Growth-Promoting Effects of Glycine-Extended Progastrin

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Peptide α amidation is required to produce some hormones, such as gastrin, from their glycine-extended precursors. This terminal posttranslational processing reaction is thought to be essential for the biological activation of many peptide hormones; only amidated gastrin exerts a physiological effect that results in gastric acid secretion. However, both amidated gastrin and glycine-extended gastrin stimulate proliferation of exocrine pancreatic cell line AR4-2J through selective receptors for the substrate and the product, respectively, of peptide α amidation. Thus, the amidation reaction may function as a determinant of the specific biological actions of products derived from prohormones.

Although characterized as a stimulant of gastric acid secretion (*1*), the peptide hormone gastrin also exerts growth-promoting effects on normal and malignant gastrointestinal tissues (*2–5*). As with many peptide hormones, gastrin is synthesized as a precursor molecule that undergoes posttranslational processing to a product amidated on the COOH-terminus, which is presumed to be the sole biologically active form (*6–9*). Posttranslational processing intermediates of gastrin, specifically glycine-extended gastrins (G-Gly), which serve as the substrates for the amidation reaction, are at least four orders of magnitude less potent than gastrin in stimulating gastric acid secretion (*10*). However, G-Gly is stored in brain (*11*) and gut tissues (*12–14*), secreted with amidated gastrin (*15–18*) into the circulation, and achieves concentrations in plasma like those of gastrin. Malignant tissues that express gastrin, such as Zollinger-Ellison tumors and colon cancers (*15, 19, 20*), contain greater concentrations of G-Gly than amidated gastrin. These observations prompted us to examine whether G-Gly functions as a growth factor in a different fashion than its relatively weak effects on the standard receptor, which recognizes gastrin amidated on the COOH-terminus.

Amidated gastrin stimulates proliferation of a tumor-derived pancreatic acinar cell line (AR4-2J) through the classical gastrin-cholecystokinin B (G-CCK_B) receptor (*21*). We compared gastrin heptadecapeptide (G17) and its glycine-extended processing intermediate, G17-Gly, for their abilities to stimulate DNA synthesis in the AR4-2J cell line. Both G17 and synthetic rat G17-Gly stimulated [^{3}H]thymidine incorporation in a dose-dependent fashion (Fig. 1), and maximal stimulation was achieved by G17 (142 ± 5% of an unstimulated control; mean ± SE, n = 8) and G17-Gly (140 ± 6%) at a concentration (0.1 nM) similar to that in plasma. A shorter form of glycine-extended gastrin, G5-17–Gly, also stimulated [^{3}H]thymidine incorporation with an efficacy similar to that of G17 (133 ± 2%), although its potency was decreased.

Growth of AR4-2J cells is dependent on cell polyamine content (*22*); amidated gastrin stimulates an increase in the activity of ornithine decarboxylase (ODC) (*23*), the rate-limiting enzyme in polyamine biosynthesis (*24*). G17 and G17-Gly stimulated ODC activity in AR4-2J cells (Fig. 2). The half-maximal effect (EC_{50}) was achieved at a concentration of approximately 0.2 nM for both peptides, and maximal effects were obtained at 1 nM. Difluoromethylornithine (DFMO; 2 mM), an irreversible inhibitor of ODC, completely abolished the stimulatory effects of both G17 and G17-Gly.

The stimulation of AR4-2J cell proliferation by amidated gastrin is mediated through G-CCK_B receptors (*21*). Therefore, we examined the effects of two different selective G-CCK_B receptor antagonists (L365,260 and PD-134308) on proliferation stimulated by G17 and G17-Gly. The antagonists by themselves had no effect on the basal growth of the AR4-2J cells (*25*). L365,260 and PD-134308, used at concentrations that result in full occupation of G-CCK_B receptors (100 nM), completely inhibited the increase in [^{3}H]thymidine incorporation induced by G17 (Fig. 3A). By contrast, neither antagonist decreased the [^{3}H]thymidine uptake stimulated by G17-Gly (Fig. 3B).

These data suggested that G-Gly might

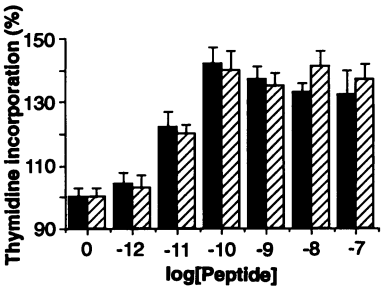

Fig. 1. Effect of G17 (black bars) and G17-Gly (hatched bars) on [^{3}H]thymidine incorporation. AR4-2J cells (*31*) grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) were plated, allowed to attach overnight, and then cultured for 24 hours in serum-free medium containing 1 mM unlabeled thymidine. After we washed the cells with serum-free medium, we treated them with increasing concentrations of G17 or G17-Gly. DNA synthesis was estimated by measurement of [^{3}H]thymidine incorporation into the trichloroacetic acid (TCA)–precipitable material. The [^{3}H]thymidine (0.1 μCi/ml; 18 Ci/mmol) was added during the last hour of the 24-hour treatment period. We then washed the cells with serum-free medium to remove unincorporated [^{3}H]thymidine. DNA was precipitated with 5% TCA at 4°C for 15 min. Precipitates were washed twice with 95% ethanol, dissolved in 1 ml of 0.1 N NaOH, and analyzed in a liquid scintillation counter. Results are expressed as percent of control, unstimulated [^{3}H]thymidine incorporation (means ± SE of six different experiments, each performed in triplicate). Unstimulated control cells incorporated 2596 ± 84 cpm of [^{3}H]thymidine.

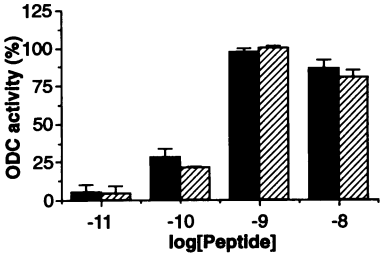

Fig. 2. Effects of G17 (black bars) and G17-Gly (hatched bars) on ODC activity. AR4-2J cells were plated in DMEM supplemented with 10% FBS at 10^5 cells per milliliter, allowed to attach overnight, and then deprived of serum for 18 hours before stimulation. Cells were then incubated with G17 or G17-Gly. ODC activity was measured in cytosolic extracts from AR4-2J cells as described (*23*). Briefly, cells were lysed by freeze-thawing them with liquid N_2 and then centrifuged at 50,000*g* for 25 min. Supernatants were incubated in the presence of pyridoral 5-phosphate (50 μM), L-[^{14}C]ornithine (0.25 μCi), and L-ornithine (0.2 mM). Protein content was measured by the method of Bradford (*32*) with the use of bovine serum albumin as standard. Released [^{14}C]CO_2 was quantitated, and ODC activity was recorded as nanomoles of CO_2 produced per hour per milligram of protein. Results represent the means ± SE of three different experiments, each performed in duplicate, and are expressed as the percent of maximum activity. The basal value of ODC activity was 3.5 ± 0.2 nanomoles of CO_2 produced per hour per milligram of protein, and maximal values after stimulation with G17-Gly and G17 were 5.1 ± 0.1 and 5.2 ± 0.3, respectively.

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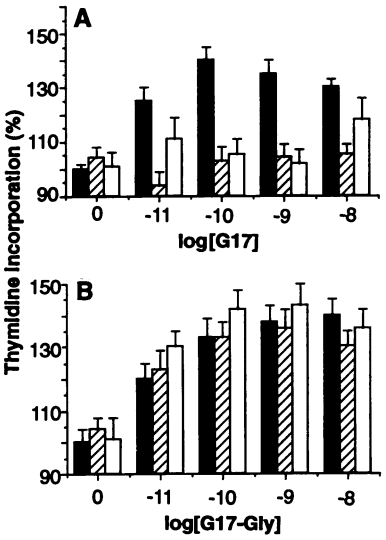

Fig. 3. Effects of the G-CCK$_B$ receptor antagonists L365,260 and PD-134308 on incorporation of [^{3}H]thymidine stimulated by (**A**) G17 or (**B**) G17-Gly in AR4-2J cells. Cells were cultured as described (Fig. 1) and treated for 24 hours with either G17 or G17-Gly alone (black bars), in the presence of L365,260 (100 nM) (hatched bars), or in the presence of PD-134308 (100 nM) (white bars). Results are expressed as percent of control, unstimulated [^{3}H]thymidine incorporation (means ± SE of six different experiments, each performed in triplicate). Unstimulated control cells incorporated 2708 ± 90 cpm and 2760 ± 150 cpm of [^{3}H]thymidine in (A) and (B), respectively.

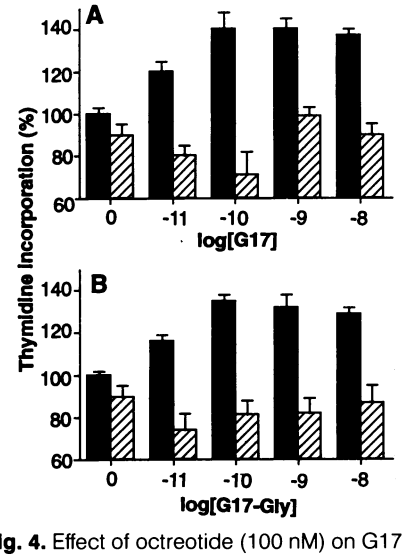

Fig. 4. Effect of octreotide (100 nM) on G17 or G17-Gly–stimulated [^{3}H]thymidine incorporation in AR4-2J cells. Cells were cultured as described (Fig. 1), treated for 24 hours with (**A**) G17 or (**B**) G17-Gly alone (black bars) or in the presence of 100 nM octreotide (hatched bars). Results are expressed as percent of control, unstimulated [^{3}H]thymidine incorporation (means ± SE of six different experiments, each performed in triplicate). Unstimulated control cells incorporated 3052 ± 110 cpm and 3284 ± 86 cpm of [^{3}H]thymidine in (A) and (B), respectively.

act through a mechanism independent of the G-CCK$_B$ receptors. Somatostatin and its long-acting analogs, such as octreotide (SMS 201–995), inhibit the proliferative action of various growth factors on different cultured cell lines (*26, 27*), including AR4-2J cells (*28*). Octreotide (100 nM) completely inhibited the increase in [^{3}H]thymidine incorporation stimulated by G17 and G17-Gly (Fig. 4). Octreotide reduced the maximal stimulation induced by G17 and G17-Gly (0.1 nM) from 140 ± 8% to 71 ± 11% and from 135 ± 3% to 81 ± 7%, respectively.

If G-Gly has a specific effect on AR4-2J proliferation that is independent of G-CCK$_B$ receptors, it should be possible to demonstrate the presence of G-Gly–selective receptors. The presence of specific G-Gly binding sites on AR4-2J cells was investigated with ^{125}I-labeled human Leu^{15}–G2-17–Gly (*29*). Leu^{15}–G2-17–Gly that was specifically bound increased in a dose-dependent manner (Fig. 5A). The equilibrium dissociation constant (K_d) was 0.45 ± 0.07 nM (n = 7), as determined by Scatchard analysis with the use of the LIGAND program of Munson and Rodbard (*30*), with a binding capacity (B_{max}) of 4.0 ± 2.0 fmol per 10^6 cells (n = 7). By comparison, other K_d and B_{max} values obtained for gastrin binding to AR4-2J cells were 1.1 ± 0.3 nM and 86.9 ± 20.1 fmol per 10^6 cells, respectively (*23*). Competitive binding studies (Fig. 5B) showed that unlabeled Leu^{15}–G2-17–Gly displaced ^{125}I-labeled Leu^{15}–G2-17–Gly bound to AR4-2J cells. However, G17, cholecystokinin octapeptide (CCK), and G-CCK$_B$ receptor antagonists L365,260 and PD-134308 had no effect. The binding of ^{125}I-labeled Leu^{15}–G2-17–Gly to AR4-2J cell mem-

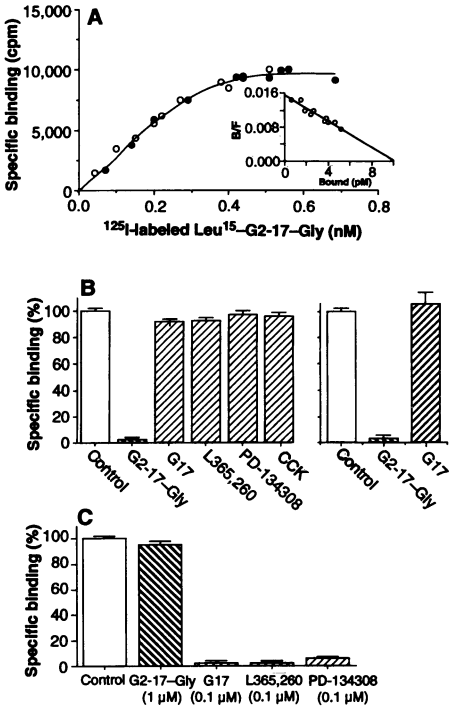

Fig. 5. Binding of ^{125}I-labeled Leu^{15}–G2-17–Gly or ^{125}I-labeled Leu^{15}–G17 to AR4-2J cells and membranes. (**A**) Human Leu^{15}–G2-17–Gly was iodinated with ^{125}I-labeled Na on Tyr^{12} with an adaptation of the chloramine T method and purified by high-pressure liquid chromatography as described (*33*). The specific activity of the label was ~1.5 μCi/pmol. Binding assays were prepared on isolated cells that were detached in phosphate-buffered saline (PBS) containing 0.02% EDTA. Cells (2 × 10^6) were incubated with the indicated concentrations of ^{125}I-labeled Leu^{15}–G2-17–Gly with or without unlabeled Leu^{15}–G2-17–Gly (1 μM) in a Krebs'-*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer (Irvine Scientific, Irvine, California) supplemented with 0.5% bovine serum albumin, 0.03% soybean trypsin inhibitor, and 0:05% bacitracin in a total volume of 1 ml at 37°C until equilibrium. Specific binding of ^{125}I-labeled Leu^{15}–G2-17–Gly was calculated per 2 × 10^6 cells as the difference between the total amount of label bound and the amount of label remaining bound in the presence of G17-Gly (1 μM). Nonspecific binding amounted to roughly 20% of the total amount of label binding in the absence of any added G17-Gly. Two saturation binding studies (open and closed circles) are shown; the results are representative of similar data obtained in five additional experiments. (Inset) A Scatchard analysis for one of the binding studies. B, bound; F, free. (**B**) Cells (2 × 10^6; left panel) were incubated with ^{125}I-labeled Leu^{15}–G2-17–Gly without (control) or with 1 μM of unlabeled Leu^{15}–G2-17–Gly; G17; L365,260; PD-134308; or CCK octapeptide, as in (A) at 37°C until equilibrium. For preparation of membranes (right panel), cells were detached and centrifuged at 600*g* for 5 min at 4°C in PBS. The pellet was resuspended in 20 mM tris (pH 7.4) containing 5 mM $MgCl_2$, 1 mM EDTA, 0.3 mM EGTA, 0.01% soybean trypsin inhibitor, and 0.05% bacitracin. The suspension was sonicated and then centrifuged again for 5 min at 600*g*. The supernatant was recentrifuged at 10,000*g* for 30 min at 4°C. For binding assays, membranes (200 μg) were incubated with ^{125}I-labeled Leu^{15}–G2-17–Gly for 2 hours at 37°C in 300 μl of the same buffer with or without 1 μM G2-17–Gly or G17. Each point represents the mean of three independent experiments, each performed in duplicate. (**C**) Binding studies with iodinated human Leu^{15}-G17 (specific activity of ~1.5 μCi/pmol) were performed on isolated cells that were detached in PBS containing 0.02% EDTA. Cells (2.5 × 10^5) were incubated with 120 pM ^{125}I-labeled Leu^{15}-G17 without (control) or with the indicated concentrations of G17; G2-17–Gly; L365,260; or PD-134308 in a total volume of 0.5 ml at 25°C until equilibrium. Specific binding of ^{125}I-labeled Leu^{15}-G17 was calculated as the difference between the total amount of label bound and the amount of label remaining bound in the presence of 1 μM G17. Each point represents the mean of three independent experiments, each performed in duplicate.

branes was also displaced by unlabeled Leu^{15}–G2-17–Gly, but not by G17 (Fig. 5B). Unlabeled G17 and both G-CCK_B receptor antagonists completely inhibited the binding of ^{125}I-labeled Leu^{15}-G17 to AR4-2J cells, but G2-17–Gly, in concentrations as high as 1 μM, had no effect on ^{125}I-labeled Leu^{15}-G17 binding (Fig. 5C).

Our results indicate that amidated gastrin and its glycine-extended posttranslational processing intermediates induce AR4-2J cell proliferation. In contrast to the difference in the potencies of amidated gastrin and G-Gly in stimulating gastric acid secretion (*11*), the two peptides appear to be equally potent in inducing cell proliferation. Moreover, selective inhibition by L365,260 and PD-134308 of the effect induced by amidated G17, but not by G17-Gly, implies that there are two different receptors that mediate the proliferative actions of the peptides. In view of the observations that both plasma and tissue concentrations of G-Gly are higher than those of amidated gastrin, growth-related receptors for G-Gly may mediate physiological or pathophysiological effects. Our data indicate that the precursor and the product of peptide α amidation may have different biological actions mediated through separate receptors.

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Effect of the Nigrostriatal Dopamine System on Acquired Neural Responses in the Striatum of Behaving Monkeys

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Dysfunction of the nigrostriatal dopamine system results in marked disorders of movement such as occur in Parkinson's disease. Functions of this dopamine-containing projection system were examined in monkeys trained in a classical conditioning task, and the effects of striatal dopamine depletion were tested. Unilateral dopamine loss substantially reduced the acquired sensory responsiveness of striatal neurons monitored electrophysiologically. This effect was ipsilateral and selective, and could be reversed by apomorphine. These results suggest that the primate nigrostriatal system modulates expression of neuronal response plasticity in the striatum during sensorimotor learning.

Understanding the neural mechanisms underlying sensorimotor learning is a cardinal goal of neurobiology. To approach this problem, we investigated neurons of the basal ganglia, central structures in the motor system. We recorded from a clearly identifiable class of neurons in the striatum (the tonically active neurons, or TANs) while monkeys underwent training in a Pavlovian conditioning task. We found that the TANs acquire responsiveness to the sensory conditioning stimuli during behavioral learning (*1*). This systematic learning-dependent plasticity of TANs opened the possibility of determining whether dopamine, a major catecholamine neurotransmitter in the striatum, affects such behaviorally contingent neural plasticity. We therefore recorded from TANs before, during, and after monkeys were trained in behavioral conditioning, and tested the effects of manipulating their dopaminergic inputs (*2*).

Before conditioning, we confirmed that only a small fraction of TANs responded to the clicks used as conditioning stimuli (51 of 305 cells, or 16.7%) (*3*). The TANs were readily identified by their characteristic 2- to 8-Hz spontaneous discharge rates, action potential waveforms, and sparse distribution at 0.5- to 1.0-mm intervals. During training, many TANs became responsive to the conditioned stimuli (Fig. 1). In all, 71.4% of cells (95 of 133) recorded in the caudate nucleus and 52.0% of cells (91 of 175) recorded in the putamen responded to the conditioned stimuli after behavioral conditioning. The responses consisted of a brief pause in tonic firing (Fig. 1D), which began about 60 ms after the conditioned stimulus, lasted about 300 ms, and was often flanked by initial and rebound excitation periods (*2*).

After the conditioned behavior was acquired, we infused 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a dopaminergic neurotoxin, into the caudate-putamen complex of one hemisphere in each monkey (*4*). Unilateral dopamine deficits were evident in the home-cage behavior of the monkeys and were confirmed histologically by immunostaining for tyrosine hydroxylase (TH) after completion of the experiments (*5*). Histology showed dose-related partial (monkey R) to massive (monkey D) loss of TH-like immunoreactivity in the caudate nucleus and putamen, with near total loss near the injection site and graded depletion beyond (Fig. 2A). Postinfusion recordings were made within 5 mm of the injection sites, in the regions of maximum depletion. TH immunostaining was also regionally reduced in the substan-

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