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(71) Applicant (for all designated States except US): UNIVERSITY OF ULSTER [GB/GB]; Coleraine, Co Londonderry BT52 1SA (GB).

(72) Inventors: and
(75) Inventors/Applicants (for US only): O’HARTE, Finbarr, Paul, Mary [GB/GB]; 2 Cambourne Park, Fivoy Road, Ballymoney, Co Antrim BT53 7PG (GB). FLATT, Peter, Raymond [GB/GB]; 18 Ballymacrea Road, Portrush, Co Antrim BT56 8NR (GB).

(74) Agent: MURGITROYD & COMPANY; 373 Scotland Street, Glasgow G5 8QA (GB).

(54) Title: PEPTIDE

(57) Abstract

The present invention provides peptides which stimulate the release of insulin. The peptides, based on GIP 1–42 include substitutions and/or modifications which enhance and influence secretion and/or have enhanced resistance to degradation. The invention also provides a process of N terminally modifying GIP and the use of the peptide analogues for treatment of diabetes.
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Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.
"Peptide"

The present invention relates to the release of insulin and the control of blood glucose concentration. More particularly the invention relates to the use of peptides to stimulate release of insulin, lowering of blood glucose and pharmaceutical preparations for treatment of type 2 diabetes.

Gastric inhibitory polypeptide (GIP) and glucagon-like peptide-1(7-36)amide (truncated GLP-1; tGLP-1) are two important insulin-releasing hormones secreted from endocrine cells in the intestinal tract in response to feeding. Together with autonomic nerves they play a vital supporting role to the pancreatic islets in the control of blood glucose homeostasis and nutrient metabolism.

Dipeptidyl peptidase IV (DPP IV; EC 3.4.14.5) has been identified as a key enzyme responsible for inactivation of GIP and tGLP-1 in serum. DPP IV is completely inhibited in serum by the addition of diprotin A (DPA, 0.1 mmol/l). This occurs through the rapid removal of
the N-terminal dipeptides Tyr\(^1\)-Ala\(^2\) and His\(^7\)-Ala\(^8\)
giving rise to the main metabolites GIP(3-42) and GLP-1(9-36)amide, respectively. These truncated peptides
are reported to lack biological activity or to even
serve as antagonists at GIP or tGLP-1 receptors. The
resulting biological half-lives of these incretin
hormones in vivo are therefore very short, estimated to
be no longer than 5 min.

In situations of normal glucose regulation and
pancreatic B-cell sensitivity, this short duration of
action is advantageous in facilitating momentary
adjustments to homeostatic control. However, the
current goal of a possible therapeutic role of incretin
hormones, particularly tGLP-1 in NIDDM therapy is
frustrated by a number of factors in addition to
finding a convenient route of administration. Most
notable of these are rapid peptide degradation and
rapid absorption (peak concentrations reached 20 min)
and the resulting need for both high dosage and precise
timing with meals. Recent therapeutic strategies have
focused on precipitated preparations to delay peptide
absorption and inhibition of GLP-1 degradation using
specific inhibitors of DPP IV. A possible therapeutic
role is also suggested by the observation that a
specific inhibitor of DPP IV, isoleucine thiazolidide,
lowered blood glucose and enhanced insulin secretion in
glucose-treated diabetic obese Zucker rats presumably
by protecting against catabolism of the incretin
hormones tGLP-1 and GIP.
Numerous studies have indicated that tGLP-1 infusion restores pancreatic B-cell sensitivity, insulin secretory oscillations and improved glycemic control in various groups of patients with IGT or NIDDM. Longer term studies also show significant benefits of tGLP-1 injections in NIDDM and possibly IDDM therapy, providing a major incentive to develop an orally effective or long-acting tGLP-1 analogue. Several attempts have been made to produce structurally modified analogues of tGLP-1 which are resistant to DPP IV degradation. A significant extension of serum half-life is observed with His\(^7\)-glucitol tGLP-1 and tGLP-1 analogues substituted at position 8 with Gly, Aib, Ser or Thr. However, these structural modifications seem to impair receptor binding and insulinotropic activity thereby compromising part of the benefits of protection from proteolytic degradation. In recent studies using His\(^7\)-glucitol tGLP-1, resistance to DPP IV and serum degradation was accompanied by severe loss of insulin-releasing activity.

GIP shares not only the same degradation pathway as tGLP-1 but many similar physiological actions, including stimulation of insulin and somatostatin secretion, and the enhancement of glucose disposal. These actions are viewed as key aspects in the antihyperglycemic properties of tGLP-1, and there is therefore good expectation that GIP may have similar potential as NIDDM therapy. Indeed, compensation by GIP is held to explain the modest disturbances of glucose homeostasis observed in tGLP-1 knockout mice. Apart from early studies, the anti-diabetic potential
of GIP has not been explored and tGLP-1 may seem more
attractive since it is viewed by some as a more potent
insulin secretagogue when infused at "so called"
physiological concentrations estimated by RIA.

The present invention aims to provide effective
analogues of GIP. It is one aim of the invention to
provide a pharmaceutical for treatment of Type 2
diabetes.

According to the present invention there is provided an
effective peptide analogue of the biologically active
GIP(1-42) which has improved characteristics for
treatment of Type 2 diabetes wherein the analogue
comprises at least 15 amino acid residues from the N
terminus of GIP(1-42) and has at least one amino acid
substitution or modification at position 1-3 and not
including Tyr\(^1\) glucitol GIP(1-42).

The structures of human and porcine GIP(1-42) are shown
below. The porcine peptide differs by just two amino
acid substitutions at positions 18 and 34.

The analogue may include modification by fatty acid
addition at an epsilon amino group of at least one
lysine residue.

The invention includes Tyr\(^1\) glucitol GIP(1-42) having
fatty acid addition at an epsilon amino group of at
least one lysine residue.
Analogues of GIP(1-42) may have an enhanced capacity to stimulate insulin secretion, enhance glucose disposal, delay glucose absorption or may exhibit enhanced stability in plasma as compared to native GIP. They also may have enhanced resistance to degradation.

Any of these properties will enhance the potency of the analogue as a therapeutic agent.

Analogues having D-amino acid substitutions in the 1, 2 and 3 positions and/or N-glycated, N-alkylated, N-acetylated or N-acylated amino acids in the 1 position are resistant to degradation in vivo.

Various amino acid substitutions at second and third amino terminal residues are included, such as GIP(1-42)Gly2, GIP(1-42)Ser2, GIP(1-42)Abu2, GIP(1-42)Aib, GIP(1-42)D-Ala2, GIP(1-42)Sar2, and GIP(1-42)Pro3.

Amino-terminally modified GIP analogues include N-glycated GIP(1-42), N-alkylated GIP(1-42), N-acetylated
GIP(1-42), N-acetyl-GIP(1-42) and N-isopropyl GIP(1-42).

Other stabilised analogues include those with a peptide isostere bond between amino terminal residues at position 2 and 3. These analogues may be resistant to the plasma enzyme dipeptidyl-peptidase IV (DPP IV) which is largely responsible for inactivation of GIP by removal of the amino-terminal dipeptide Tyr1-Ala2.

In particular embodiments, the invention provides a peptide which is more potent than human or porcine GIP in moderating blood glucose excursions, said peptide consisting of GIP(1-42) or N-terminal fragments of GIP(1-42) consisting of up to between 15 to 30 amino acid residues from the N-terminus (i.e. GIP(1-15) – GIP(1-3)) with one or more modifications selected from the group consisting of:

(a) substitution of Ala2 by Gly
(b) substitution of Ala2 by Ser
(c) substitution of Ala2 by Abu
(d) substitution of Ala2 by Aib
(e) substitution of Ala2 by D-Ala
(f) substitution of Ala2 by Sar
(g) substitution of Glu3 by Pro
(h) modification of Tyr1 by acetylation
(i) modification of Tyr1 by acylation
(j) modification of Tyr1 by alkylation
(k) modification of Tyr1 by glycation
(l) conversion of Ala2-Glu3 bond to a psi [CH2NH] bond
conversion of Ala2-Glu3 bond to a stable peptide
isotere bond
(n) (n-isopropyl-H) 1GIP.

The invention also provides the use of Tyr1-glucitol
GIP in the preparation of a medicament for the
treatment of diabetes.

The invention further provides improved pharmaceutical
compositions including analogues of GIP with improved
pharmacological properties.

Other possible analogues include certain commonly
encountered amino acids, which are not encoded by the
 genetic code, for example, beta-alanine (beta-ala), or
other omega-amino acids, such as 3-amino propionic, 4-
amino butyric and so forth, ornithine (Orn), citrulline
(Cit), homoarginine (Har), t-butylalanine (t-BuA), t-
butylglycine (t-BuG), N-methylisoleucine (N-MeIle),
phenylglycine (Phg), and cyclohexylalanine (Cha),
norleucine (Nle), cysteic acid (Cya) and methionine
sulfoxide (MSO), substitution of the D form of a
neutral or acidic amino acid or the D form of tyrosine
for tyrosine.

According to the present invention there is also
provided a pharmaceutical composition useful in the
treatment of diabetes type II which comprises an
effective amount of the peptide as described herein, in
admixture with a pharmaceutically acceptable excipient.
The invention also provides a method of N-terminally modifying GIP or analogues thereof the method comprising the steps of synthesizing the peptide from the C terminal to the penultimate N terminal amino acid, adding tyrosine to a bubbler system as a F-moc protected Tyr(tBu)-Wang resin, deprotecting the N-terminus of the tyrosine and reacting with the modifying agent, allowing the reaction to proceed to completion, cleaving the modified tyrosine from the Wang resin and adding the modified tyrosine to the peptide synthesis reaction.

Preferably the agent is glucose, acetic anhydride or pyroglutamic acid.

The invention will now be demonstrated with reference to the following non-limiting example and the accompanying figures wherein:

Figure 1a illustrates degradation of GIP by DPP IV.

Figure 1b illustrates degradation of GIP and Tyr¹ glucitol GIP by DPP IV.

Figure 2a illustrates degradation of GIP human plasma.

Figure 2b illustrates degradation of GIP and Tyr¹-glucitol GIP by human plasma.
Figure 3 illustrates electrospray ionization mass spectrometry of GIP, Tyr₁-glucitol GIP and the major degradation fragment GIP(3-42).

Figure 4 shows the effects of GIP and glycated GIP on plasma glucose homeostasis.

Figure 5 shows effects of GIP on plasma insulin responses.

Figure 6 illustrates DPP-IV degradation of GIP 1-42.

Figure 7 illustrates DPP-IV degradation of GIP (Abu²).

Figure 8 illustrates DPP-IV degradation of GIP (Sar³).

Figure 9 illustrates DPP-IV degradation of GIP (Ser³).

Figure 10 illustrates DPP-IV degradation of N-Acetyl-GIP.

Figure 11 illustrates DPP-IV degradation of glycated GIP.

Figure 12 illustrates human plasma degradation of GIP.

Figure 13 illustrates human plasma degradation of GIP (Abu²).

Figure 14 illustrates human plasma degradation of GIP (Sar³).
Figure 15 illustrates human plasma degradation of GIP (Ser²).

Figure 16 illustrates human plasma degradation of glycated GIP.

Figure 17 shows the effects of various concentrations of GIP 1-42 and GIP (Abu²) on insulin release from BRIN-BD11 cells incubated at 5.6mM glucose.

Figure 18 shows the effects of various concentrations of GIP 1-42 and GIP (Abu²) on insulin release from BRIN-BD11 cells incubated at 16.7mM glucose.

Figure 19 shows the effects of various concentrations of GIP 1-42 and GIP (Sar²) on insulin release from BRIN-BD11 cells incubated at 5.6mM glucose.

Figure 20 shows the effects of various concentrations of GIP 1-42 and GIP (Sar²) on insulin release from BRIN-BD11 cells incubated at 16.7mM glucose.

Figure 21 shows the effects of various concentrations of GIP 1-42 and GIP (Ser²) on insulin release from BRIN-BD11 cells incubated at 5.6mM glucose.

Figure 22 shows the effects of various concentrations of GIP 1-42 and GIP (Ser²) on insulin release from BRIN-BD11 cells incubated at 16.7mM glucose.
Figure 23 shows the effects of various concentrations of GIP 1-42 and N-Acetyl-GIP 1-42 on insulin release from BRIN-BD11 cells incubated at 5.6mM glucose.

Figure 24 shows the effects of various concentrations of GIP 1-42 and N-Acetyl-GIP 1-42 on insulin release from BRIN-BD11 cells incubated at 16.7mM glucose.

Figure 25 shows the effects of various concentrations of GIP 1-42 and glycated GIP 1-42 on insulin release from BRIN-BD11 cells incubated at 5.6mM glucose.

Figure 26 shows the effects of various concentrations of GIP 1-42 and glycated GIP 1-42 on insulin release from BRIN-BD11 cells incubated at 16.7mM glucose.

Figure 27 shows the effects of various concentrations of GIP 1-42 and GIP (Gly^2) on insulin release from BRIN-BD11 cells incubated at 5.6mM glucose.

Figure 28 shows the effects of various concentrations of GIP 1-42 and GIP (Gly^2) on insulin release from BRIN-BD11 cells incubated at 16.7mM glucose.

Figure 29 shows the effects of various concentrations of GIP 1-42 and GIP (Pro^3) on insulin release from BRIN-BD11 cells incubated at 5.6mM glucose.

Figure 30 shows the effects of various concentrations of GIP 1-42 and GIP (Pro^3) on insulin release from BRIN-BD11 cells incubated at 16.7mM glucose.
Example 1

Preparation of N-terminally modified GIP and analogues thereof.

The N-terminal modification of GIP is essentially a three-step process. Firstly, GIP is synthesised from its C-terminal (starting from a Fmoc-Gln (Trt)-Wang resin, Novabiochem) up to the penultimate N-terminal amino-acid (Ala2) on an automated peptide synthesizer (Applied Biosystems, CA, USA). The synthesis follows standard Fmoc peptide chemistry protocols. Secondly, the N-terminal amino acid of native GIP (Tyr) is added to a manual bubbler system as a Fmoc-protected Tyr(tBu)-Wang resin. This amino acid is deprotected at its N-terminus (piperidine in DMF (20% v/v)) and allowed to react with a high concentration of glucose (glycation, under reducing conditions with sodium cyanoborohydride), acetic anhydride (acetylation), pyroglutamic acid (pyroglutamyl) etc. for up to 24 h as necessary to allow the reaction to go to completion. The completeness of reaction will be monitored using the ninhydrin test which will determine the presence of available free a-amino groups. Thirdly, (once the reaction is complete) the now structurally modified Tyr is cleaved from the wang resin (95% TFA, and 5% of the appropriate scavengers. N.B. Tyr is considered to be a problematic amino acid and may need special consideration) and the required amount of N-terminally modified-Tyr consequently added directly to the automated peptide synthesiser, which will carry on the synthesis, thereby stitching the N-terminally modified-
Tyr to the α-amino of GIP(Ala2), completing the synthesis of the GIP analogue. This peptide is cleaved off the Wang resin (as above) and then worked up using the standard Buchner filtering, precipitation, rotary evaporation and drying techniques.

Example 2

The following example investigates preparation of Tyr\(^1\)-glycitol GIP together with evaluation of its antihyperglycemic and insulin-releasing properties in vivo. The results clearly demonstrate that this novel GIP analogue exhibits a substantial resistance to aminopeptidase degradation and increased glucose lowering activity compared with the native GIP.

Research Design and Methods

Materials. Human GIP was purchased from the American Peptide Company (Sunnyvale, CA, USA). HPLC grade acetonitrile was obtained from Rathburn (Walkersburn, Scotland). Sequencing grade trifluoroacetic acid (TFA) was obtained from Aldrich (Poole, Dorset, UK). All other chemicals purchased including dextran T-70, activated charcoal, sodium cyanoborohydride and bovine serum albumin fraction V were from Sigma (Poole, Dorset, UK). Diprotin A (DPA) was purchased from Calbiochem-Novabiochem (UK) Ltd. (Beeston, Nottingham, UK) and rat insulin standard for RIA was obtained from Novo Industria (Copenhagen, Denmark). Reversed-phase
Sep-Pak cartridges (C-18) were purchased from Millipore-Waters (Milford, MA, USA). All water used in these experiments was purified using a Milli-Q, Water Purification System (Millipore Corporation, Milford, MA, USA).

**Preparation of Tyr$^1$-glucitol GIP.** Human GIP was incubated with glucose under reducing conditions in 10 mmol/l sodium phosphate buffer at pH 7.4 for 24 h. The reaction was stopped by addition of 0.5 mol/l acetic acid (30 µl) and the mixture applied to a Vydac (C-18) (4.6 x 250mm) analytical HPLC column (The Separations Group, Hesperia, CA, USA) and gradient elution conditions were established using aqueous/TFA and acetonitrile/TFA solvents. Fractions corresponding to the glycated peaks were pooled, taken to dryness under vacuum using an AES 1000 Speed-Vac concentrator (Life Sciences International, Runcorn, UK) and purified to homogeneity on a Supelcosil (C-8) (4.6 x 150mm) column (Supelco Inc., Poole, Dorset, UK).

**Degradation of GIP and Tyr$^1$-glucitol GIP by DPP IV.** HPLC-purified GIP or Tyr$^1$-glucitol GIP were incubated at 37°C with DPP-IV (5mU) for various time periods in a reaction mixture made up to 500 µl with 50 mmol/l triethanolamine-HCl, pH 7.8 (final peptide concentration 1 µmol/l). Enzymatic reactions were terminated after 0, 2, 4 and 12 hours by addition of 5 µl of 10% (v/v) TFA/water. Samples were made up to a final volume of 1.0 ml with 0.12% (v/v) TFA and stored at -20°C prior to HPLC analysis.
Degradation of GIP and Tyr\(^1\)-glucitol GIP by human plasma. Pooled human plasma (20 μl) taken from six healthy fasted human subjects was incubated at 37°C with GIP or Tyr\(^1\)-glucitol GIP (10 μg) for 0 and 4 hours in a reaction mixture made up to 500 μl, containing 50 mmol/l triethanolamine/HCL buffer pH 7.8. Incubations for 4 hours were also performed in the presence of diprotin A (5 μU). The reactions were terminated by addition of 5 μl of TFA and the final volume adjusted to 1.0 ml using 0.1% v/v TFA/water. Samples were centrifuged (13,000g, 5 min) and the supernatant applied to a C-18 Sep-Pak cartridge (Millipore-Waters) which was previously primed and washed with 0.1% (v/v) TFA/water. After washing with 20 ml 0.12% TFA/water, bound material was released by elution with 2 ml of 80% (v/v) acetonitrile/water and concentrated using a Speed-Vac concentrator (Runcorn, UK). The volume was adjusted to 1.0ml with 0.12% (v/v) TFA/water prior to HPLC purification.

HPLC analysis of degraded GIP and Tyr\(^1\)-glucitol GIP. Samples were applied to a Vydac C-18 widepore column equilibrated with 0.12% (v/v) TFA/H\(_2\)O at a flow rate of 1.0 ml/min. Using 0.1% (v/v) TFA in 70% acetonitrile/H\(_2\)O, the concentration of acetonitrile in the eluting solvent was raised from 0% to 31.5% over 15 min, to 38.5% over 30 min and from 38.5% to 70% over 5 min, using linear gradients. The absorbance was monitored at 206 nm and peak areas evaluated using a
model 2221 LKB integrator. Samples recovered manually
were concentrated using a Speed-Vac concentrator.

Electrospray ionization mass spectrometry (ESI-MS).
Samples for ESI-MS analysis containing intact and
degradation fragments of GIP (from DPP IV and plasma
incubations) as well as Tyr\(^{1}\)-glucitol GIP, were further
purified by HPLC. Peptides were dissolved
(approximately 400 pmol) in 100 \(\mu\)l of water and applied
to the LCQ benchtop mass spectrometer (Finnigan MAT,
Hemel Hempstead, UK) equipped with a microbore C-18
HPLC column (150 x 2.0mm, Phenomenex, UK, Ltd,
Macclesfield). Samples (30\(\mu\)l direct loop injection)
were injected at a flow rate of 0.2ml/min, under
isocratic conditions 35\% (v/v) acetonitile/water. Mass
spectra were obtained from the quadripole ion trap mass
analyzer and recorded. Spectra were collected using
full ion scan mode over the mass-to-charge (m/z) range
150-2000. The molecular masses of GIP and related
structures were determined from ESI-MS profiles using
prominent multiple charged ions and the following
equation \(M_r = iM_i - iM_h\) (where \(M_r\) = molecular mass; \(M_i\) =
m/z ratio; \(i\) = number of charges; \(M_h\) = mass of a
proton).

In vivo biological activity of GIP and Tyr\(^{1}\)-glucitol
GIP. Effects of GIP and Tyr\(^{1}\)-glucitol GIP on plasma
glucose and insulin concentrations were examined using
10-12 week old male Wistar rats. The animals were
housed individually in an air conditioned room and
22±2\(^{\circ}\)C with a 12 hour light/12 hour dark cycle.
Drinking water and a standard rodent maintenance diet
(Trouw Nutrition, Belfast) were supplied ad libitum. Food was withdrawn for an 18 hour period prior to intraperitoneal injection of glucose alone (18 mmol/kg body weight) or in combination with either GIP or Tyr\(^1\)-glucitol GIP (10 nmol/kg). Test solutions were administered in a final volume of 8 ml/kg body weight. Blood samples were collected at 0, 15, 30 and 60 minutes from the cut tip of the tail of conscious rats into chilled fluoride/heparin microcentrifuge tubes (Sarstedt, Nümbrecht, Germany). Samples were centrifuged using a Beckman microcentrifuge for about 30 seconds at 13,000 g. Plasma samples were aliquotted and stored at -20\(^\circ\)C prior to glucose and insulin determinations. All animal studies were done in accordance with the Animals (Scientific Procedures) Act 1986.

Analyses. Plasma glucose was assayed by an automated glucose oxidase procedure using a Beckman Glucose Analyzer II [33]. Plasma insulin was determined by dextran charcoal radioimmunoassay as described previously [34]. Incremental areas under plasma glucose and insulin curves (AUC) were calculated using a computer program (CAREA) employing the trapezoidal rule [35] with baseline subtraction. Results are expressed as mean ± SEM and values were compared using the Student’s unpaired t-test. Groups of data were considered to be significantly different if P<0.05.

**Results**
Degradation of GIP and Tyr\textsuperscript{1}-glucitol GIP by DPP IV.

Figure 1 illustrates the typical peak profiles obtained from the HPLC separation of the products obtained from the incubation of GIP (Fig 1a) or Tyr\textsuperscript{1}-glucitol GIP (Fig 1b) with DPP IV for 0, 2, 4 and 12 hours. The retention times of GIP and Tyr\textsuperscript{1}-glucitol GIP at t=0 were 21.93 minutes and 21.75 minutes respectively. Degradation of GIP was evident after 4 hours incubation (54% intact), and by 12 hours the majority (60% of intact GIP was converted to the single product with a retention time of 21.61 minutes. Tyr\textsuperscript{1}-glucitol GIP remained almost completely intact throughout 2-12 hours incubation. Separation was on a Vydac C-18 column using linear gradients of 0% to 31.5% acetonitrile over 15 minutes, to 38.5% over 30 minutes and from 38.5 to 70% acetonitrile over 5 minutes.

Degradation of GIP and Tyr\textsuperscript{1}-glucitol GIP by human plasma. Figure 2 shows a set of typical HPLC profiles of the products obtained from the incubation of GIP or Tyr\textsuperscript{1}-glucitol GIP with human plasma for 0 and 4 h. GIP (Fig 2a) with a retention time of 22.06 min was readily metabolised by plasma within 4 hours incubation giving rise to the appearance of a major degradation peak with a retention time of 21.74 minutes. In contrast, the incubation of Tyr\textsuperscript{1}-glucitol GIP under similar conditions (Fig 2b) did not result in the formation of any detectable degradation fragments during this time with only a single peak being observed with a retention time of 21.77 minutes. Addition of diprotin A, a specific inhibitor of DPP IV, to GIP during the 4 hours incubation completely inhibited degradation of
the peptide by plasma. Peaks corresponding with intact
GIP, GIP (3-42) and Tyr\(^1\)-glucitol GIP are indicated.
A major peak corresponding to the specific DPP IV
inhibitor tripeptide DPA appears in the bottom panels
with retention time of 16-29 min.

Identification of GIP degradation fragments by ESI-MS.
Figure 3 shows the monoisotopic molecular masses
obtained for GIP, (panel A), Tyr\(^1\)-glucitol GIP (panel
B) and the major plasma degradation fragment of GIP
(panel C) using ESI-MS. The peptides analyzed were
purified from plasma incubations as shown in Figure 2.
Peptides were dissolved (approximately 400 pmol) in
100\(\mu\)l of water and applied to the LC/MS equipped with a
microbore C-18 HPLC column. Samples (30\(\mu\)l direct loop
injection) were applied at a flow rate of 0.2ml/min,
under isocratic conditions 35% acetonitrile/water.
Mass spectra were recorded using a quadripole ion trap
mass analyzer. Spectra were collected using full ion
scan mode over the mass-to-charge (m/z) range 150-2000.
The molecular masses (M\(_r\)) of GIP and related structures
were determined from ESI-MS profiles using prominent
multiple charged ions and the following equation
M\(_r\)=iM\(_1\)-iM\(_h\). The exact molecular mass (M\(_r\)) of the
peptides were calculated using the equation M\(_r\) = iM\(_1\) -
iM\(_h\) as defined in Research Design and Methods. After
spectral averaging was performed, prominent multiple
charges species (M+3H)\(^3+\) and (M+4H)\(^4+\) were detected from
GIP at m/z 1661.6 and 1246.8, corresponding to intact
M\(_r\) 4981.8 and 4983.2 Da, respectively (Fig. 3A).
Similarly, for Tyr\(^1\)-glucitol GIP ((M+4H)\(^4+\) and (M+5H)\(^5+\))
were detected at m/z 1287.7 and 1030.3, corresponding
to intact molecular masses of $M^r$ 5146.8 and 5146.5 Da, respectively (Fig. 3B). The difference between the observed molecular masses of the quadruply charged GIP and the N-terminally modified GIP species (163.6 Da) indicated that the latter peptide contained a single glucitol adduct corresponding to Tyr$^1$-glucitol GIP. 

Figure 3C shows the prominent multiply charged species (M+3H)$^{3+}$ and (M+4H)$^{4+}$ detected from the major fragment of GIP at m/z 1583.8 and 1188.1, corresponding to intact $M^r$ 4748.4 and 4748 Da, respectively (Figure 3C). This corresponds with the theoretical mass of the N-terminally truncated form of the peptide GIP(3-42). This fragment was also the major degradation product of DPP IV incubations (data not shown).

**Effects of GIP and Tyr$^1$-glucitol GIP on plasma glucose homeostasis.** Figures 4 and 5 show the effects of intraperitoneal (ip) glucose alone (18mmol.kg) (control group), and glucose in combination with GIP or Tyr$^1$-glucitol GIP (10mmol/kg) on plasma glucose and insulin concentrations.

(4A) Plasma glucose concentrations after i.p. glucose alone (18mmol/kg) (control group), or glucose in combination with either GIP of Tyr$^1$-glucitol GIP (10mmol/kg). The time of injection is indicated by the arrow (0 min). (4B) Plasma glucose AUC values for 0-60 min post injection. Values are mean ± SEM for six rats. **P<0.01, ***P<0.001 compared with GIP and Tyr$^1$-glucitol GIP; †P<0.05, ††P<0.01 compared with non-glucated GIP.
Plasma insulin concentrations after i.p. glucose along (18 mmol/kg) (control group), or glucose in combination with either with GIP or glycated GIP (10nmol/kg). The time of injection is indicated by the arrow. (5B) Plasma insulin AUC values were calculated for each of the 3 groups up to 90 minutes post injection. The time of injection is indicated by the arrow (0 min). Plasma insulin AUC values for 0-60 min post injection. Values are mean ± SEM for six rats.

*P<0.05, **P<0.001 compared with GIP and Tyr\(^1\)-glucitol GIP; †P<0.05, ††P<0.01 compared with non-glycated GIP.

Compared with the control group, plasma glucose concentrations and area under the curve (AUC) were significantly lower following administration of either GIP or Tyr\(^1\)-glucitol GIP (Figure 4A, B). Furthermore, individual values at 15 and 30 minutes together with AUC were significantly lower following administration of Tyr\(^1\)-glucitol GIP as compared to GIP. Consistent with the established insulin-releasing properties of GIP, plasma insulin concentrations of both peptide-treated groups were significantly raised at 15 and 30 minutes compared with the values after administration of glucose alone (Figure 5A). The overall insulin responses, estimated as AUC were also significantly greater for the two peptide-treated groups (Figure 5B).

Despite lower prevailing glucose concentrations than the GIP-treated group, plasma insulin response, calculated as AUC, following Tyr\(^1\)-glucitol GIP was significantly greater than after GIP (Figure 5B). The significant elevation of plasma insulin at 30 minutes is of particular note, suggesting that the insulin-
releasing action of Tyr\textsuperscript{1}-glucitol GIP is more
protracted than GIP even in the face of a diminished
glycemic stimulus (Figures 4A, 5A).

Discussion

The forty two amino acid GIP is an important incretin
hormone released into the circulation from endocrine K-
cells of the duodenum and jejunum following ingestion
of food. The high degree of structural conservation
of GIP among species supports the view that this
peptide plays and important role in metabolism.
Secretion of GIP is stimulated directly by actively
transported nutrients in the gut lumen without a
notable input from autonomic nerves. The most
important stimulants of GIP release are simple sugars
and unsaturated long chain fatty acids, with amino
acids exerting weaker effects. As with tGLP-1, the
insulin-releasing effect of GIP is strictly glucose-
dependent. This affords protection against
hypoglycemia and thereby fulfils one of the most
desirable features of any current or potentially new
antidiabetic drug.

The present results demonstrate for the first time that
Tyr\textsuperscript{1}-glucitol GIP displays profound resistance to serum
and DPP IV degradation. Using ESI-MS the present study
showed that native GIP was rapidly cleaved \textit{in vitro} to
a major 4748.4 Da degradation product, corresponding to
GIP(3-42) which confirmed previous findings using
matrix-assisted laser desorption ionization time-of-
flight mass spectrometry. Serum degradation was
completely inhibited by diprotin A (Ile-Pro-Ile), a
specific competitive inhibitor of DPP IV, confirming
this as the main enzyme for GIP inactivation in vivo.
In contrast, Tyr¹-glucitol GIP remained almost
completely intact after incubation with serum or DPP IV
for up to 12 hours. This indicates that glycation of
GIP at the amino-terminal Tyr¹ residue masks the
potential cleavage site from DPP IV and prevents
removal of the Tyr¹-Ala² dipeptide from the N-terminus
preventing the formation of GIP(3-42).

Consistent with in vitro protection against DPP IV,
administration of Tyr¹-glucitol GIP significantly
enhanced the antihyperglycemic activity and
insulin-releasing action of the peptide when
administered with glucose to rats. Native GIP enhanced
insulin release and reduced the glycemic excursion as
observed in many previous studies. However, aminoterminal glycation of GIP increased the insulin-
releasing and antihyperglycemic actions of the peptide
by 62% and 38% respectively, as estimated from AUC
measurements. Detailed kinetic analysis is difficult
due to necessary limitation of sampling times, but the
greater insulin concentrations following Tyr¹-glucitol
GIP as opposed to GIP at 30 minutes post-injection is
indicative of a longer half-life. The glycemic rise
was modest in both peptide-treated groups and glucose
concentrations following injection of Tyr¹-glucitol GIP
were consistently lower than after GIP. Since the
insulinotropic actions of GIP are glucose-dependent, it
is likely that the relative insulin-releasing potency
of Tyr\textsuperscript{1}-glucitol GIP is greatly underestimated in the
present in vivo experiments.

In vitro studies in the laboratory of the present
inventors using glucose-responsive clonal B-cells
showed that the insulin-releasing potency of Tyr\textsuperscript{1}-
glutitol GIP was several order of magnitude greater
than GIP and that its effectiveness was more sensitive
to change of glucose concentrations within the
physiological range. Together with the present in vivo
observations, this suggests that N-terminal glycation
of GIP confers resistance to DPP IV degradation whilst
enhancing receptor binding and insulin secretory
effects on the B-cell. These attributes of Tyr\textsuperscript{1}-
glutitol GIP are fully expressed in vivo where DPP IV
resistance impedes degradation of the peptide to GIP(3-
42), thereby prolonging the half-life and enhancing
effective concentrations of the intact biologically
active peptide. It is thus possible that glycated GIP
is enhancing insulin secretion in vivo both by enhanced
potency at the receptor as well as improving DPP IV
resistance. Thus numerous studies have shown that GIP
(3-42) and other N-terminally modified fragments,
including GIP(4-42), and GIP (17-42) are either weakly
effective or inactive in stimulating insulin release.
Furthermore, evidence exists that N-terminal deletions
of GIP result in receptor antagonist properties in GIP
receptor transfected Chinese hamster kidney cells [9],
suggesting that inhibition of GIP catabolism would also
reduce the possible feedback antagonism at the receptor
level by the truncated GIP(3-42).
In addition to its insulinotopic actions, a number of other potentially important extrapancreatic actions of GIP may contribute to the enhanced antihyperglycemic activity and other beneficial metabolic effects of Tyr\(^1\)-glucitol GIP. These include the stimulation of glucose uptake in adipocytes, increased synthesis of fatty acids and activation of lipoprotein lipase in adipose tissue. GIP also promotes plasma triglyceride clearance in response to oral fat loading. In liver, GIP has been shown to enhance insulin-dependent inhibition of glycogenolysis. GIP also reduces both glucagon-stimulated lipolysis in adipose tissue as well as hepatic glucose production. Finally, recent findings indicate that GIP has a potent effect on glucose uptake and metabolism in mouse isolated diaphragm muscle. This latter action may be shared with tGLP-1 and both peptides have additional benefits of stimulating somatostatin secretion and slowing down gastric emptying and nutrient absorption.

In conclusion, this study has demonstrated for the first time that the glycation of GIP at the amino-terminal Tyr\(^1\) residue limits GIP catabolism through impairment of the proteolytic actions of serum peptidases and thus prolongs its half-life in vivo. This effect is accompanied by enhanced antihyperglycemic activity and raised insulin concentrations in vivo, suggesting that such DPP IV resistant analogues should be explored alongside tGLP-1 as potentially useful therapeutic agents for NIDDM. Tyr\(^1\)-glucitol GIP appears to be particularly interesting in this regard since such amino-terminal
modification of GIP enhances rather than impairs
glucose-dependent insulinotropic potency as was
observed recently for tGLP-1.

Example 3

This example further looked at the ability of
additional N-terminal structural modifications of GIP
in preventing inactivation by DPP and in plasma and
their associated increase in both the insulin-releasing
potency and potential therapeutic value. Native human
GIP, glycated GIP, acetylated GIP and a number of GIP
analogues with N-terminal amino acid substitutions were
tested.

Materials and Methods

Reagents

High-performance liquid chromatography (HPLC) grade
acetonitrile was obtained from Rathburn (Walkersburn,
Scotland). Sequencing grade trifluoroacetic acid (TFA)
was obtained from Aldrich (Poole, Dorset, UK).
Dipeptidyl peptidase IV was purchased from Sigma
(Poole, Dorset, UK), and Diprotin A was purchased from
Calbiochem Novabiochem (Beeston, Nottingham, UK). RPMI
1640 tissue culture medium, foetal calf serum,
penicillin and streptomycin were all purchased from
Gibco (Paisley, Strathclyde, UK). All water used in
these experiments was purified using a Milli-Q, Water
Purification System (Millipore, Millford, MA, USA).
All other chemicals used were of the highest purity available.

Synthesis of GIP and N-terminally modified GIP analogues

GIP, GIP(Abu2), GIP(Sar2), GIP(Ser2), GIP(Gly2) and GIP(Pro3) were sequentially synthesised on an Applied Biosystems automated peptide synthesizer (model 432A) using standard solid-phase Fmoc procedure, starting with an Fmoc-Gln-Wang resin. Following cleavage from the resin by trifluoroacetic acid: water, thioanisole, ethanedithiol (90/2.5/5/2.5, a total volume of 20 ml/g resin), the resin was removed by filtration and the filtrate volume was decreased under reduced pressure. Dry diethyl ether was slowly added until a precipitate was observed. The precipitate was collected by low-speed centrifugation, resuspended in diethyl ether and centrifuged again, this procedure being carried out at least five times. The pellets were then dried in vacuo and judged pure by reversed-phase HPLC on a Waters Millennium 2010 chromatography system (Software version 2.1.5.). N-terminal glycated and acetylated GIP were prepared by minor modification of a published method.

Electrospray ionization-mass spectrometry (ESI-MS) was carried out as described in Example 2.

Degradation of GIP and novel GIP analogues by DPP IV and human plasma was carried out as described in Example 2.
Culture of insulin secreting cells

BRIN-BD11 cells [30] were cultured in sterile tissue culture flasks (Corning, Glass Works, UK) using RPMI-1640 tissue culture medium containing 10% (v/v) foetal calf serum, 1% (v/v) antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin) and 11.1 mM glucose. The cells were maintained at 37°C in an atmosphere of 5% CO₂ and 95% air using a LEBC incubator (Laboratory Technical Engineering, Nottingham, UK).

Acute tests for insulin secretion

Before experimentation, the cells were harvested from the surface of the tissue culture flasks with the aid of trypsin/EDTA (Gibco), seeded into 24-multiwell plates (Nunc, Roskilde, Denmark) at a density of 1.5 x 10⁵ cells per well, and allowed to attach overnight at 37°C. Acute tests for insulin release were preceded by 40 min pre-incubation at 37°C in 1.0 ml Krebs Ringer bicarbonate buffer (115 mM NaCl, 4.7 mM KCl, 1.28 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 10 mM NaHCO₃, 5 g/l bovine serum albumin, pH 7.4) supplemented with 1.1 mM glucose. Test incubations were performed (n=12) at two glucose concentrations (5.6 mM and 16.7 mM) with a range of concentrations (10⁻¹³ to 10⁻⁴ M) of GIP or GIP analogues. After 20 min incubation, the buffer was removed from each well and aliquots (200 μl) were used for measurement of insulin by radioimmunoassay [31].

Statistical analysis
Results are expressed as mean ± S.E.M. and values were compared using the Student’s unpaired t-test. Groups of data were considered to be significantly different if P< 0.05.

Results and Discussion

Structural identification of GIP and GIP analogues by ESI-MS

The monoisotopic molecular masses of the peptides were determined using ESI-MS. After spectral averaging was performed, prominent multiple charged species (M+3H)3+ and (M+4H)4+ were detected for each peptide. Calculated molecular masses confirmed the structural identity of synthetic GIP and each of the N-terminal analogues.

Degradation of GIP and novel GIP analogues by DPP-IV

Figs. 6-11 illustrate the typical peak profiles obtained from the HPLC separation of the reaction products obtained from the incubation of GIP, GIP(Abu2), GIP(Sar2), GIP(Ser2), glycated GIP and acetylated GIP with DPP IV, for 0, 2, 4, 8 and 24 h. The results summarised in Table 1 indicate that glycated GIP, acetylated GIP, GIP(Ser2) are GIP(Abu2)
more resistant than native GIP to in vitro degradation with DPP IV. From these data GIP(Sar2) appears to be less resistant.

Degradation of GIP and GIP analogues by human plasma
Figs. 12-16 show a representative set of HPLC profiles obtained from the incubation of GIP and GIP analogues with human plasma for 0, 2, 4, 8 and 24 h. Observations were also made after incubation for 24 h in the presence of DPA. These results are summarised in Table 2 are broadly comparable with DPP IV incubations, but conditions which more closely mirror in vivo conditions are less enzymatically severe. GIP was rapidly degraded by plasma. In comparison, all analogues tested exhibited resistance to plasma degradation, including GIP(Sar2) which from DPP IV data appeared least resistant of the peptides tested. DPA substantially inhibited degradation of GIP and all analogues tested with complete abolition of degradation in the cases of GIP(Abu2), GIP(Ser2) and glycated GIP. This indicates that DPP IV is a key factor in the in vivo degradation of GIP.

Dose-dependent effects of GIP and novel GIP analogues on insulin secretion

Figs. 17-30 show the effects of a range of concentrations of GIP, GIP(Abu2), GIP(Sar2), GIP(Ser2), acetylated GIP, glycated GIP, GIP(Gly2) and GIP(Pro3) on insulin secretion from BRIN-BD11 cells at 5.6 and 16.7 mM glucose. Native GIP provoked a prominent and dose-related stimulation of insulin secretion. Consistent with previous studies [28], the glycated GIP analogue exhibited a considerably greater insulino tropic response compared with native peptide. N-terminal acetylated GIP exhibited a similar pattern and the GIP(Ser2) analogue also evoked a strong
response. From these tests, GIP(Gly2) and GIP(Pro3) appeared to the least potent analogues in terms of insulin release. Other stable analogues tested, namely GIP(Abu2) and GIP(Sar2), exhibited a complex pattern of responsiveness dependent on glucose concentration and dose employed. Thus very low concentrations were extremely potent under hyperglycaemic conditions (16.7 mM glucose). This suggests that even these analogues may prove therapeutically useful in the treatment of type 2 diabetes where insulinotropic capacity combined with in vivo degradation dictates peptide potency.
Table 1: % Intact peptide remaining after incubation with DPPIV

<table>
<thead>
<tr>
<th>Peptide</th>
<th>% Intact peptide remaining after time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>GIP 1-42</td>
<td>100</td>
</tr>
<tr>
<td>Glycated GIP</td>
<td>100</td>
</tr>
<tr>
<td>GIP (Abu²)</td>
<td>100</td>
</tr>
<tr>
<td>GIP (Ser²)</td>
<td>100</td>
</tr>
<tr>
<td>GIP (Sar²)</td>
<td>100</td>
</tr>
<tr>
<td>N-Acetyl-GIP</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 2: % Intact peptide remaining after incubation with human plasma

<table>
<thead>
<tr>
<th>Peptide</th>
<th>% Intact peptide remaining after incubations with human plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>GIP 1-42</td>
<td>100</td>
</tr>
<tr>
<td>Glycated GIP</td>
<td>100</td>
</tr>
<tr>
<td>GIP (Abu²)</td>
<td>100</td>
</tr>
<tr>
<td>GIP (Ser²)</td>
<td>100</td>
</tr>
<tr>
<td>GIP (Sar²)</td>
<td>100</td>
</tr>
</tbody>
</table>

Tables represent the percentage of intact peptide (i.e. GIP 1-42) relative to the major degradation product GIP 3-42. Values were taken from HPLC traces performed in triplicate and the mean and S.E.M. values calculated. DPA is diprotin A, a specific inhibitor of DPPIV.
1. A peptide analogue of GIP (1-42) comprising at least 15 amino acid residues from the N terminal end of GIP (1-42) having a least one amino acid substitution or modification at position 1-3 and not including Tyr¹ glucitol GIP (1-42).

2. A peptide analogue as claimed in claim 1 including modification by fatty acid addition at an epsilon amino group of at least one lysine residue.

3. A peptide analogue of biologically active GIP (1-42) wherein the analogue is Tyr¹ glucitol GIP (1-42) modified by fatty acid addition at an epsilon amino group of at least one lysine residue.

4. A peptide analogue as claimed in any of the preceding claims wherein the substitution or modification is chosen from the group comprising D-amino acid substitutions in 1, 2 and/or 3 positions and/or N terminal glycation, alkylation, acetylation or acylation.

5. A peptide analogue as claimed in any of the preceding claims wherein the amino acid in the 2 or 3 position is substituted by lysine, serine, 4-amino butyric, Aib, D-alanine, Sarcosine or Proline.

6. An analogue as claimed in any of the preceding claims wherein the N terminus is modified by one
of the group of modifications include glycation,
alkylation, acetylation or by the addition of an
isopropyl group.

7. Use of an analogue as claimed in any of the
preceding claims in the preparation of a
medicament for the treatment of diabetes.

8. A pharmaceutical composition including an analogue
as claimed in any of the preceding claims.

9. A pharmaceutical composition as claimed in claim 8
in admixture with a pharmaceutically acceptable
excipient.

10. A method of N-terminally modifying GIP or
analогues thereof the method comprising the steps
of synthesising the peptide from the C terminal to
the penultimate N terminal amino acid, adding
tyrosine as a F-moc protected Tyr(tBu)-Wang resin,
deprotecting the N-terminus of the tyrosine and
reacting with modifying agent, allowing the
reaction to proceed to completion, cleaving the
modified tyrosine from the Wang resin and adding
the modified tyrosine to the peptide synthesis
reaction.

11. A method as claimed in claim 10 wherein the
modifying agent is chosen from the group
comprising glucose, acetic anhydride or
pyroglutamic acid.
Fig. 6 HPLC traces showing DPPIV degradation of GIP 1-42
Fig. 7 HPLC traces showing DPP IV degradation of GIP (Abu²)
Fig. 8 HPLC traces showing DPPIV degradation of GIP (Sar²)
Fig. 9 HPLC traces showing DPPIV degradation of GIP (Ser²)
Fig. 10 HPLC traces showing DPPIV degradation of N-Acetyl-GIP
Fig. 11 HPLC traces showing DPPIV degradation of glycated GIP
Fig. 12. HPLC traces showing human plasma degradation of GIP
Fig. 13. HPLC traces showing human plasma degradation of GIP (Abu²)
Fig. 14. HPLC traces showing human plasma degradation of GIP (Sar²)
Fig. 15 HPLC traces showing human plasma degradation of GIP(Ser²)
Fig. 16. HPLC traces showing human plasma degradation of glycated GIP
Fig.17. Graph showing the effects of various concentrations of GIP and GIP (Abu\(^3\)) on insulin release from BRIN-BD11 cells incubated at 5.6 mM glucose

- **5.6 mM glucose control**
- **GIP**
- **GIP (Abu\(^3\))**

**Insulin secretion (ng/10^6 cells/20 min)**

<table>
<thead>
<tr>
<th>Peptide concentration (M)</th>
<th>10^{-13}</th>
<th>10^{-12}</th>
<th>10^{-11}</th>
<th>10^{-10}</th>
<th>10^{-9}</th>
<th>10^{-8}</th>
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</tr>
</tbody>
</table>

Values are means ± S.E.M. for 12 separate observations. *p<0.05, **p<0.01, ***p<0.001 compared to control (5.6mM glucose alone). *p<0.05, **p<0.01, ***p<0.001 compared to GIP (Abu\(^3\)) at the same concentration.
Fig. 18. Graph showing the effects of various concentrations of GIP and GIP (Abu³) on insulin release from BRIN-BD11 cells incubated at 16.7 mM glucose.

Values are means ± S.E.M. for 12 separate observations. *p<0.05, **p<0.01, ***p<0.001 compared to control (16.7 mM glucose alone). *p<0.05, **p<0.01, ***p<0.001 compared to GIP (Abu³) at the same concentration.
Fig.19. Graph showing the effects of various concentrations of GIP and GIP (Sar²) on insulin release from BRIN-BD11 cells incubated at 5.6 mM glucose.

Values are means ± S.E.M. for 12 separate observations. *P<0.05, **P<0.01, ***P<0.001 compared to control (5.6mM glucose alone). *P<0.05, **P<0.01, ***P<0.001 compared to GIP (Sar²) at the same concentration.
Fig. 20. Graph showing the effects of various concentrations of GIP and GIP (Sar²) on insulin release from BRIN-BD11 cells incubated at 16.7 mM glucose.

Insulin secretion (pg/10⁶ cells/20 min)

Peptide concentration (M)

Values are means ± S.E.M. for 12 separate observations. *P<0.05, **P<0.01, ***P<0.001 compared to control (16.7 mM glucose alone). ΔP<0.05, ΔΔP<0.01, ΔΔΔP<0.001 compared to GIP (Sar²) at the same concentration.
Fig.21. Graph showing the effects of various concentrations of GIP and GIP (Ser²) on insulin release from BRIN-BD11 cells incubated at 5.6 mM glucose.

Values are means ± S.E.M. for 12 separate observations. *P<0.05, **P<0.01, ***P<0.001 compared to control (5.6 mM glucose alone). *P<0.05, **P<0.01, ***P<0.001 compared to GIP (Ser²) at the same concentration.
Fig. 22. Graph showing the effects of various concentrations of GIP and GIP (Ser²) on insulin release from BRIN-βD11 cells incubated at 16.7 mM glucose.

Values are means ± S.E.M. for 12 separate observations. *P<0.05, **P<0.01, ***P<0.001 compared to control (16.7 mM glucose alone). *P<0.05, **P<0.01, ***P<0.001 compared to GIP (Ser²) at the same concentration.
Fig. 23 Graph showing the effects of various concentrations of GIP 1-42 and N-Acetyl-GIP 1-42 on insulin release from BRIN-BD11 cells incubated at 5.6 mM glucose

- Control
- GIP 1-42
- N-Acetyl-GIP 1-42
Fig. 24 Graph showing the effects of various concentrations of GIP 1-42 and N-Acetyl-GIP 1-42 on insulin release from BRIN-BD11 cells incubated at 16.7 mM glucose.
Fig. 25 Graph showing the effects of various concentrations of GIP 1-42 and glycated GIP 1-42 on insulin release from BRIN-BD11 cells incubated at 5.6 mM glucose.
Fig. 26 Graph showing the effects of various concentrations of GIP 1-42 and glycated GIP 1-42 on insulin release from BRIN-BD11 cells incubated at 16.7 mM glucose.
Fig. 27. Graph showing the effects of various concentrations of GIP and GIP (Gly²) on insulin release from BRIN-BD11 cells incubated at 5.6 mM glucose.

Values are means ± S.E.M. for 12 separate observations. *P < 0.05, **P < 0.01, ***P < 0.001 compared to control (5.6 mM glucose alone). ΔP < 0.05, ΔΔP < 0.01, ΔΔΔP < 0.001 compared to GIP (Gly²) at the same concentration.
Fig. 28 Graph showing the effects of various concentrations of GIP and GIP (Gly²) on insulin release from BRIN-BD11 cells incubated at 16.7 mM glucose

- □ 16.7 mM glucose control
- GIP
- GIP (Gly²)

Values are means ± S.E.M. for 12 separate observations. *P<0.05, **P<0.01, ***P<0.001 compared to control (16.7 mM glucose alone). *P<0.05, **P<0.01, ***P<0.001 compared to GIP (Gly²) at the same concentration.
Fig. 29 Graph showing the effects of various concentrations of GIP and GIP (Pro5) on insulin release from BRIN-BD11 cells incubated at 5.6 mM glucose.

- 5.6 mM glucose control
- GIP
- GIP (Pro5)

Insulin secretion (ng/10^6 cells/20 min)

Peptide concentration (M)

Values are means ± S.E.M. for 12 separate observations. *P<0.05, **P<0.01, ***P<0.001 compared to control (5.6mM glucose alone). ∆P<0.05, ∆∆P<0.01, ∆∆∆P<0.001 compared to GIP (Pro5) at the same concentration.
Fig. 30 Graph showing the effects of various concentrations of GIP and GIP (Proα) on insulin release from BRIN-BD11 cells incubated at 16.7 mM glucose.

Values are means ± S.E.M. for 12 separate observations. *P< 0.05, **P< 0.01, ***P< 0.001 compared to control (16.7 mM glucose alone). ^P< 0.05, ^^^P< 0.01, ^^^^^P< 0.001 compared to GIP (Proα) at the same concentration.