findings depicted in Fig. 1, the H510 SCLC line has been used in additional experiments to characterize the Ca2+-mobilizing effects of gastrin I and CCK-8.

Repeated additions of gastrin I caused the homologous desensitization of Ca2+ mobilization. Furthermore, the addition of gastrin I attenuated the increase in [Ca2+]i induced by CCK-8, and reciprocally, brief exposure to CCK-8 prevented the [Ca2+]i response induced by gastrin I. Neither gastrin I nor CCK-8 prevented the increase in [Ca2+]i induced through a distinct neuropeptide receptor such as bradykinin (data not shown). These results suggest that gastrin I and CCK-8 induced Ca2+ mobilization in H510 through a common receptor.

Gastrin I, gastrin II, des(SO3)CCK-8, and CCK-8 increased the peak level of [Ca2+]i in a concentration-dependent fashion (Fig. 2). The concentrations required to induce half-maximum stimulation by these agonists were 7, 2.5, 5, and 2.5 nM, respectively. Thus, the receptors expressed by H510 cells recognize gastrin and CCK agonists with approximately equal apparent affinities.

Effect of Various CCK/Gastrin Antagonists. In order to gain insight into the receptor that mediates the [Ca2+]i response to gastrin and CCK-8 in H510 cells we tested the effect of various receptor antagonists. Proglumide, a derivative of glutamic acid, and benzotript, a tryptophan derivative, block the binding and biological effects of both gastrin and CCK analogues (reviewed in Ref. 17). Accordingly, the Ca2+-mobilizing effects of either gastrin II or CCK-8 were prevented by the addition of
either proglumide or benzotript (Fig. 3). These antagonists, however, do not discriminate between different types of CCK/gastrin receptors. Recently, a CCK receptor antagonist has been identified that is selective for gastrin/CCKB receptors as compared to pancreatic CCKA receptors (18, 19). Fig. 4 shows that the addition of the specific gastrin/CCKB receptor antagonist L-365260 (15 nM) completely blocked the increase in [Ca\(^{2+}\)]\(_i\) induced by 5 nM gastrin II. The antagonist inhibited the [Ca\(^{2+}\)]\(_i\) response by either gastrin II or CCK-8 in a concentration-dependent manner; 50% inhibition concentration values were 4 and 1 nM, respectively. In contrast, the CCKA preferring antagonist L-364,718 (15 nM) had little effect on the increase in [Ca\(^{2+}\)]\(_i\) induced by 5 nM gastrin II (data not shown). The fact that gastrin and CCK agonists were able to induce Ca\(^{2+}\) mobilization with comparable potencies (Fig. 2) and that L-365260 was a potent inhibitor of these responses (Fig. 4) indicates that H510 cells express a gastrin/CCKB-type receptor.

Gastrin Stimulates Clonal Growth in SCLC. The preceding findings indicate that the H510 SCLC cell line can provide a useful model system for determining whether gastrin can act as a direct growth factor in vitro. Consequently, we attempted to determine whether gastrin can stimulate colony formation by this SCLC cell line in agarose-containing medium. Fig. 5 shows that gastrin I and CCK-8 caused a striking increase in the ability of these cells to form colonies in semisolid medium. Gastrin increased both the number and the size of colonies, in a dose-dependent manner (Fig. 6). This increase in clonal growth was comparable to that induced by other growth factors. Gastrin I, gastrin II, des(SO\(_3\))CCK-8, and CCK-8 stimulated colony formation at comparable concentrations (Fig. 7). In contrast, CCK-1020, which lacks the COOH-terminal sequence critical for receptor binding, neither increased [Ca\(^{2+}\)]\(_i\) nor stimulated colony formation. The ability of gastrin to induce colony growth was attenuated by the addition of the specific gastrin/CCKB receptor antagonist L-365260 (results not shown).

The peptides [D-Arg\(_1\), D-Phe\(_5\), D-Trp\(_7\), 9, Leu\(_{11}\)] substance P and [Arg\(_6\), D-Trp\(_7\), 9, MePhe\(_8\)] substance P (6—11) have been shown to inhibit signal transduction and cell proliferation by multiple neuropeptides that induce Ca\(^{2+}\) mobilization in SCLC cells (11, 13, 20). These broad-spectrum neuropeptide antagonists inhibited both the [Ca\(^{2+}\)]\(_i\) response and the stimulation of colony formation induced by gastrin (data not shown).

**DISCUSSION**

In the present study we demonstrate that the addition of gastrin at nanomolar concentrations to the SCLC line H510 causes a rapid mobilization of Ca\(^{2+}\) from intracellular stores. The magnitude of the [Ca\(^{2+}\)]\(_i\) signal evoked by gastrin was more
Gastrin has been postulated as a cellular growth factor, but compelling in vitro evidence of this has been difficult to obtain. SCLC cell lines, including H510, are able to form colonies in semisolid medium, and their clonogenic ability is markedly increased by multiple neuropeptides (21). The SCLC cell line H510 provides an excellent model system in which to determine whether gastrin can act as a direct growth factor. Consequently, we determined the effect of gastrin on the ability of these cells to form colonies in agarose-containing medium. Here we demonstrate that gastrin markedly stimulates the clonal growth of H510 cells. Gastrin I, gastrin II, des-(SO3)CCK-8, and CCK-8 induce colony formation at comparable concentrations, in agreement with the effects obtained on Ca2+ mobilization. 

pronounced than that elicited by other Ca2+-mobilizing peptides in this cell line. Thus, the SCLC cell line H510 has been identified as a useful model system for studying the effects of gastrin in vitro.

Gastrin and CCK share a common COOH-terminal pentapeptide and consequently bind to common cell surface receptors. The CCKA receptors have a 500-fold higher affinity for CCK-8 than for gastrin. In contrast, CCKB receptors bind either CCK-8 or gastrin with approximately equal apparent affinities (17). Here we demonstrate that gastrin I, gastrin II, CCK-8, and des-(SO3)CCK-8 induce rapid Ca2+ mobilization in SCLC H510 at comparable concentrations. Furthermore, the selective gastrin/CCKB antagonist L360265 (18, 19) blocked the increase in [Ca2+]i induced by either gastrin or CCK-8. Thus, the effects of gastrin and CCK in SCLC H510 are mediated by gastrin/CCKB receptors.

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These results demonstrate that gastrin acts as a direct growth factor in vitro and show, for the first time, that this hormonal peptide can stimulate the proliferation of cells outside the gastrointestinal tract.

Lung cancer remains the commonest fatal malignancy in the developed world. SCLC constitutes 25% of the total and follows an aggressive clinical course, despite initial chemosensitivity (22). Identification of the factors that stimulate the proliferation of SCLC cells will be important in the design of alternative and more effective therapeutic strategies. SCLC is characterised by its ability to secrete many hormones and neuropeptides including GRP, neurotensin, and vasopressin (23—25). Results from our laboratory show that, at optimal concentrations, neurotensin, vasopressin, GRP, galanin, and bradykinin induce comparable increases in SCLC clonal growth in responsive cell lines (21). Consequently, it has been hypothesized that SCLC growth is regulated by multiple autocrine and/or paracrine circuits involving Ca\(^{2+}\)-mobilizing neuropeptides (12, 21, 26). Interestingly, SCLC cells have been shown to express gastrin and CCK peptides (27, 28). Thus, the findings presented here demonstrating that gastrin and CCK-8 can act as direct growth factors for the SCLC cell line H510 further extend the hypothesis that SCLC growth may be regulated by multiple autocrine and paracrine loops involving neuropeptides.

REFERENCES

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