Carboxyl-terminal Modification of a Gastrin Releasing Peptide Derivative Generates Potent Antagonists*

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Gastrin releasing peptide (GRP) is a 27-residue peptide hormone which is analogous to the amphibian peptide bombesin. GRP serves a variety of physiological functions and has been implicated as an autocrine factor in the growth regulation of small cell lung cancer cells. We have developed a series of potent GRP antagonists by modification of the COOH terminus of N-acetyl-GRP-20-27. The most potent member of this series, N-acetyl-GRP-20-26-OCH2CH3, exhibits an IC50 of 4 nM in a competitive binding inhibition assay. This compound blocks GRP-stimulated mitogenesis in Swiss 3T3 mouse fibroblasts, inhibits GRP-dependent release of gastrin in vitro, and blocks GRP-induced elevation of [Ca2+]i in H345 small cell lung cancer cells. These results demonstrate that while residues 20-27 of GRP influence binding of the parent peptide to its receptor, the COOH-terminal amino acid is primarily responsible for triggering the subsequent biological response.

Gastrin releasing peptide (GRP) is a peptide hormone containing 27 amino acids which is structurally analogous to the amphibian peptide bombesin (1). GRP stimulates a variety of biological responses, including the release of gastrin and bombesin (1). GRP serves a variety of physiological functions and has been implicated as an autocrine factor in the growth regulation of small cell lung cancer cells. We have developed a series of potent GRP antagonists by modification of the COOH terminus of N-acetyl-GRP-20-27. The most potent member of this series, N-acetyl-GRP-20-26-OCH2CH3, exhibits an IC50 of 4 nM in a competitive binding inhibition assay. This compound blocks GRP-stimulated mitogenesis in Swiss 3T3 mouse fibroblasts, inhibits GRP-dependent release of gastrin in vitro, and blocks GRP-induced elevation of [Ca2+]i in H345 small cell lung cancer cells. These results demonstrate that while residues 20-27 of GRP influence binding of the parent peptide to its receptor, the COOH-terminal amino acid is primarily responsible for triggering the subsequent biological response.

MATERIALS AND METHODS

Preparation of Ligands—Peptide ligands were synthesized by standard solid-phase methodology on benzhydrylamine or amino acyl phenylacetic acid methyl resins using commercially available Boc amino acids containing the normal side chain protecting groups. Cleavage and deprotection of the resin-bound peptide was achieved by treatment with liquid HF containing anisole as a scavenger. Purification of the crude material was achieved by reverse-phase HPLC on a Vydac C18 support. The correct ratios for the given sequence, and were consistent with the predicted sequence. N-Acetyl-GRP-20-26-NHEt was synthesized from the corresponding His-ethyl amide utilizing coupling procedures analogous to those described above.

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† The abbreviations used are: GRP, gastrin releasing peptide; Et, ethyl; IC50, concentration of ligand required to reduce the measured response to 50% of the maximal response; HEPSS, 2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; HPLC, high performance liquid chromatography; SCLC, small cell lung cancer.
filter apparatus (Skatron, Inc., Sterling, VA). A 50-fold stimulation of [3H]thymidine uptake was typically observed upon treatment of these cells with 3 nM GRP or 3 nM N-acetyl-GRP-20–27. Mitogenesis inhibition studies were conducted by co-administration of the antagonist and 3 nM N-acetyl-GRP-20–27.

Measurement of [Ca2+]i, flux in SCLC cells was performed essentially as described (23, 24). H345 SCLC cells were obtained from E. Sausville (National Cancer Institute, Naval Oncology Research Branch, Bethesda, MD). Approximately 10^6 cells, maintained in suspension culture as described (23), were harvested by gravity, washed once with RPMI 1640, and resuspended in 2 ml RPMI 1640. Fura-2/acetoxymethyl ester (Molecular Probes) was added at a final concentration of 1.2 nmol/10^6 cells from a fresh 10 mM stock in dimethyl sulfoxide. After 15 min at 37 °C, the cells were diluted to 10 ml and incubated at 37 °C for 1 h. The cells were then centrifuged and resuspended in HEPES/saline (140 mM NaCl, 5 mM KCl, 5 mM glucose, 1 mM CaCl2, 20 mM HEPES, pH 7.4) at a density of 5 × 10^6 cells/ml. The cells were kept on ice until used. Fluorescence measurements were performed at 37 °C in an Aminco SPF-500 fluorimeter with a stirred cell attachment. The excitation wavelength was 340 nm and the emission wavelength was 510 nm.

The effect of GRP antagonists on gastrin release in vivo was measured in rats. Female Sprague-Dawley rats (=150 g) were anesthetized by intramuscular injection of ketamine. One hundred microliters of sample was then injected intraperitoneally. After 5 min, blood samples were taken by cardiac puncture and immediately stored on ice. Gastrin concentration in serum was measured in duplicate by radioimmunoassay (Cambridge Medical Diagnostics). Each data point represents the mean value obtained from four separate rats. In a time course experiment, the maximal gastrin response to injection of GRP was observed 5 min after injection of the sample (data not shown).

**RESULTS**

The smallest GRP derivative to maintain full biological activity and potency compared to the parent human GRP is N-acetyl-GRP-20–27. Deletion of the COOH-terminal methionine from this derivative yielded a weak GRP antagonist. This peptide, N-acetyl-GRP-20–26-amide, displayed an IC50 of 1.6 nM in competitive binding inhibition studies, yet did not initiate a mitogenic response in mouse 3T3 fibroblasts at concentrations as high as 100 μM (Table I). This peptide derivative blocked GRP-dependent mitogenic stimulation in Swiss 3T3 mouse fibroblasts and is the smallest GRP antagonist reported to date (25). Substitution of a carboxylate for the COOH-terminal carboxamide or deletion of the NH2-terminal acetyl group or Leu26 from this antagonist generated derivatives with substantially reduced affinity for the GRP receptor in 3T3 cells (data not shown). Substitution of other amino acids for the methionine at position 27 of N-acetyl-GRP-20–27 generated peptides which were agonists (Table I).

These initial studies demonstrated that deletion of Met27 from N-acetyl-GRP-20–27 generates an antagonist with substantially reduced affinity for the GRP receptor compared to the parent molecule. It was anticipated that addition of alkyl substituents to the COOH terminus of this antagonist might restore receptor binding potency without regenerating mitogenic activity. A series of COOH-terminal esters of N-acetyl-GRP-20–26 were investigated first, due to their ease of synthesis. All of the esters synthesized proved to be mitogenic antagonists, with the ethyl and propyl derivatives both having IC50 values of approximately 4 nM in the binding inhibition assay (Table I). This value represents an approximately 400-fold improvement over N-acetyl-GRP-20–26-amide. Analysis of a series of binding inhibition curves at varying concentrations of the tritiated radioligand by the method of Schild demonstrated that the ethyl ester is a competitive GRP antagonist with pA2 = 8.5 (data not shown) (26). Values for inhibition of GRP-dependent stimulation of mitogenesis in Swiss 3T3 mouse fibroblasts were consistent with the binding inhibition values (Fig. 1). It is apparent from the data in Table I that longer aliphatic substituents on the ester linkage result in a significant loss of potency. The ethyl amide derivative of N-acetyl-GRP-20–26 was prepared for comparison with the corresponding ethyl ester. Despite the fact that the carboxyl terminus of this derivative more closely mimics the peptide bond between Leu26-Met27 of the native molecule, the ethyl amide exhibited lower potency than the corresponding ethyl ester (Table I).

**Table I**

**Potency of GRP derivatives in 3T3 mouse fibroblasts**

The compounds listed were tested in competitive binding inhibition, mitogenic stimulation, and mitogenic inhibition assays in Swiss 3T3 mouse fibroblasts as described under “Materials and Methods.” Numerical values represent the concentration of the compound which reduced binding of radioligand to 50% of the value observed in vehicle-treated controls. A plus (+) symbol represents a positive response; a minus (−) symbol indicates the peptide had no effect. Compounds which did not stimulate mitogenesis at their IC50 also had no effect at concentrations up to 30 μM.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Binding inhibition IC50</th>
<th>Mitogenic at IC50</th>
<th>Mitogenic inhibition IC50</th>
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</thead>
<tbody>
<tr>
<td>Acetyl-His-Trp-Ala-Val-Gly-His-Leu-Met-NH₂</td>
<td>1.5 ND*</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>-NH₂</td>
<td>1570 ND*</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>-Gly-NH₂</td>
<td>607 ND*</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>-Ala-NH₂</td>
<td>82 ND*</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>-Phe-NH₂</td>
<td>540 ND*</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>-O-CH₃</td>
<td>7.7 ND*</td>
<td>-</td>
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<td>-O-CH₂CH₃</td>
<td>3.9 ND*</td>
<td>-</td>
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<tr>
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<td>3.5 ND*</td>
<td>-</td>
<td></td>
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<tr>
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<td>6.8 ND*</td>
<td>-</td>
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<tr>
<td>-N-CH₃CH₂</td>
<td>53 ND*</td>
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</tbody>
</table>

*ND, not determined.
Generation of GRP Antagonists

**FIG. 1.** Comparison of mitogenic inhibition and binding inhibition by N-acetyl-GRP-20-26-OEt in 3T3 fibroblasts. Binding inhibition data (○) and mitogenic inhibition data (□) were obtained as described under "Materials and Methods." Mitogenic inhibition data were obtained using 3 nM N-acetyl-GRP-20-27 as the competing mitogen.

**FIG. 2.** Inhibition of GRP-dependent increase in [Ca\(^{2+}\)]\(_i\) in H345 SCLC cells by N-acetyl-GRP-20-26-OEt. Data were obtained as described under "Materials and Methods." Discontinuities in the data traces represent opening of the cuvette chamber for sample addition. Panel A, fluorescence response resulting from the administration (at arrow 1) of 100 nM GRP. A subsequent addition (at 2) yielded no response (23). Panel B, co-administration of 1 μM N-acetyl-GRP-20-26-OEt and 100 nM GRP resulted in no elevation of [Ca\(^{2+}\)]\(_i\). Subsequent addition of 100 nM GRP had no effect. Panel C, co-administration of 1 μM GRP-1-16 and 100 nM GRP resulted in a full [Ca\(^{2+}\)]\(_i\) response. As with GRP alone, a subsequent addition of 100 nM GRP had no effect.

As one of the most potent derivatives in the series, the ethyl ester, N-acetyl-GRP-20-26-OEt, was subjected to further study. The potential clinical utility of this derivative depends on its ability to block the effect of GRP in human SCLC cells and to function as a GRP antagonist in vivo. The effect of N-acetyl-GRP-20-26-OEt on human SCLC cells was determined by examining GRP-dependent effects on the concentration of intracellular calcium ([Ca\(^{2+}\)]\(_i\)). The addition of 100 nM human GRP to a suspension of H345 SCLC cells elicits a rapid transient increase in [Ca\(^{2+}\)]\(_i\), which can be monitored using the fluorescent dye fura-2 (23, 24) (Fig. 2A). N-acetyl-GRP-20-26-OEt does not elicit any calcium re-

**FIG. 3.** Dose-response of the inhibition of GRP-dependent [Ca\(^{2+}\)]\(_i\) response by N-acetyl-GRP-20-26-OEt. Data were obtained by co-administration of 100 nM GRP and varying concentrations of N-acetyl-GRP-20-26-OEt, as described in the legend to Fig. 2. Percent inhibition was calculated from the peak fluorescent response at each concentration of antagonist versus a control with GRP alone. Half-maximal inhibition of the response to 100 nM GRP occurs at approximately 400 nM N-acetyl-GRP-20-26-OEt.

**FIG. 4.** GRP-dependent gastrin release in rats. Data were obtained as described under "Materials and Methods." Each data point represents the average of four rats. A dose-dependent increase in serum gastrin concentrations is observed in response to injection of GRP (○), but not N-acetyl-GRP-20-26-OEt (□).

**FIG. 5.** Dose-response of the inhibition of GRP-dependent gastrin release in rats by N-acetyl-GRP-20-26-OEt. Co-administration of varying concentrations of N-acetyl-GRP-20-26-OEt with 7.2 nmol of GRP yields a significant (p < 0.05) inhibition of the GRP-dependent stimulation of gastrin release.
response in H345 cells at concentrations up to 5 μM. This derivative blocks the GRP-dependent increase in [Ca^{2+}], with an IC_{50} of approximately 400 nM (Fig. 2B, Fig. 3). GRP-1-16, which does not bind to the GRP receptor (16), does not elicit a calcium response and does not block the effect of co-administered GRP (Fig. 2C). These results demonstrate that N-acetyl-GRP-20-26-OEt is a GRP antagonist in H345 SCLC cells.

The in vivo efficacy of N-acetyl-GRP-20-26-OEt was tested by monitoring the GRP-dependent increase of serum gastrin concentration in rats (27, 28). Intraperitoneal injection of 7.2 nmol of human GRP caused a 2- to 3-fold elevation in serum gastrin within 5 min (Fig. 4). Injection of up to 195 nmol of N-acetyl-GRP-20-26-OEt elicited no significant gastrin response. Co-injection of N-acetyl-GRP-20-26-OEt with GRP significantly reduced the gastrin response, compared to injection of GRP alone (Fig. 5). These results indicate that N-acetyl-GRP-20-26-OEt is also an effective GRP antagonist in vivo.

DISCUSSION

We have demonstrated that the ethyl ester derivative of N-acetyl-GRP-20-26: (a) competitively blocks binding of a GRP radioligand and GRP-induced mitogenesis in Swiss 3T3 mouse fibroblasts; (b) inhibits GRP-induced calcium effects in H345 SCLC cells; and (c) inhibits GRP-dependent elevation of serum gastrin in rats. Delineation of the specific modifications responsible for conversion of a potent GRP agonist (N-acetyl-GRP-20-27) to a potent antagonist (N-acetyl-GRP-20-26-amide, N-acetyl-GRP-20-26-OR, or N-acetyl-GRP-20-26-NEt) is central to the understanding of how GRP interacts with its receptor. The similarity in the antagonist/agonist ratio required to inhibit 50% of the biological response to the two in vitro systems tested implies that the same structural features of GRP regulate high affinity binding and initiation of a biological response in the mouse 3T3 and human SCLC receptors. We have demonstrated that amino acid substitutions for the methionine at position 27 of N-acetyl-GRP-20-27 generate peptides that are agonists. Therefore the methionine side chain is not uniquely responsible for the initiation of a biological response by GRP (16). Recent studies by Coy et al. (14) have suggested that the COOH-terminal peptide bond of bombesin (corresponding to the Leu^{25}-Met^{27} bond of GRP) may play a role in establishing a β-turn structure which is important for the initiation of a biological response by this peptide. The effects of the chemical modifications outlined in the current study on the three-dimensional structure of the resultant peptides as they interact with the GRP receptor are unknown. Our data and that of Coy et al. (14) suggest, however, that no single amino acid residue or peptide bond is individually the sole determinant of whether a GRP derivative is an agonist or an antagonist. The unifying structural feature of the antagonists described in the current work is the lack of a carboxamide group corresponding to an amino acid at position 27 of GRP. While other structural features may also be important, it seems reasonable to propose that this amide functionality plays a significant role in triggering the biological response elicited by GRP.

It is apparent from the work described above that the COOH-terminal methionine of GRP plays an essential role in the initiation of a GRP-dependent biological response in Swiss 3T3 mouse fibroblasts, in gastrin-releasing cells, and in H345 SCLC cells. Fundamentally similar results have been reported for cholecystokinin and gastrin, where deletion of the COOH-terminal phenylalanine generates competitive cholecystokinin gastrin antagonists (29, 30). Addition of phenethylamine or phenylethyl moieties to the COOH terminus of the truncated gastrin derivative yields antagonists with improved potency, presumably by mimicking the side chain of the deleted phenylalanine. The authors hypothesize that the COOH-terminal carboxamide of gastrin is responsible for the initiation of a biological response to this peptide (31, 32). The analogies between the structural modifications which generate antagonists from gastrin, cholecystokinin, and GRP implies that this approach may represent a useful strategy for the preparation of antagonists to other COOH-terminal amidated peptide hormones.

Acknowledgments—We are grateful to V. M. Garasky for synthesis of N-acetyl-GRP-20-26-NHEt, D. Coy for providing a sample of [Leu^{31}-32]-bombesin, L. Wassel for determination of amino acid compositions, and M. Rosenblatt and R. Freidinger for helpful discussions.

REFERENCES