Glucagon-like peptide I stimulates insulin gene expression and increases cyclic AMP levels in a rat islet cell line

(Insulinothrop/cyclic AMP)


*Laboratory of Molecular Endocrinology, Massachusetts General Hospital and 1Howard Hughes Medical Institute, Harvard Medical School, Boston, MA 02114; and 2Departments of Biochemistry and Medicine, University of Massachusetts Medical School, Worcester, MA 01613

Communicated by Francis O. Schmitt, January 20, 1987 (received for review October 29, 1986)

ABSTRACT Insulin secretion is controlled by a complex set of factors. Although blood glucose levels serve as the major stimulus of insulin secretion in mammals, insulin release is also modulated by amino acids, catecholamines, glucagon, and other, intestinal hormones. The identification of factors that modulate insulin production has engendered much interest because of their potential importance in the altered dynamics of insulin secretion in response to glucostatic characteristics of maturity-onset diabetes mellitus. Decoding of the glucagon gene has uncovered two additional glucagon-like peptides encoded in proglucagon, the polypeptide precursor of glucagon. One of these peptides, glucagon-like peptide I, is processed from proglucagon in two forms, of 31 and 37 amino acids. We report that the smaller of the two glucagon-like peptides potently increases cAMP levels, insulin mRNA transcripts, and insulin release in cultured rat insulinoma cells. These results indicate that glucagon-like peptide I may be a physiologic modulator of insulin gene expression.

The endocrine secretions of the pancreatic islets in mammals are governed by a complex number of controlling factors that include blood-borne metabolites (for example, glucose, amino acids, and catecholamines) and local neural and paracrine influences (1). The major islet hormones (insulin, glucagon, somatostatin) interact among their specific cell types (B, A, and D cells, respectively) to modulate secretory responses mediated by the metabolites (1). Although insulin secretion is predominantly controlled by blood levels of glucose, glucagon and somatostatin stimulate and inhibit glucose-mediated insulin secretory responses, respectively. Certain peptides such as vasoactive intestinal peptide have insulin-releasing properties and are located in peptidergic neurons of the pancreas (2). Thus, insulin secretion may be influenced by hormonal mediators acting locally by paracrine mechanisms as well as by vagal, sympathetic, and peptidergic innervation. In addition to the proposed metabolic, endocrine, and neural regulation of insulin secretion, there is evidence to support the existence of insulinotropic factors termed "incretin" that originate from the intestine (3). This concept of an entero-insular axis originates from the observations that glucose taken orally is a much more potent stimulus of insulin secretion than is a comparable amount of glucose given intravenously (4).

Analysis of the cDNAs and genes encoding proprorglucagon, a biosynthetic precursor of glucagon, reveals the presence of two additional glucagon-like peptides, GLP-I and GLP-II, each flanked by pairs of basic amino acids characteristic of the sites that are cleaved during the posttranslational processing of prohormones (5-10). In addition, the GLP-I of 37 amino acids, GLP-I-(1-37), formed by cleavage at the two basic residues (see amino acid sequence below; standard one-letter symbolism is used, and colons represent sites of cleavage), contains a single arginine at position 6 that serves as a processing site to produce a 31 amino acid peptide, GLP-I-(7-37) (11, 12).

1
7
37

KR: HDEFER: HAEFTTSDVSSYLEGQAKEFIAWLKVGRG: RR

Several observations raise the possibility that one or more of the forms of GLP-I is a bioactive peptide. (i) The amino acid sequence of GLP-I is highly conserved among the anglerfish and mammalian proprorglucagons, and the GLP-I sequences identified by decoding of the rat, hamster, human, and bovine cDNAs are identical (5-10). (ii) GLP-I is encoded by a separate exon in the glucagon gene, suggesting a separate and distinct functional activity (13). (iii) Liberation of GLP-I by posttranslational cleavage from proprorglucagon occurs in intestine (11, 14, 15) and, to a lesser extent, in pancreas (11). Several lines of evidence obtained earlier suggest that GLP-I may regulate insulin secretion. The amino acid sequence of GLP-I is similar to the sequences of both glucagon and gastrointestinal inhibitory peptide, two known stimulators of insulin release (16, 17). The synthetic peptide analog GLP-I-(1-36)-NH2 stimulates adenylate cyclase activity in brain and pituitary membranes (18). Recently, this amided analog of GLP-I was shown to weakly stimulate insulin release from isolated rat pancreatic islets in the presence of glucose (17). The studies described in this report implicate a smaller form of glucagon-like peptide I, GLP-I-(7-37), as a mediator of insulin gene expression.

MATERIALS AND METHODS

Reagents. Tissue culture media and antibiotics were from Gibco. Chemical reagents were from Fisher or Sigma. Nylon membranes were from Schleicher & Schuell. Cyclic AMP antibody and tracer were from Meloy Laboratories (Springfield, VA). Synthetic oligonucleotide cDNAs were synthesized on an Applied Biosystems 380A synthesizer. The chemical synthesis of GLP-I-(1-37) and GLP-I-(7-37) has been described (11). GLP-I-(1-36)-NH2 was obtained from Peninsula Laboratories (Belmont, CA). RIN 1046-38 cells (18) were grown in Dulbecco's modified Eagle's medium (DMEM), containing 4.5 g of glucose per liter, supplemented with 10% fetal bovine serum, 100 units of penicillin per ml, and 100 μg of streptomycin per ml under an atmosphere of 5% CO2. GLP-I and glucagon were dissolved at 1 mM in water and 1.0 M acetic acid, respectively. Dilutions of peptide for

Abbreviations: GLP-I and GLP-II, glucagon-like peptides I and II.
incubation with insulinoma cells were made directly in culture medium.

**Assay of cAMP Formation.** For measurement of cAMP formation, 5 x 10^5 cells were seeded into individual wells of multiwell tissue culture plates, and cells were grown to 50% confluence. Incubations were carried out in DMEM (4.5 g of glucose per liter) supplemented with 0.1% bovine serum albumin. After incubation with test peptide for 10 min, the reactions were terminated by the addition of ice-cold 95% ethanol and the contents of the wells were scraped, frozen, and centrifuged to remove cell debris. A minimum of four wells were assayed for each experimental condition, and each experiment was repeated on at least three separate occasions. Aliquots (usually 1/50th of total well contents) of the wells were assayed for cAMP by radioimmunoassay (19). Statistical analyses of the results were performed using the unpaired two-tailed t test to compare the differences between experimental observations and controls.

**RNA Analyses.** Total cellular RNA was extracted by lysing the cells in guanidinium isothiocyanate, followed by centrifugation on a cesium chloride cushion (20). For blot analyses, RNA was denatured in glyoxal, size-separated by agarose gel electrophoresis, and transferred to nylon membranes. Following prehybridization in 1 M NaCl/1% sodium dodecyl sulfate (NaDodSO4)/10% dextran sulfate, blots were hybridized for 24 hr in the same solution with 32P-end-labeled synthetic cDNAs (5 x 10^6 cpm/ml) complementary to the rat insulin-I coding sequence. After three 20-min washes in 0.3 M NaCl/0.03 M sodium citrate, pH 7/1% NaDodSO4, the membranes were subjected to autoradiography with an enhancing screen for 4–6 hr at −70°C. Each lane was corrected for the total content of RNA by rehybridizing the blot with a radiolabeled actin cDNA and normalizing the hybrid images of insulin to those of actin. Autoradiographic band images were quantitated by scanning with an LKB laser densitometer.

**High-Pressure Liquid Chromatography.** HPLC was performed on a Waters chromatography system, on a column (0.75 cm x 7.5 cm) of ion-exchange Protein-Pak DEAE-52. The solvent system was 0.02 M Tris-HCl (pH 8.2) (solvent A) and 0.02 M Tris-HCl (pH 8.7)/0.5 M NaCl (solvent B); the peptides were eluted with a linear gradient of 0–70% solvent B over a period of 25 min.

**Radioimmunoassays.** GLP-I radioimmunoassays were carried out using antibody B-5 (11) at a dilution of 1:10,000. Radiodination of GLP-I, conditions of incubation, and separation of bound and free peptide have been described (11). The levels of insulin in the cell culture medium were measured by radioimmunoassay (21).

**RESULTS**

To investigate the insulinotropic properties of GLP-I, we synthesized GLP-I-(1-37) and GLP-I-(7-37). That both peptides are liberated from proglucagon by posttranslational processing in cells in which the glucagon gene is expressed was shown in recent studies by the chromatographic detection of both GLP-I-(1-37) and GLP-I-(7-37) in extracts of rat intestine and pancreas (11), a glucagon-producing cell line (22), and in two cell lines transfected with a glucagon fusion gene (12). We also tested a third peptide, GLP-I-(1-36)-NH2, that is available commercially but which we have not detected in either cell lines or tissue extracts (11, 12, 22). We examined the effects of these three glucagon-like peptides on cAMP formation, insulin mRNA levels, and insulin release in the RIN 1046-38 cell line derived from a rat islet insulinoma (21). Because glucagon and peptides related to glucagon are known to act on their target cells through cAMP-dependent pathways (19), we measured cAMP levels in RIN 1046-38 cells after stimulation with glucagon and the glucagon-like peptides (Table 1). At the relatively high concentration of 0.5 μM, all three of the GLP-1s and glucagon increased cAMP levels. Not shown in Table 1 are the results of the experiment with 0.1 M GLP-I-(1-36)-NH2 control, 41.3 ± 7.1 fmol of cAMP; peptide, 70.2 ± 2.8 fmol of cAMP (n = 4, P < 0.01). At 5 nM, GLP-I-(7-37) increased cAMP levels at least 4-fold and was still active at 50 pM. In contrast, the effects of glucagon, GLP-I-(1-37), and GLP-I-(1-36)-NH2 on the formation of cAMP were negligible at these concentrations.

The glucagon-like peptides increased the levels of insulin mRNA during 24-hr incubations (Fig. 1, Table 2). The increase in insulin mRNA levels was consistently greater in response to the shorter, 31-amino acid peptide: 3-fold higher than control values at 24 hr. These stimulatory effects on insulin mRNA levels and on the release of insulin were observed in the presence of high (25 mM) and not low (5.5 mM) concentrations of glucose. Evidence that the stimulatory actions of GLP-I are relatively specific for insulin mRNA was obtained by demonstrating that (i) GLP-I-(7-37) had negligible effects on levels of actin and angiotensinogen mRNAs in the insulinoma cell line; (ii) glucagon and GLP-II had no effects on insulin mRNA levels; and (iii) GLP-I-(7-37), when added to the rat islet glucagon-producing cell line 1056A (22) and two pituitary cell lines, one producing prolactin (GH4) and the other corticotropin (AtT-20), had no

**Table 1. Stimulation of cAMP formation by glucagon and glucagon-like peptides in RIN 1046-38 cells**

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Peptide conc., M</th>
<th>No. of plates</th>
<th>cAMP, fmol* (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>1</td>
<td>No peptide</td>
<td>8</td>
<td>15.4 ± 0.7*</td>
</tr>
<tr>
<td></td>
<td>5 x 10^-12</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 x 10^-11</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 x 10^-10</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>No peptide</td>
<td>4</td>
<td>43.6 ± 4.1</td>
</tr>
<tr>
<td></td>
<td>5 x 10^-4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 x 10^-7</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 x 10^-10</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 x 10^-9</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>No peptide</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>5 x 10^-10</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 x 10^-9</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 x 10^-8</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 x 10^-7</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

Statistical significance between control (no peptide) and experimental observations (by unpaired two-tailed t test) is given in parentheses. NS, not significant.

*Per 1/50th of cell extract per plate.
effects on the levels of glucagon, prolactin, and corticotropin mRNAs, respectively (data not shown).

To test the effectiveness of GLP-I in stimulating insulin release, we added GLP-I-(7-37) to RIN 1046-38 cells and measured insulin accumulation in the medium (Table 3). Incubation of GLP-I-(7-37) with RIN 1046-38 cells increased the secretion of insulin into the culture medium. This effect was seen within 1 hr after addition of the peptide to the culture medium, indicating that GLP-I-(7-37) stimulates insulin secretion and not just biosynthesis. No effect on insulin secretion was observed after incubation with GLP-II.

To assess the stability of the 37 amino acid peptide in the experimental conditions, we incubated GLP-I-(1-37) for 24 hr in culture medium alone or in medium supplemented with either 0.1% bovine serum albumin or 10% fetal bovine serum. Aliquots of media were analyzed by high-pressure liquid chromatography and radioimmunoassay. Before incubation, no GLP-I-(7-37) was detected in the preparation of GLP-I-(1-37) (Fig. 2). However, after incubation of GLP-I-(1-37) in conditioned medium containing 0.1% bovine serum albumin, a small peak of GLP-I-(7-37) appeared, indicating that cleavage of GLP-I-(1-37) to the smaller, more active GLP-I-(7-37) occurs under these experimental conditions.

**DISCUSSION**

The ability of GLP-I to stimulate insulin release, cAMP formation, and insulin mRNA levels demonstrates that the peptide is an insulinotropic peptide. Moreover, GLP-I-(7-37) appears to be a more potent insulinotropic peptide than GLP-I-(1-37). Examination of the amino acid sequences of GLP-I, glucagon, and gastric inhibitory polypeptide reveals that the first six amino acids of GLP-I, ending in arginine, are only weakly homologous to the amino termini of glucagon and gastric-inhibitory peptide. However, alignment of GLP-I with these peptides beginning with the histidine residue at amino acid 7 indicates that GLP-I-(1-37), glucagon, and gastric-inhibitory peptide are identical at 8 of the first 11 residues (17). Several different peptides encoded in prohormones are cleaved after single basic arginine residues by a monobasic-specific endopeptidase (23), lending support to the evidence that the 31 amino acid GLP-I-(7-37), and not GLP-I-(1-37), may be the more potent bioactive peptide. Additional evidence for a biologic role of GLP-I-(7-37) comes from an examination of the processing of proglucagon in pancreas and intestines (11), a glucagon-producing clonal cell line (22), and transfected cell lines (12). Analyses of tissue and cell extracts by high-pressure liquid chromatography consistently reveal the presence of at least three forms of GLP-I, two of which correspond to GLP-I-(7-37) and GLP-I-(1-37). Further, in the perfused rat pancreas, GLP-I-(7-37) stimulates insulin release at a concentration as low as 50 pM, whereas no insulin-releasing activity was observed with GLP-I-(1-37) at a concentration 10,000 times higher (0.5 μM) (24). Thus, several lines of evidence suggest that GLP-I-(7-37) is a potent insulinotropic peptide.

The greater potency of the shorter, 31 amino acid peptide may be partially accounted for by the "blocking" of an active site at the amino terminus by the first 6 residues of the 1-37 peptide. Earlier studies of the structure-function relationships of glucagon indicate that the amino terminus is involved in the signal transduction and biological potency of the peptide, whereas the carboxyl terminus is primarily responsible for recognition and binding to receptors (25). Hence, GLP-I-(1-37) may be an inactive precursor, which undergoes further processing to expose a new amino-terminal region that confers bioactivity. Studies of GLP-I bioactivity that use the amino-terminally extended peptide may underestimate the true biological potency of the peptide (16, 17).

The biphasic dose-response curve for the stimulation of cAMP by GLP-I-(7-37) in RIN 1046-38 cells is strikingly similar to the observations of Hoosein and Gurd (18), who found that GLP-I-(1-36)-NH2 at 1 nM maximally stimulated adenylate cyclase activity in brain membranes and higher doses of GLP-I (0.01–1 μM) paradoxically produced less adenylate cyclase stimulation. The high dose inhibition of cAMP formation observed with GLP-I-(7-37) is similar to previous observations for a number of different activators of
adenylate cyclase including glucagon, secretin, β-adrenergic agonists, and prostaglandins (18, 26, 27).

The increase in insulin mRNA levels following cAMP accumulation in RIN insulinoma cells has been observed before. Cholera toxin, an activator of adenylate cyclase, stimulates insulin secretion and insulin gene transcription in RIN-5F cells (28). Moreover, cholera toxin also stabilizes insulin mRNA levels in RIN-5F cells (29). Activation of adenylate cyclase may increase insulin mRNA levels by both transcriptional and posttranscriptional mechanisms. That GLP-I increased insulin mRNA levels is not surprising, because it stimulates cAMP formation in RIN 1046-38 cells. However, the attribution of the GLP-I-mediated increase in insulin mRNA levels solely to an increase in intracellular cAMP must be considered in the light of recent observations in which dibutyryl-cAMP had no effect on insulin gene expression in the RIN 1056A cell line (30). Whether or not there is a causal relationship between the increase in intracellular cAMP and the GLP-I-mediated increase in insulin mRNA levels is unknown. The relative effectiveness of GLP-I as a mediator of insulin release is probably underestimated using the rat insulinoma cell line. Insulin release and biosynthesis in the RIN 1046-38 cell line is not responsive to glucose, unlike isolated islets or primary cultures of islet cells in which elevations in glucose levels in the bathing medium elicit prompt insulin-secretory responses (31).

These observations suggest that GLP-I is a regulator of insulin biosynthesis. The identification of multiple molecular forms of GLP-I in intestinal extracts, and in smaller amounts in pancreas, raises physiological questions of how GLP-I

Table 2. Densitometric quantitation of effects of glucagon-like peptides on levels of insulin and actin mRNAs in RIN 1046-38 cells

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Peptide conc., M</th>
<th>Arbitrary densitometric units*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control†</td>
<td>GLP-I-(1-37)</td>
</tr>
<tr>
<td>Insulin</td>
<td>5 × 10⁻⁷</td>
<td>1.28 ± 0.18</td>
</tr>
<tr>
<td>Actin</td>
<td>5 × 10⁻⁷</td>
<td>0.68 ± 0.03</td>
</tr>
</tbody>
</table>

**Experiment 1**

| Insulin | 5 × 10⁻⁷       | 5.90 (6.86, 4.99) | 7.00 (5.58, 8.41) |
| Actin  | 5 × 10⁻¹¹      | 2.69 (3.23, 2.15) | 2.11 (1.86, 2.36) |
|        | 5 × 10⁻¹⁰      | 6.70 (7.92, 5.50) |
|        | 5 × 10⁻⁹       | 8.50 (7.59, 9.38) |
|        | 5 × 10⁻⁸       | 7.90 (8.40, 7.40) |

**Experiment 2**

| Insulin | 5 × 10⁻⁷       | 5.56 ± 0.43  | 13.87 ± 0.40‡ |
| Actin  | 5 × 10⁻⁷       | 3.29 ± 0.08  | 4.36 ± 0.44  |

**Experiment 3**

* Determined by scanning of autoradiograms of RNA blots. Values from experiments 1 and 3 are means ± SEM of triplicate plates of cells; values from experiment 2 are means of duplicates (individual values are given in parentheses). Statistical significance between control and experimental observations were calculated by Student's unpaired two-tailed t test.

† No peptide added.
‡ P < 0.02.
§ P < 0.05.
¶ P < 0.001.

Table 3. Stimulation of insulin release from RIN 1046-38 cells by GLP-I-(7-37)

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Insulin, microunits/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 hr</td>
</tr>
<tr>
<td>None (control)</td>
<td>166 ± 7</td>
</tr>
<tr>
<td>0.5 µM GLP-I-(7-37)</td>
<td>381 ± 63</td>
</tr>
</tbody>
</table>

GLP-I-(7-37) was incubated with 10⁶ cells for 1 or 24 hr. Insulin released into the culture medium was measured by radioimmunoassay. Data represent the mean ± SEM of three separate determinations. Statistical significance between control and experimental observations was calculated by Student's unpaired two-tailed t test.
exerts its insulinotropic effects. Furthermore, whether GLP-I acts via a receptor separate from that for glucagon or serves as a potent agonist for the glucagon receptor remains to be determined. The contribution of GLP-I to the incretin effect, as well as its possible role in the impaired insulin secretion in patients with non-insulin-dependent diabetes mellitus, merits further investigation.

Note Added in Proof. Recently, Holst et al. (32) reported that GLP-I-(7-37)-NH2 at concentrations as low as 0.1 nM increased insulin secretion from the isolated perfused pig pancreas.

We thank Esther Hoomis for typing the manuscript. This research was supported in part by National Institutes of Health Grants AM30834, AM30846, and AM32520. D.J.D. is a Centennial Fellow of the Medical Research Council of Canada, and J.P. is supported by the Swiss National Medical Foundation.