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Effects of carbamylcholine chloride on human antral gastrin mRNA levels

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Abstract: The effects of the muscarinic receptor agonist, carbamylcholine chloride (carbachol), on gastrin release and gastrin mRNA levels in human antral mucosa (n = 15) were determined. During a-2-h incubation period, carbachol (10^{-6}–10^{-4} M) decreased gastrin mRNA levels to 71 ± 8% (10^{-6} M), 40 ± 8% (10^{-5} M), and 33 ± 5% (10^{-4} M) of control levels. Carbachol (10^{-5} M) decreased intracellular gastrin (from 1634 ± 103 to 1272 ± 126 pg/mg tissue protein), while it increased gastrin release into the medium (from 609 ± 48 to 918 ± 68 pg/ml per mg tissue protein). After 6- and 9-h culture, carbachol gradually increased gastrin mRNA levels, by 96 ± 12% and 126 ± 23%, respectively. Atropine sulfate (10^{-5} M) completely inhibited the carbachol-induced changes. Cycloheximide markedly decreased tissue gastrin concentration, but increased gastrin mRNA levels, whereas it had no effects on gastrin release. These findings suggested that carbachol may have a time-related biphasic action on human antral gastrin biosynthesis.

Key words: carbachol, gastrin gene

Introduction

The gastrointestinal regulatory peptide, gastrin, plays a central role in the physiological regulation of gastric acid secretion.[1] Following stimulation, acid secretion is modulated by a negative feedback loop, in which antral acidification inhibits the further release of gastrin.[1] Gastrin release is significantly enhanced by the tonic parasympathetic pathway. However, the precise mechanism by which the muscarinic effect modulates gastrin biosynthesis in humans is still unclear. To clarify this mechanism, we report in the present study that, in addition to its effects on gastrin release, the muscarinic agonist carbamylcholine chloride has a time-related biphasic action on gastrin mRNA levels in human antral mucosa in vitro.

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Materials and methods

Tissue culture system

Tissue culture was performed essentially as described previously.[2] Briefly, antral mucosa was obtained from 15 patients (7 females and 8 males; age, 40–55 years) with gastric cancer at gastrectomy at Kochi Medical School Hospital. The excised antral tissue was immediately washed three times in ice-cold Hank's balanced salt solution (Sigma Chemical Co., St. Louis, Mo.) containing 100 U/ml penicillin and 100 mg/ml streptomycin. Antral mucosal strips were sectioned into fragments of 1–2 mm^3. The operative time required to prepare the antral explants was about 30 min after gastrectomy. Sterile plastic culture dishes (Falcon Plastics Division, Bio-Quest, Oxnard, Calif.) containing antral tissue fragments were incubated at 37°C for various periods in Dulbecco's minimal essential medium (DMEM; Sigma) containing fetal bovine serum (5%) and gassed with 95% O_2–5% CO_2.

Effects of carbamylcholine chloride and atropine sulfate on gastrin mRNA levels

The effects of the muscarinic receptor agonist, carbamylcholine chloride (carbachol; Sigma), and the muscarinic receptor antagonist, atropine sulfate (Tanabe Pharmaceutical Co. Ltd., Tokyo, Japan) on gastrin mRNA levels were examined by adding them

to the incubation medium. Antral mucosal fragments were harvested at various intervals, either for total RNA extraction followed by Northern blotting, or for slot blot hybridizaiton.

Effects of cycloheximide on gastrin mRNA levels

The effects of cycloheximide, an inhibitor of protein synthesis, were examined by incubating antral mucosa under basal conditions, both with and without carbachol. Antral mucosal tissue was preincubated with 10 μg/ml cycloheximide for 1 h, and then incubated in the presence or absence of carbachol for 2 h. After 2 h, the tissues were harvested and the concentration of gastrin mRNA was measured by dot blot hybridization.

RNA extraction

RNA was extracted from antral mucosa, using a modification of the guanidium isothiocyanate method of Chirgwin et al.[3] Briefly, the antral tissue was homogenized in 4 M guanidium isothiocyanate, 25 mM sodium citrate, 100 mM 2-mercaptoethanol, and 0.5% sodium N-lauroylsarcosine. The homogenate was then loaded onto a cushion of 5.7 M cesium chloride and centrifuged for 18 h at 121 000 g. The RNA pellet was extracted twice with chloroform:n-butano (4:1). RNA in the aqueous phase was precipitated in ethanol, and RNA yields were quantitated by absorption at 260 and 280 nm. A260/A280 ratios of 1.95–2.0 indicated that the samples were essentially free of contaminating protein.

Hybridization probes

A cDNA (376 bp) encoding human gastrin precursor was obtained from the pHG 53 plasmid, as reported previously;[2] the plasmid was kindly donated by Dr. Y. Hayashizaki (Division of Biochemistry, National Cardiovascular Institute, Osaka, Japan).[4,5] Human β-actin third exon DNA was used as an internal control.

Dot and Northern blot analysis

For the dot blot analysis of gastrin mRNA, total RNA was denatured in 50% deionized formamide, 6% formaldehyde at 60°C for 15 min and then spotted onto a nylon membrane (Hybond-N; Amersham International plc, Amersham, England). For Northern blotting, RNA was denatured and separated on a 0.8% agarose/formaldehyde gel. The gel was stained with 1 mg/ml ethidium bromide and photographed to show the relative amounts of 28S and 18S rRNA in each sample. The RNA was then transferred onto a nylon membrane. RNA analyzed by dot and by Northern blotting was hybridized to ^{32}P-labelled gastrin precursor cDNA or β-actin DNAs in 20 mM sodium phosphate, pH 6.5, 5 × NaCl/Cit (1 × NaCl/Cit = 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7), 50% (v/v) formamide, 10 × Denhardt's solution, and 100 μg/ml herring sperm DNA, at 42°C for 16 h. After hybridization, the membrane was washed three times in 2 × NaCl/Cit/0.1% sodium dodecyl sulfate (SDS) at room temperature and once in 0.16 × NaCl/Cit/0.1% SDS at 50°C. The bands were visualized by autoradiography. The filter was exposed to Kodak XAR-5 film for the indicated period at −70°C with an intensifying screen. The band intensity was quantified using a Shimadzu dual-wavelength TLC scanner model CS-930 (Shimadzu, Kyoto, Japan).

Tissue gastrin measurement

Extracts were obtained by boiling weighed pieces of antrum in 10 volumes of water for 10 min. Gastrin concentrations in the aqueous supernatants were determined by radioimmunoassay, using commercially available gastrin assay kits (Dainabot, Tokyo, Japan), as described previously.[2,6] Gastrin secreted into the culture medium was also measured. The antibody was specific for gastrin 17 and did not crossreact with the larger gastrin polypeptide. Values were expressed as picogram equivalents of synthetic human gastrin 17.

Statistical analysis

The results are expressed as means ± SEM for each sample, except where noted. The generalized Wilcoxon test was used to compare gastrin mRNA levels in the carbachol and control culture systems. The effects of carbachol, cycloheximide, and atropine sulfate on gastrin release, content, and mRNA levels were analyzed by two-way analysis of variance (ANOVA). A two-tailed P value of <0.05 was considered to indicate statistical significance.

Results

Effects of culture period on gastrin mRNA levels in the control study

We studied the effect of time on gastrin mRNA levels in tissue culture under control conditions. Steady-state gastrin mRNA levels were determined after incubating antral mucosa for 30 min, and for 1, 2, 4, 6, and 12 h. A decrease in the gastrin mRNA level was identified at 60 min, and the basal gastrin mRNA levels were virtually steady (65%–75% of those at 0 h) during the entire period 1- to 12-h (Fig. 1). We then regarded

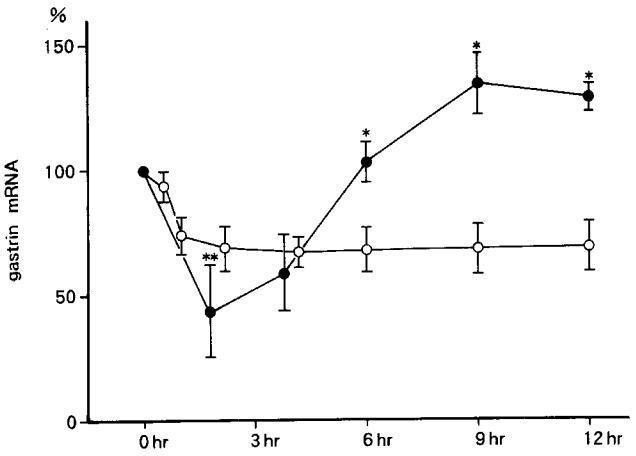

Fig. 1. Time-dependent effects of carbachol on gastrin mRNA levels. In the control experiments ($n = 15$), a decrease in the gastrin mRNA level was determined as early as 60 min, and the basal gastrin mRNA levels were virtually steady (65%–75% of those at 0 h) during the entire period (1–12 h). During the 2-h incubation period, carbachol (10^{-5} M) significantly decreased gastrin mRNA levels ($n = 15$) compared with those in controls ($P < 0.05$). After 6-h incubation, gastrin mRNA levels gradually increased (at 6-h culture they were 98 ± 17% and at 9-h culture they were 128 ± 22% of those at 0 h), and were significantly higher than those in controls ($P < 0.01$). The results are expressed as means ± SEM. The generalized Wilcoxon test was used to compare gastrin mRNA levels in the carbachol and control culture systems. A two-tailed P value of <0.05 was considered to indicate statistical significance. $^{*}P < 0.01$; $^{**}P < 0.05$. *Open circles*, Control culture; *closed circles*, carbachol (10^{-5} M)

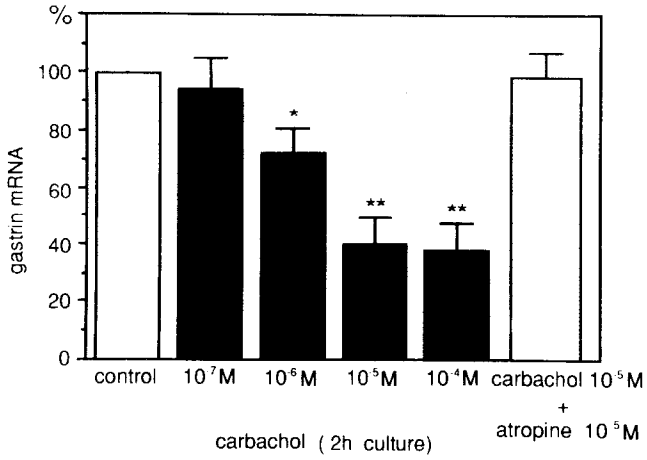

Fig. 2. Dose-dependent effects of carbachol on mRNA levels during 2-h incubation. Carbachol decreased gastrin mRNA levels during the 2-h incubation period: at 10^{-7} M, gastrin mRNA levels were 94 ± 12% of control; at 10^{-6} M, 71 ± 8% of control; at 10^{-5} M, 40 ± 8% of control; and at 10^{-4} M, 33 ± 5% of control. Atropine sulfate (10^{-5} M) abolished these changes. The results are expressed as means ± SEM ($n = 15$). The effects of carbachol and atropine sulfate on gastrin mRNA levels were analyzed by two-way analysis of variance (*ANOVA*). A two-tailed P value of <0.05 was considered to indicate statistical significance. $^{*}P < 0.05$; $^{**}P < 0.01$

gastrin mRNA levels during the 2-h incubation as steady state levels.

Effects of carbachol on gastrin mRNA levels

Antral mucosal strips were incubated in the presence of increasing concentrations of carbachol (10^{-7}–10^{-4} M). At all concentrations examined, carbachol caused a significant and progressive decrease in gastrin mRNA levels at the 2-h incubation period: at 10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} M, gastrin mRNA levels were 92 ± 14% (P:NS), 72 ± 10% ($P < 0.05$), 40 ± 8% ($P < 0.01$), and 36 ± 10% of the control values ($P < 0.01$), respectively (Fig. 2). Atropine sulfate (10^{-5} M) inhibited the carbachol-induced changes (Figs. 2, 3). With 10^{-5} M carbachol, gastrin mRNA levels gradually increased after 6 h, reaching 98 ± 17%, and, after 9 h, reaching 128 ± 22% of the control value (Fig. 1).

Effects of cycloheximide on gastrin mRNA levels

In response to incubation with cycloheximide, basal gastrin mRNA levels increased by 34 ± 9% ($P < 0.01$) at 2-h incubation. With carbachol and cycloheximide, gastrin mRNA levels did not change significantly, being 108 ± 14% (P:NS) of control values, while carbachol decreased gastrin mRNA levels (40 ± 8%) (Fig. 3).

Effects of carbachol, atropine sulfate, and cycloheximide on gastrin release and on intracellular gastrin content

During the 2-h incubation, carbachol (at 10^{-5} M) increased gastrin release (from 608 ± 48 to 918 ± 68 pg/ml per mg tissue protein) from human antral tissue; the carbachol-induced gastrin release was associated with a decrease in intracellular gastrin content (from 1634 ± 168 to 1272 ± 126 pg/mg tissue protein). Incubation in the presence of the muscarinic antagonist, atropine, did not alter basal gastrin release (Fig. 4), but it completely inhibited the carbachol-induced gastrin release (Fig. 5). Cycloheximide had no effect on gastrin release (Fig. 4), but markedly decreased tissue gastrin levels (Fig. 5). During all culture periods, gastrin release was significantly increased compared with control levels, and, during the 6-h incubation, the concentration of gastrin in the tissue increased progres sively (data not shown).

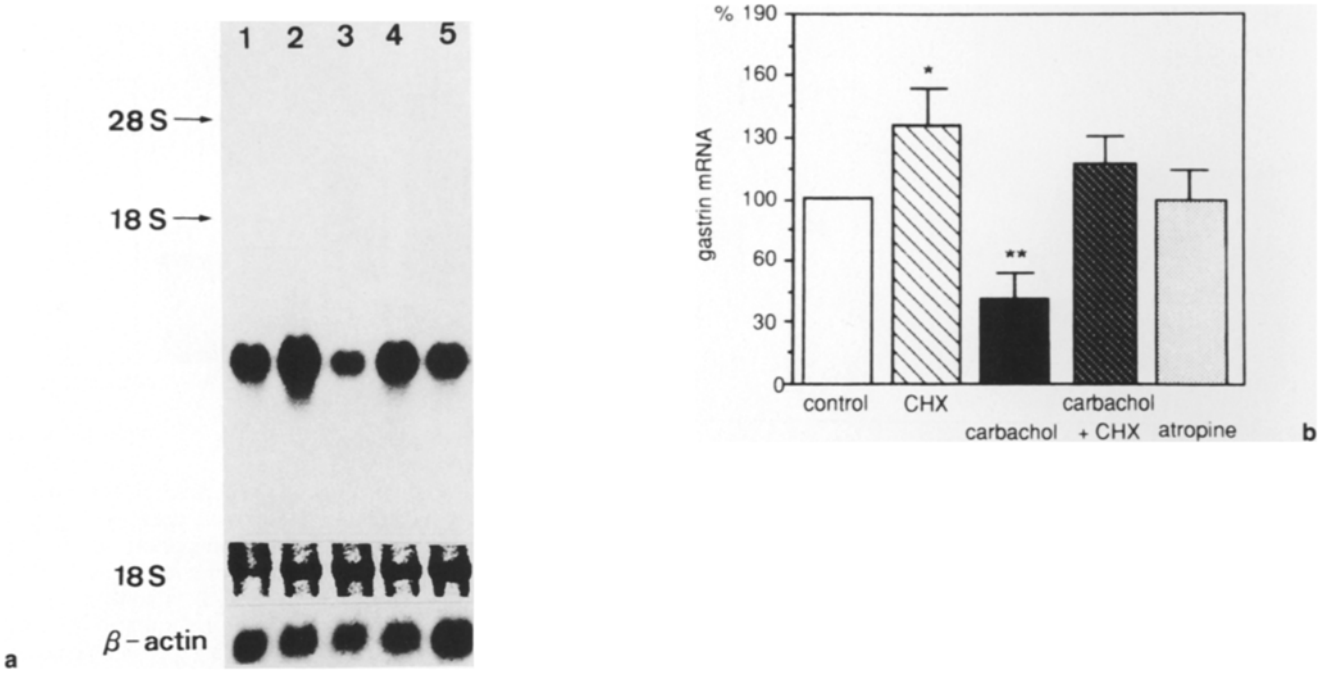

Fig. 3a,b. Northern blot (**a**) and dot blot (**b**) analyses for the effects of carbachol, cycloheximide, and atropine sulfate on gastrin mRNA during 2-h culture. **a** Northern blot analysis. Cycloheximide (10 μg/ml) increased gastrin mRNA levels (*lane 2*) compared with the control (*lane 1*) on Northern blots after 2-h culture. Carbachol (10^{-5} M) decreased these levels (*lane 3*). With carbachol and cycloheximide (10 μg/ml), gastrin mRNA levels did not change significantly compared with the control (*lane 4*). Atropine sulfate (10^{-5} M) inhibited the carbachol-induced changes (*lane 5*). **b** Dot blot analysis. Cycloheximide (*CHX*; 10 μg/ml) increased gastrin mRNA levels (134 ± 9%) ($P < 0.05$). Carbachol (10^{-5} M) significantly ($P < 0.01$) decreased gastrin mRNA levels (40 ± 8%). With carbachol and cycloheximide, gastrin mRNA levels did not change significantly, being 108 ± 12% (P: NS) compared with the control. Atropine sulfate (10^{-5} M) inhibited the carbachol-induced changes. The results are expressed as means ± SEM ($n = 15$). The effects of carbachol, atropine sulfate, and cycloheximide on gastrin mRNA levels were analyzed by two-way ANOVA. A two-tailed P value of <0.05 was considered to indicate statistical significance. $^*P < 0.05$; $^{**}P < 0.01$

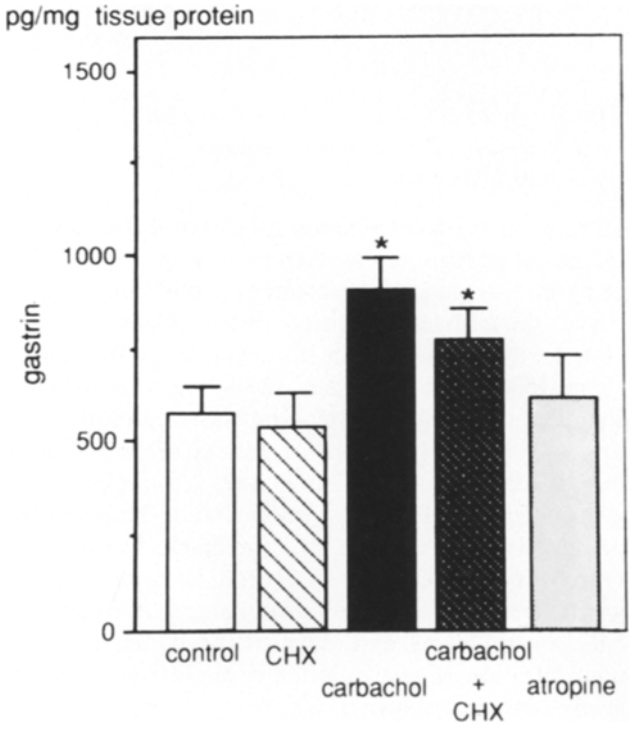

Fig. 4. Effects of carbachol, cycloheximide, and atropine on the release of gastrin. During the 2-h incubation, carbachol (10^{-5} M) increased gastrin release (from 608 ± 48 to 918 ± 68 pg/ml per mg tissue protein) from human antral tissue; atropine (10^{-5} M) completely inhibited he carbachol-induced gastrin release. Cycloheximide (10 μg/ml) had no significant effects on gastrin release. The results are expressed as means ± SEM ($n = 15$). The effects of carbachol, atropine sulfate, and cycloheximide on gastrin mRNA levels were analyzed by two-way ANOVA. A two-tailed P value of <0.05 was considered to indicate statistical significance. $^*P < 0.05$

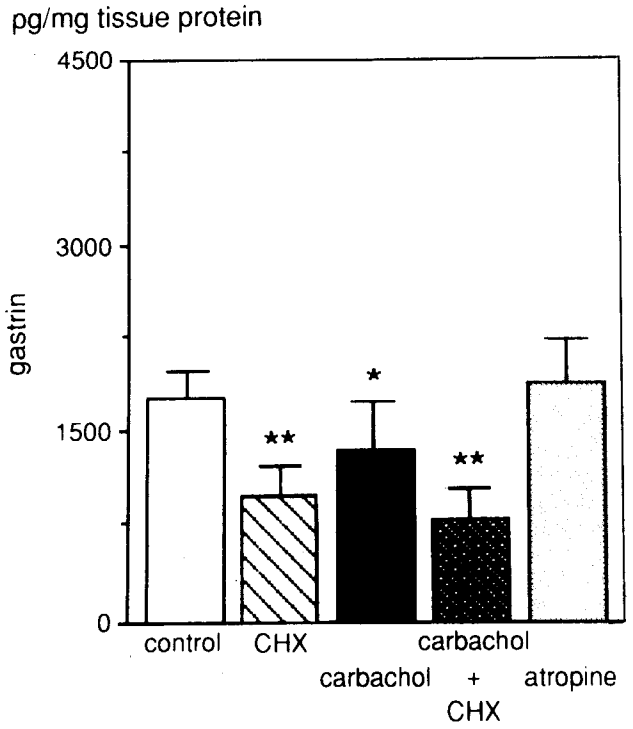

Fig. 5. Effects of carbachol, cycloheximide, and atropine on intracellular gastrin content. During the 2-h incubation, carbachol (10^{-5} M) decreased the intracellular gastrin content (from 1634 ± 168 to 1272 ± 126 pg/mg tissue protein). Atropine (10^{-5} M) completely inhibited the carbachol-induced changes. Cycloheximide (10 μg/ml) had no effect on gastrin release, but markedly decreased tissue gastrin content (from 1634 ± 168 to 863 ± 226 pg/mg tissue protein). With carbachol and cycloheximide, tissue gastrin content decreased from 1634 ± 168 to 763 ± 234 pg/mg tissue protein. The results are expressed as means ± SEM (n = 15). The effects of carbachol, atropine sulfate, and cycloheximide on gastrin levels were analyzed by two-way ANOVA. A two-tailed P value of <0.05 was considered to indicate statistical significance. $^{*}P < 0.05$; $^{**}P < 0.01$

Discussion

Gastrin exerts a wide range of activities on the mucosa and smooth muscle of the gastrointestinal tract. The most important actions are stimulation of gastric acid secretion and regulation of growth of the oxyntic mucosa of the stomach.[7] The regulation of gastrin release has been studied extensively, but there are few reports of gastrin biosynthesis in the human antrum under differing physiological or pharmacological conditions. In the study of gastrin biosynthesis, investigators have examined the incorporation of radiolabelled amino acids[8,9] and the post-translational processing of progastrin,[10] and have measured gastrin levels, using anti-gastrin antibody.[11] Recently, mammalian gastrin cDNA and genes have been isolated from various species and characterized.[4,5,12–15] Gastrin gene expression has been induced by neutralizing gastric pH,[16,17] by extracellular calcium and membrane depolarization in rat insulinoma cells,[16] and by direct stimulation with dietary proteins and amino acids.[18] The expression was inhibited by somatostatin[19–21] and by starvation.[18] However, the effects of various stimulators on gastrin release seem to be different from their effects on its biosynthesis. Luminal nutrients, such as peptone, phenylalanine, and tryptophan do concurrently stimulate both gastrin release and gene expression.[18] Although gastrin secretion in vivo is stimulated within 2 h of an omeprazole injection, the increase in gastrin mRNA is seen only after 24 h of achlorhydria.[21] Brand and Stone[21] emphasized that changes in gastric pH modulated somatostatin secretion and synthesis to mediate paracrine inhibition of gastrin gene expression in adjacent G cells.

The influence of the vagus nerve on gastrin release in vivo is complex; in experiments using somatostatin and bombesin,[7] the vagus nerve has been shown to have both stimulatory and inhibitory effects on gastrin release through paracrine pathways. Cholinergic agonists are strong stimulants of gastrin release in the rat and dog, presumably through binding to specific receptors on gastrin G cells.[22–24] Antral mucosal tissue culture, which permits the direct assessment of gastrin cell function, has been used to demonstrate the effects of the cholinergic agent, carbachol, on gastrin synthesis and secretion.[8,9] In the rat, carbachol stimulated both gastrin secretion and synthesis in a dose-dependent manner, maximal stimulation occurring at a concentration of 1×10^{-5} M carbachol.[9] Abello et al.[11] recently reported that carbachol reduced intracellular gastrin content in rat pancreatic gastrin-producing cells during 2-h culture, although the agent increased gastrin release in the cells. In this present study, during 2-h incubation, carbachol at (10^{-5} M) increased gastrin release from human antral tissue, and this carbachol-induced gastrin release seeemed to be associated with decreased intracellular gastrin. Atropine had no effect on basal gastrin release and inhibited the carbachol-induced gastrin release. It thus appears that carbachol may have a time-related biphasic action on gastrin gene expression, although the reason is not clear from the findings in the present study. Karnik and Wolfe[20] reported that somatostatin stimulated gastrin mRNA turnover and that carbachol stimulated gastrin gene transcription in dog antral mucosa. A possible explanation for these findings that carbachol may enhance gastrin mRNA degradation at 2 h, masking its stimulatory effect on gastrin gene transcription. A similar delay in the rise of proopiomelanocortin (POMC) mRNA levels compared with the stimulation of adrenocorticotrophic hormone secretion is also seen in

pituitary corticotrophs when they are released from glucocorticoid inhibition by adrenalectomy.[25] The slow accumulation of mRNA occurs because the gene transcription rate is low compared with the stability of the cytophlasmic pool of POMC mRNA.[26] We found here that, when the antral mucosa was incubated under basal and cabachol-induced conditions in the presence of cycloheximide, gastrin mRNA levels were significantly increased, despite the decreased tissue gastrin content. This finding suggested that cycloheximide stabilized gastrin mRNA by preserving the polysome structure or by inhibiting the synthesis of selective gastrin RNAse.[20] Carbachol induces diacylglycerol and cytosolic calcium as second messengers. In the present study, the role of these second messengers was not determined. Future studies, using homogeneous populations of cells, rather than intact antral mucosa, and nuclear run-on assays, will be needed.

In conclusion, this study indicated that carbachol may have a time-related biphasic action on human antral gastrin mRNA levels.

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