Synergistic induction of ornithine decarboxylase by asparagine and gut peptides in intestinal crypt cells

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Wang, J ian-Ying, J i Li, Anami R. Patel, Stephen Summers, Li Li, and Barbara L. Bass. Synergistic induction of ornithine decarboxylase by asparagine and gut peptides in intestinal crypt cells. Am. J. Physiol. 274 (Cell Physiol. 43): C1476-C1484, 1998.—The objective of this study was to determine whether the amino acid asparagine stimulates the activity of ornithine decarboxylase (ODC) synergistically with epidermal growth factor (EGF) or gastrin in IEC-6 cells, a line of normal rat small intestinal crypt cells. Cells were grown in DMEM containing 5% dialyzed fetal bovine serum, and serum was deprived for 24 h before experiments. Exposure to EGF or gastrin alone increased ODC activity 4.5- to 6-fold. Asparagine alone increased the enzyme activity 10- to 13-fold in IEC-6 cells. Simultaneous addition of asparagine and EGF or gastrin, however, increased ODC activity more than 40-fold. In contrast, there was no synergistic induction of ODC activity when gastrin and EGF were added together. Increased ODC activity in cells treated with asparagine and EGF or gastrin was associated with an increase in ODC mRNA and protein levels. The rate of transcription of the ODC gene was significantly increased by exposure to EGF or gastrin. Asparagine alone had little or no effect on the rate of transcription of the ODC gene. When given together with EGF or gastrin, asparagine also had no additional effect on the transcription rate of the ODC gene. The half-life of mRNA for ODC in unstimulated IEC-6 cells was ~30 min and increased to more than 2 h in cells exposed to asparagine, although neither gastrin nor EGF prolonged the stability of ODC mRNA. The half-life of mRNA for ODC after combined addition of asparagine and EGF or gastrin was extended to ~2 h, similar to asparagine alone. Combined addition of asparagine and EGF or gastrin also significantly increased DNA synthesis compared with cells exposed to each of the three agents alone. In conclusion, 1) simultaneous addition of asparagine and EGF or gastrin increases ODC activity in a synergistic manner and 2) asparagine increases ODC mRNA levels through completely distinct mechanisms from EGF or gastrin. EGF or gastrin specifically stimulates transcription of the ODC gene, whereas asparagine affects a posttranscriptional process.

polyamines; cell proliferation; intestine; epidermal growth factor; gastrin; transcription

INCREASING EVIDENCE HAS demonstrated that cell proliferation in the intestinal mucosa is dependent on the supply of polyamines to the dividing cells (18, 35). Intracellular polyamine levels are highly regulated and completely depend on the activation or inhibition of ornithine decarboxylase (ODC), which is the first rate-limiting step in polyamine biosynthesis. ODC catalyzes the conversion of ornithine to putrescine, the diamine precursor of spermidine and spermine in eukaryotic cells (12, 27, 30, 33). An increase in ODC activity is paralleled by large increases in newly synthesized polyamines, which occur in concert with increases in the expression of a number of early response genes (22, 23, 34). In addition to the general effect of metabolic hormones, the amount of ODC in the gastrointestinal mucosa is also regulated by growth-related gut peptides and luminal factors present in the diet or secreted from digestive glands (11, 35). It has been shown that an increase in the activator protein-1 transcription factors is necessary for the activation of the ODC gene (14, 17) and that downregulation of gene expression is influenced by methylation in the promoter region (7).

Epidermal growth factor (EGF) and gastrin are additional important gut peptides that stimulate cell proliferation in the mucosa under physiological condition (11). Administration of EGF or gastrin significantly increases ODC activity and intracellular polyamine levels in intestinal epithelial cells, changes that precede an increased rate of DNA synthesis (11, 20, 33). Inhibition of polyamine biosynthesis by blocking ODC activity with α-difluoromethylornithine (DFMO) prevents EGF or gastrin-stimulated cell proliferation. Luminal amino acids have been shown to markedly increase ODC activity and mucosal growth when administered intragastrically (11, 29). Of normal luminal constituents, type A amino acids are much more effective than other amino acids for inducing ODC activity and mucosal growth (2, 9). Asparagine is the most potent type A amino acid. We have recently demonstrated that asparagine significantly stimulates ODC gene expression in normal small intestinal crypt cells and that increased ODC mRNA levels result at least partly from a delay in the rate of mRNA degradation (36). To our knowledge, there are no studies concerning the interactive effects between gut peptides and luminal amino acids on expression of the ODC gene in small intestinal epithelial cells.

In the following study, we investigated the influence of combined addition of the amino acid asparagine and gut peptides EGF or gastrin on ODC activity and expression of the ODC gene in cultured normal rat intestinal crypt cells (IEC-6 cell line). First, we examined whether there is a synergistic induction of ODC by combined addition of asparagine and EGF or gastrin. Second, we examined the molecular mechanism underlying the observed synergistic induction of ODC due to gut peptides and amino acid by comparing changes in the enzyme activity with changes in the amount of ODC mRNA and its transcription and posttranscriptional stabilization of ODC mRNA and protein. Third, we examined the effect of synergistic induction of ODC activity by combined asparagine and EGF or gastrin on
cell division. Some of these data have been published in abstract form (32).

MATERIALS AND METHODS

Chemicals and supplies. Disposable cultureware was purchased from Corning Glass Works (Corning, NY). Tissue culture media and dialyzed fetal bovine serum (dFBS) were from Gibco (Grand Island, NY). Biochemicals and human EGF were purchased from Sigma (St. Louis, MO). Pentagastrix was from Research Plus (Bayonne, NJ). The DNA probes used in these experiments included pBR 322 containing mouse ODC cDNA (American Type Culture Collection (ATCC) 63075), pHcGAP containing human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (ATCC 57090), and plasmid pSV2neo (ATCC 37149). The ODC probe from the mouse is well characterized and has been used routinely in studies of ODC gene expression in the rat, having >75% homology between the species (3, 14). The primary antibody, monoclonal anti-ODC antibody (O-1136), and the secondary antibody, anti-mouse IgG conjugated to Sigma horseradish peroxidase (A-8924), were purchased from Sigma Chemical. L-[1-14C]ornithine (sp act 51.6 mCi/µmol) was purchased from NEN (Boston, MA), and [α-32P]dCTP (3,000 Ci/µmol) and [α-32P]UTP (800 Ci/µmol) were from Amersham (Arlington Heights, IL). DFMO was the kind gift of the Merrell Dow Research Institute (Cincinnati, OH).

Cell culture and general experimental protocol. The IEC-6 cell line was derived from normal rat intestine and was developed and characterized by Quarone et al. (24). IEC-6 cells originated from intestinal crypt cells, as judged by morphological and immunological criteria. They are nontumorigenic and retain the undifferentiated character of epithelial stem cells.

Stock cells were maintained in T-150 flasks in DMEM supplemented with 5% heat-inactivated FBS, 10 µg insulin, and 0.05 µg gentamicin sul fate/ml. Flasks were incubated at 37°C in a humidified atmosphere of 90% air-10% CO2. Stock cells were subcultured once a week at a 1:20 medium. The dishes were placed on ice, the monolayers were washed three times with ice-cold D-PBS, and then different solutions were added according to the assays to be conducted.

In the second series of studies, we examined possible mechanisms involved in the observed synergistic induction of ODC by asparagine and EGF or gastrin. First, we investigated the effect of combined addition of asparagine and EGF or pentagastrin on the rate of ODC gene transcription in IEC-6 cells. After exposure to experimental medium for 3 h, nuclei were isolated and the rate of transcription of the ODC gene was measured by nuclear run-on transcription analysis. Second, we examined the effect of combined addition of asparagine and EGF or pentagastrin on the posttranscriptional regulation of ODC mRNA. The half-life of ODC mRNA was measured after the treatment. Actinomycin D (5 µg/ml) was added to cultures 3 h following exposure to asparagine plus EGF or pentagastrin. Control cells received actinomycin D without being stimulated with asparagine and EGF or pentagastrin. ODC mRNA levels were assayed 0, 20, 60, 90, 120, 240, and 480 min after the addition of actinomycin D.

In the third study, the effect of combined addition of asparagine and EGF or pentagastrin on the proliferation of the IEC-6 cells was examined. Cells were plated at a concentration of 1.2 × 10^3/well in 24-well plates and grown in DMEM containing 5% dFBS for 48 h. Serum was deprived from the medium for 24 h before experiments. Cells were harvested 20 h after exposure to asparagine alone or asparagine plus EGF or pentagastrin, and DNA synthesis was measured by an [3H]thymidine incorporation technique.

Assay for ODC activity. ODC activity was determined by a radiometric technique in which the amount of 14CO2 liberated from L-[1-14C]ornithine was estimated (25). Samples were collected as described above and placed in 0.5 ml of 20 mM Triss buffer (pH 7.4) containing 0.05 mM EDTA, 0.05 mM pyrophosphate, and 5 mM dithiothreitol. The cells were frozen and thawed three times, scraped, and transferred to microcentrifuge tubes. Cells were sonicated for 20 s and centrifuged at 12,000 g at 4°C for 15 min. The ODC activity of an aliquot of the supernatant was determined during incubation in stopped vials in the presence of 7.6 n mole of [14C]ornithine (sp act 51.6 mCi/µmol) for 3 h at 37°C. The 14CO2 liberated by the decarboxylation of ornithine was trapped on a piece of filter paper impregnated with 20 µl of 2 N NaOH, which was suspended in a center well above the reaction mixture. The reaction was stopped by the addition of trichloroacetic acid to a final concentration of 10%. The 14CO2 trapped in the filter paper was measured by liquid scintillation spectroscopy at a counting efficiency of 95%. Aliquots of the 12,000-g supernatant were assayed for total protein, using the method described by Bradford (1). Enzymatic activity was expressed as picomoles of CO2 per milligram of protein per hour.

Western blot analysis of ODC protein. Cell samples, dissolved in SDS sample buffer (250 mM Tris·HCl, pH 6.8, 2% SDS, 20% glycerol, and 5% mercaptoethanol), were sonicated at 20 s and centrifuged at 2,000 rpm for 15 min. The supernatant was boiled for 10 min and then subjected to electrophoresis on 7.5% acrylamide gels according to Laemmli (15). Each lane was loaded with 20 µg protein equivalents. Briefly, after the transfer of protein onto nitrocellulose filters, the filters were incubated for 1 h in 5% nonfat dry milk in 1× PBS-Tween 20 (PBS-T; 15 mM NaH2PO4, 80 mM Na2HPO4, 1.5 mM NaCl, pH 7.5, and 0.5% [vol/vol] Tween 20). Immunological evaluation was then performed for 1 h in 1% BSA-PBS-T buffer containing 1 µg/ml monoclonal antibody against ODC protein. The filters were subsequently washed with 1×
PBS-T and incubated for 1 h with goat anti-mouse IgG antibody conjugated to peroxidase. After extensive washing with 1× PBS-T, the immunocomplexes on the filters were reacted for 1 min with chemiluminescence reagent (DuPont NEN, NEL-100). Finally, the filters were placed in a plastic sheet protector and exposed to autoradiography film for 30 or 60 s.

RNA isolation and Northern blot analysis. Total RNA was extracted with guanidinium isothiocyanate solution and purified by CsCl density gradient ultracentrifugation as described by Chirgwin (4). Briefly, the monolayer of cells was washed with D-PBS and lysed in 4 M guanidinium isothiocyanate. The lysates were brought to 2.4 M CsCl concentration and centrifuged through a 5.7 M CsCl cushion at 150,000 g at 20°C for 24 h. After centrifugation, the supernatant was aspirated and the tube was cut ~0.5 cm from the bottom with a flame scalpel. The resulting RNA pellet was dissolved in Tris-HCl (pH 7.5) containing 1 mM EDTA, 5% sodium laurylsarcosine, and 5% phenol (added just before use). The purified RNA was precipitated from the aqueous phase by the addition of 0.1 vol of 3 M sodium acetate and 2.5 vol of ethanol in sequence. Final RNA was dissolved in water and estimated from its ultraviolet absorbance at 260 nm using a conversion factor of 40 units. In most cases, 30 µg of total cellular RNA were denatured and fractionated electrophoretically, using a 1.2% agarose gel containing 3% formaldehyde, and transferred by blotting to nitrocellulose filters. Blots were prehybridized for 24 h at 42°C with 5× Denhardt’s solution-5× standard saline sperm DNA. DNA probes for ODC and GAPDH were labeled with [α-32P]dCTP using a standard nick translation procedure. Hybridization was carried out overnight at 42°C in the same solution containing 10% dextran sulfate and 32P-labeled DNA probes. Blots were washed with two changes of 1× sodium chloride/sodium citrate (SSC)-0.1% sodium chloride/sodium citrate (SSC)-0.1% SDS at room temperature. After the final wash, the filters were autoradiographed with intensifying screens at ~70°C. The signals were quantitated by densitometry analysis of the autoradiograms.

Nuclear run-on assays. Nuclei were prepared according to established methods of deBustros et al. (5). Briefly, IEC-6 cells were suspended in buffer A (20 mM Tris-HCl, pH 7.4, 10 mM NaCl, and 3 mM MgCl2). Nonidet P-40 was added at a final concentration of 0.1%, and the suspension was homogenized in a sterile Dounce homogenizer. Nuclei were pelleted at 1,000 g and washed once in buffer A. The nuclear pellet was resuspended in 40% glycerol, 50 mM Tris-HCl, pH 8.3, 5 mM MgCl2, and 0.1 mM EDTA at 1 µg of DNA/ml and frozen at ~8°C until analysis. Nuclear isolated as described were reproductively intact and free of cellular debris as assessed by phase contrast microscopy. Nuclear transcription activity was determined by measurement of [α-32P]UTP incorporation in RNA transcripts elongated in vitro as described by McNight and Palmiter (19). Nuclear transcription assays were carried out in a transcription buffer composed of 35% glycerol, 10 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 80 mM KCl, 0.1 mM EDTA, 0.5 mM dithiothreitol, 4 mM ATP, GTP, and CTP, and 200 µCi of [α-32P]UTP (Amersham; 800 Ci/mmol) at 26°C for 10 min. The RNA was extracted by a modification of the guanidinium method (4) as described in RNA isolation and Northern blot analysis.

Im mobilization of DNA plasmids and hybridization. The cDNAs for ODC (AVA-I), GARPDH (BamH I, Hind III), and pSV2neo were linearized by digestion with respective enzymes, boiled, and blotted (20 µg of cDNA/blot) onto nitrocellulose membrane (GeneScreen, DuPont). The GAPDH served as a positive control and pSV2neo as a negative control. After these membranes were dried at 80°C for 2 h, they were prehybridized overnight and then hybridized with α-32P-labeled RNA isolated from nuclear transcription experiments for 24 h at 42°C. Filters were washed in 2× SSC-1% SDS at 65°C for 1 h and then in 0.1× SSC-0.1% SDS at room temperature for 1 h. Filters were exposed to Kodak XAR-2 films at ~70°C. Quantitative results were obtained by densitometric scanning and are expressed with reference to the signal for GAPDH.

Measurement of DNA synthesis. DNA synthesis was measured with the use of the [3H]thymidine incorporation technique, as previously described (31). Cells in 24-well plates were pulsed with 1 µCi/ml tritiated thymidine for 4 h before harvest. Cells were washed twice with cold D-PBS solution and then incubated in cold 10% TCA for 30 min at 4°C. After rinsing twice with cold 10% TCA, cells were dissolved in 0.5 ml of 0.5 N NaOH at 37°C in humidified air for 90 min. The incorporation of [3H]thymidine into DNA was determined by counting the aliquot of cell lysate in a Beckman liquid scintillation counter. The protein content of an aliquot of cell lysate was determined by the method as described by Bradford (1). DNA synthesis was expressed as disintegrations per minute per microgram of protein.

Polyamine analysis. The cellular polyamine content was analyzed by HPLC as described previously (34). In brief, after monolayers were washed three times with ice-cold D-PBS, we added 0.5 M perchloric acid and then froze the monolayers at ~80°C until ready for extraction, dansylation, and HPLC. The standard curve encompassed 0.31–10 µM. Values that fell more than 25% below the curve were considered not detectable. Protein was determined by the Bradford method (1).

Statistics. All data are expressed as means ± SE from six dishes. Autoradiographic results were repeated three times. The significance of the difference between means was determined by analysis of variance. The level of significance was determined using the Duncan’s multiple range test (8).

RESULTS

Synergistic effect of asparagine and EGF or gastrin on the induction of ODC activity. In these studies, IEC-6 cells were grown in DMEM in the absence of 5% FBS for 24 h before experiments. This preincubation period served to partially synchronize the cells in a quiescent state. Basal ODC activity was low at the end of this incubation (8.9 ± 0.5 pmol·mg protein−1·h−1). Exposure of IEC-6 cells to EGF (10 ng/ml) or pentagastrin (10−5 M) alone increased ODC activity 4.5- to 6-fold above basal levels. Asparagine, when added alone, induced a 10- to 13-fold increase in ODC activity. Gastrointestinal hormones, when added alone, induced a 3-fold increase in ODC activity. Combined addition of asparagine and EGF or gastrin increased ODC activity in IEC-6 cells. There was no 40-fold increase in ODC activity 3 h after the treatment with asparagine and EGF (Fig. 1). Similarly, simultaneous administration of asparagine and pentagastrin also increased ODC activity to 35-fold. The synergistic induction of ODC activity occurred 2 h after treatment and remained significantly elevated 6 h thereafter. The maximum increase in ODC activity occurred 3 h after administration of asparagine and EGF or pentagastrin, and the enzyme activity returned to control values by 8 h (data not shown). In contrast, there was no synergistic effect on induction of ODC activity when EGF and pentagastrin were given together without asparagine (Fig. 1).
Increases in ODC activity in cells exposed to asparagine and EGF or pentagastrin were paralleled by increases in cellular polyamine levels (Table 1). Treatment with asparagine, EGF, or pentagastrin alone significantly increased the cellular putrescine content, although it had no effect on spermidine and spermine levels. Simultaneous addition of asparagine and EGF or pentagastrin, however, significantly increased putrescine content above those of cells exposed to any of the three agents alone. Cellular spermidine content was also significantly increased when asparagine was administered together with EGF or pentagastrin.

Fig. 1. Ornithine decarboxylase (ODC) activity in IEC-6 cells exposed to asparagine (ASP, 10 mM), epidermal growth factor (EGF; 10 ng/ml), or pentagastrin (PG, 10^{-5} M) alone and ASP plus EGF or PG. Cells were grown in DMEM containing 5% dialyzed FBS for 48 h and serum deprived for 24 h before experiments. ODC activity was assayed 2 h (A) and 3 h (B) after treatments. Values represent means ± SE of data from 6 cultures. *,† P < 0.05 compared with control (Con) and ASP alone, respectively.

Table 1. Cellular polyamine concentrations in IEC-6 cells exposed to asparagine and asparagine plus EGF or pentagastrin

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Putrescine (nmol/mg protein)</th>
<th>Spermidine (nmol/mg protein)</th>
<th>Spermine (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.14 ± 0.02</td>
<td>1.6 ± 0.3</td>
<td>3.1 ± 0.4</td>
</tr>
<tr>
<td>ASP (10^{-2} M)</td>
<td>0.18 ± 0.02*</td>
<td>1.7 ± 0.05</td>
<td>3.2 ± 0.5</td>
</tr>
<tr>
<td>EGF (10 ng/ml)</td>
<td>0.175 ± 0.02*</td>
<td>1.73 ± 0.04</td>
<td>3.3 ± 0.4</td>
</tr>
<tr>
<td>PG (10^{-5} M)</td>
<td>0.178 ± 0.02*</td>
<td>1.68 ± 0.04</td>
<td>3.2 ± 0.5</td>
</tr>
<tr>
<td>ASP plus EGF</td>
<td>0.29 ± 0.04†</td>
<td>1.98 ± 0.1†</td>
<td>3.42 ± 0.4</td>
</tr>
<tr>
<td>ASP plus PG</td>
<td>0.28 ± 0.03†</td>
<td>1.88 ± 0.08†</td>
<td>3.3 ± 0.3</td>
</tr>
</tbody>
</table>

Values are means ± SE of data from 6 cultures. Cellular polyamine levels were determined 3 h by HPLC analysis after treatments. ASP, asparagine; EGF, epidermal growth factor; PG, pentagastrin. *,† P < 0.05 compared with control and asparagine alone, respectively.

Changes in the amount of ODC mRNA and protein in IEC-6 cells treated with asparagine and EGF or gastrin. Increased ODC activity in IEC-6 cells in response to the combined action of asparagine and EGF or pentagastrin was associated with a rise in steady-state levels for ODC mRNA and protein. As can be seen in Figs. 2 and 3, administration of EGF, pentagastrin, or asparagine alone significantly increased the basal level of ODC mRNA and protein. Consistent with the changes in the enzyme activity, simultaneous administration of asparagine and EGF or pentagastrin significantly increased ODC mRNA and protein levels above those of cells treated with asparagine, EGF, or pentagastrin alone. No changes in ODC mRNA and protein levels occurred in cells treated with combined EGF and pentagastrin compared with those of cells exposed to EGF or pentagastrin alone (data not shown).

Fig. 2. Relative ODC mRNA levels in IEC-6 cells described in Fig. 1. RNA was harvested at 3 h after treatments and determined by Northern blotting analysis. Total cellular RNA (30 µg) was blotted and hybridized with 32P-labeled ODC cDNA probe. Hybridization to labeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe served as marker for equal loading of lanes. Values were means from 3 separate experiments, and relative levels of mRNA for ODC were corrected for RNA loading as measured by densitometry of GAPDH. *,† P < 0.05 compared with control and asparagine alone, respectively.

Fig. 3. Western immunoblotting analysis of ODC in cell extracts from cells described in Fig. 1. Whole cell lysates were harvested at 3 h after treatments and subjected to electrophoresis on 7.5% acrylamide gel and Western immunoblotting. Protein (20 µg) was applied to each lane, and ODC protein was identified by probing nitrocellulose with specific antibody as described in MATERIALS AND METHODS. Size of observed ODC protein molecular mass is ~53 kDa, and the position is indicated by ODC. Three experiments were performed that showed similar results.
Effect of combined asparagine and EGF or gastrin on the transcription of the ODC gene. Nuclear run-on transcription assay was employed to test whether asparagine and EGF or pentagastrin, when added together or separately, stimulate the synthesis of ODC mRNA and whether the increased ODC mRNA levels result from an increased rate of gene transcription. The results presented in Fig. 4 clearly show that the transcription rate of the ODC gene was significantly stimulated by combined asparagine and EGF or pentagastrin. The rate of ODC gene transcription was increased by approximately threefold in cells exposed to asparagine and EGF or pentagastrin. Treatment with either EGF or pentagastrin alone also significantly increased ODC gene transcription in IEC-6 cells. On the other hand, asparagine, when given alone, did not affect the rate of transcription of the ODC gene. Furthermore, when asparagine was administered together with EGF or pentagastrin, the transcription rate of the ODC gene was indistinguishable from that of cells treated with EGF or pentagastrin alone.

Effect of simultaneous administration of asparagine and EGF or gastrin on the half-life of ODC mRNA and activity. To examine the possibility that the synergistic induction of ODC was regulated posttranscriptionally, the effect of combined addition of asparagine and EGF or pentagastrin on the stability of ODC mRNA was tested. The half-life of ODC mRNA in control cells was compared with cells treated with asparagine alone or asparagine plus EGF or pentagastrin. As can be seen in Figs. 5 and 6, ODC mRNA levels declined rapidly after administration of actinomycin D. In control cells, basal levels of ODC mRNA decreased with a half-life of 32 min. ODC mRNA from cells stimulated with 10 mM asparagine decreased at a slower rate, with a half-life of 120 min. The half-life of ODC mRNA after combined addition of asparagine and EGF or pentagastrin was extended to 125 min, similar to asparagine alone. There were no statistically significant differences in the half-life of ODC mRNA between cells exposed to asparagine alone and those treated with combined asparagine and EGF or pentagastrin. We also demonstrated that EGF or pentagastrin alone did not significantly prolong the stability of ODC mRNA over basal half-life (data not shown).
Because data presented in Fig. 3 and in other sources (6) have demonstrated that the amount of ODC protein correlates well with the increase in ODC activity, we examined the role of stabilization of the enzyme protein in the synergistic induction of ODC by measurement of ODC activity. The ODC activity of IEC-6 cells declined rapidly after the administration of cycloheximide. The half-life of ODC activity in control IEC-6 cells was 21 min and increased to 43 min in cells treated with asparagine alone. Simultaneous administration of asparagine and EGF or pentagastrin had a slight additional effect on the half-life of ODC activity (Fig. 7). When asparagine was administered together with EGF or pentagastrin, ODC activity decreased at a slower rate, with a half-life of \( \sim 50 \) min. Therefore, half-life of ODC enzyme activity was increased by \( \sim 20\% \) in cells stimulated with asparagine and EGF or pentagastrin compared with cells exposed to asparagine alone, although these differences were not statistically significant. Neither EGF nor pentagastrin alone increased the half-life of ODC activity (data not shown).

DISCUSSION

Numerous studies have demonstrated that a single amino acid, asparagine, is essential for inducing maximal ODC activity in a number of tumor cells and primary cultures (3, 25). When hepatocytes and differentiated mouse neuroblastoma cells are maintained in a salt-glucose solution, serum or growth factors alone do not induce ODC activity; the maximal stimulation of ODC activity requires the presence of both asparagine and serum or growth hormones (3). The effect of asparagine is rather specific because other A and N system amino acids are only marginally effective (2, 9). A nonmetabolized amino acid analog, \( \alpha \)-aminoisobutyric acid, has also been shown to stimulate ODC activity (13), indicating that asparagine itself, not its metabolite, results in the induction of enzyme activity. These studies suggest the possibility that the stimulation of ODC activity by various growth factors or hormones under physiological conditions may involve the action of asparagine.

It is of interest and important to investigate the interactive effect between asparagine and growth-related gut peptides on the regulation of ODC activity.

Effect of combined addition of asparagine and EGF or gastrin on DNA synthesis. To elucidate the role of synergistic induction of ODC activity in cell proliferation, we examined the rate of DNA synthesis in IEC-6 cells exposed to asparagine and EGF or gastrin. Addition of asparagine, EGF, or pentagastrin alone increased the rate of DNA synthesis \(~1.8\text{-fold above basal levels. Simultaneous addition of asparagine and EGF or pentagastrin significantly increased DNA synthesis above that of cells exposed to asparagine, EGF, or pentagastrin alone (Fig. 8). The rates of DNA synthesis were 2.9- and 2.8-fold after combined administration of asparagine and EGF or pentagastrin, respectively. On the other hand, combined addition of EGF and pentagastrin did not significantly increase DNA synthesis compared with EGF or pentagastrin alone.

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Effect of combined addition of asparagine and EGF or gastrin on DNA synthesis. To elucidate the role of synergistic induction of ODC activity in cell proliferation, we examined the rate of DNA synthesis in IEC-6 cells exposed to asparagine and EGF or gastrin. Addition of asparagine, EGF, or pentagastrin alone increased the rate of DNA synthesis \(~1.8\text{-fold above basal levels. Simultaneous addition of asparagine and EGF or pentagastrin significantly increased DNA synthesis above that of cells exposed to asparagine, EGF, or pentagastrin alone (Fig. 8). The rates of DNA synthesis were 2.9- and 2.8-fold after combined administration of asparagine and EGF or pentagastrin, respectively. On the other hand, combined addition of EGF and pentagastrin did not significantly increase DNA synthesis compared with EGF or pentagastrin alone.

DISCUSSION

Numerous studies have demonstrated that a single amino acid, asparagine, is essential for inducing maximal ODC activity in a number of tumor cells and primary cultures (3, 25). When hepatocytes and differentiated mouse neuroblastoma cells are maintained in a salt-glucose solution, serum or growth factors alone do not induce ODC activity; the maximal stimulation of ODC activity requires the presence of both asparagine and serum or growth hormones (3). The effect of asparagine is rather specific because other A and N system amino acids are only marginally effective (2, 9). A nonmetabolized amino acid analog, \( \alpha \)-aminoisobutyric acid, has also been shown to stimulate ODC activity (13), indicating that asparagine itself, not its metabolite, results in the induction of enzyme activity. These studies suggest the possibility that the stimulation of ODC activity by various growth factors or hormones under physiological conditions may involve the action of asparagine.

It is of interest and important to investigate the interactive effect between asparagine and growth-related gut peptides on the regulation of ODC activity.
in intestinal epithelial cells because polyamine biosynthesis plays a critical role in the control of normal mucosal growth. ODC in small intestinal mucosa has a high basal activity compared with most tissues and significantly increases in response to a variety of chronic and acute mitogenic stimuli (11, 18). The rapid and striking increases in ODC and polyamine levels are absolutely required for the stimulation of intestinal mucosal growth. Small intestinal mucosal ODC activity increases during normal development, after partial resection, during adaptation to lactation, after refeeding, and during repair after injury (11, 18, 35). All of these instances are associated with increased mucosal polyamine levels and cell division. Inhibition of ODC by treatment with DFMO dramatically decreases the levels of cellular polyamines and significantly inhibits normal mucosal growth unless exogenous polyamines are added.

Refeeding fasted rats significantly stimulates ODC activity in the mucosa of the small intestine and colon (16, 28). The increase in ODC activity is 40-fold in mucosa from the intact jejunum but just 4-fold in mucosa of bypassed segments after refeeding (28, 29). These findings indicate that the regulation of ODC activity in small intestinal mucosa is controlled by both luminal factors and humoral factors and that the activation of the enzyme after refeeding is produced predominantly by direct stimulation from luminal contents. Of normal luminal constituents, only simple amines and amino acids stimulate mucosal ODC activity when administered intragastrically (29). Asparagine appears to be the most potent type A amino acid for inducing ODC in intestinal mucosa, and type A amino acids are much more effective than others (16, 21). Asparagine also has been shown to stimulate ODC activity in a number of experimental systems, including mouse N-8 neuroblastoma cells (3), PC-12 cells, and fibroblast (NIH/3T3), KRC-7, and Reuber H-35 hepatoma cells (26).

The current study clearly shows that, in small intestinal crypt cells maintained in DMEM, asparagine stimulates ODC activity synergistically with EGF and gastrin (Fig. 1). As we have reported previously (36), the concentration of 10 mM asparagine is optimal in IEC-6 cells, probably because it ensures an intracellular level of asparagine, which satisfies a threshold value for the stimulation of ODC in the presence of EGF or gastrin. Although doses of asparagine > 10 mM also increase enzyme activity, none of the values reached is significantly higher than the response to 10 mM asparagine with or without the gut peptides (data not shown). The physiological concentration of intracellular asparagine is in the 0.001 mM range, which can be achieved with extracellular levels in the 10 mM range (26). It also has been demonstrated that asparagine at 10 mM has no effect on extracellular and intracellular pH and on cell volume in IEC-6 cells and LoVo cells (9, 18, 36). Therefore, the synergistic induction of ODC activity in the cells exposed to asparagine and EGF or gastrin is not simply due to changes in cellular pH or osmolality.

As can be seen in Fig. 4, the rate of transcription of the ODC gene is significantly increased by combined administration of asparagine and EGF or gastrin, indicating that the synergistic induction of ODC is at least partially regulated at the level of gene transcription. Administration of EGF or gastrin alone also significantly stimulates the transcription rate of the ODC gene in IEC-6 cells. Asparagine, when given alone, does not significantly affect the rate of transcription of the ODC gene. These data indicate that the increased synthesis of ODC mRNA in cells exposed to combined asparagine and EGF or gastrin primarily results from the action of the gut peptides rather than the amino acid. These results are consistent with data from other investigators (3, 31) who have demonstrated that, in primary cultures of rat hepatocytes, asparagine by itself slightly increases ODC gene transcription, which is significantly potentiated in the presence of glucagon. Because the rate of transcription of the ODC gene is two- to threefold higher in cells treated with asparagine and EGF or gastrin than that seen in the control, it is not enough to explain the increase in the level of ODC mRNA and the synergistic induction of ODC activity following simultaneous addition of the amino acid and the gut peptides.

Figures 5 and 6 clearly show that combined administration of asparagine and EGF or gastrin increases the half-life of the induced ODC mRNA by more than four times in the presence of actinomycin D, suggesting that posttranscriptional stabilization of ODC mRNA is one of the key factors increasing the levels of ODC mRNA in IEC-6 cells exposed to asparagine and EGF or gastrin. The half-life of ODC mRNA after treatment with asparagine alone also is prolonged to 120 min, similar to those from cells treated with combined asparagine and EGF or gastrin together. In contrast, administration of EGF or gastrin alone did not significantly affect the stability of ODC mRNA in IEC-6 cells. These results indicate that the prolonged half-life of the induced ODC mRNA in cells exposed to asparagine and EGF or gastrin is produced predominantly by asparagine. Our results are similar to those in DF-40 neuroblastoma cells where the half-life of ODC mRNA is increased to 20-fold after exposure to asparagine (3).

Results presented in Fig. 7 show that combined administration of asparagine and EGF or gastrin also stabilizes the ODC protein, as indicated by an increase in the half-life of enzyme activity. The half-life of ODC activity of IEC-6 cells is increased to more than two times when asparagine is given together with EGF or gastrin. The stabilizing effect on the enzyme protein partly explains the differences between increased ODC activity and elevated ODC mRNA, because the 35- to 40-fold increase in activity induced by combined addition of asparagine and EGF or gastrin cannot be accounted for solely by the 8- to 9-fold increase in the level of ODC mRNA. Although the exact mechanism involved in the posttranslational modification of ODC protein in cells exposed to asparagine is unclear (6, 7), similar changes in ODC half-life have been demon-
strated in various cells (10, 13, 36) and also in mucosal tissue (18).

In summary, these results indicate that exposure to the luminal amino acid asparagine stimulates ODC activity synergistically with the gut peptides EGF or gastrin in normal small intestinal crypt cells. The increased ODC activity in cells treated with asparagine and EGF or gastrin is associated with a rise in steady-state levels for ODC mRNA and protein. In this investigation of the mechanism involved in the synergistic induction of ODC, our results indicate that asparagine increases ODC mRNA levels through completely distinct processes from EGF and gastrin. Asparagine prolongs the half-life of ODC mRNA but has no effect on the mRNA transcription. EGF and gastrin stimulate the rate of ODC gene transcription without having an effect on the stability of the mRNA. Because the transcription rate of the ODC gene and the stability of ODC mRNA and protein are significantly increased after combined addition of asparagine and EGF or gastrin, the synergistic induction of ODC is a complex process, which includes changes at the transcriptional, posttranscriptional, and posttranslational levels. Our results also indicate that combined addition of asparagine and EGF or gastrin significantly increases DNA synthesis in IEC-6 cells. These findings suggest that the synergistic induction of ODC activity by combined asparagine and gut peptides plays an important role in the regulation of cell proliferation in the intestinal mucosa under physiological condition.

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