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GIP(Lys[16]PAL) and GIP(Lys[37]PAL): Novel Long-Acting Acylated Analogues of Glucose-Dependent Insulinotropic Polypeptide with Improved Antidiabetic Potential

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Glucose-dependent insulinotropic polypeptide (GIP) is a physiological insulin releasing peptide. We have developed two novel fatty acid derivatized GIP analogues, which bind to serum albumin and demonstrate enhanced duration of action in vivo. GIP(Lys[16]PAL) and GIP(Lys[37]PAL) were resistant to dipeptidyl peptidase IV (DPP IV) degradation. In vitro studies demonstrated that GIP analogues retained their ability to activate the GIP receptor through production of cAMP and to stimulate insulin secretion. Intraperitoneal administration of GIP analogues to obese diabetic (ob/ob) mice significantly decreased the glycemic excursion and elicited increased and prolonged insulin responses compared to native GIP. A protracted glucose-lowering effect was observed 24 h following GIP(Lys[37]PAL) administration. Once a day injection for 14 days decreased nonfasting glucose, improved glucose tolerance, and enhanced the insulin response to glucose. These data demonstrate that fatty acid derivatized GIP peptides represent a novel class of long-acting stable GIP analogues for therapy of type 2 diabetes.

Introduction

Glucose-dependent insulinotropic polypeptide (GIP) is a gastrointestinal hormone secreted from enteroendocrine K-cells in response to nutrient absorption following feeding.[1] Although for some time it was believed to be purely an incretin hormone moderating pancreatic β-cell insulin release, several studies have indicated that GIP has wider effects on islet function and acts at other extrapancreatic sites, which further enhance its antidiabetic potential.[2,3] This unique repertoire of biological effects provides an opportunity to develop GIP into an effective antidiabetic agent, especially since its insulinotropic effects are strictly glucose-dependent thus avoiding hypoglycemia.[4]

The pharmacokinetic profile and therapeutic attractiveness of GIP are compromised by the ubiquitous enzyme dipeptidyl peptidase IV (DPP IV; EC 3.4.15.5).[5] DPP IV degrades numerous regulatory peptides, and in the case of GIP, specifically hydrolyzes the amino-terminal Tyr[1]-Ala[2] residues to produce the degradation product GIP(3-42).[6] GIP(3-42) does not stimulate insulin release and was initially believed to be inactive.[7] However, recent studies have demonstrated that this major degradation product may act as a functional GIP receptor antagonist in vivo.[8] Therefore, it is anticipated that the development of DPP IV resistant analogues of GIP would not only extend the biological half-life of the peptide but also curtail production of GIP(3-42), thereby alleviating the risk of antagonism at the GIP receptor. Indeed the insulin-releasing and antidiabetic potential of several amino-terminally modified GIP analogues have been demonstrated both in animal models and in preliminary studies using patients with type 2 diabetes mellitus.[9−14] Furthermore, although many populations of type 2 diabetic patients show decreased responsiveness to GIP infusion,[15−18] it is clear from animal studies that amino-terminally modified analogues of GIP may overcome a large part of any possible defect.[9−13] In fact, this reduced GIP-induced insulin secretion in patients with type 2 diabetes is much less evident following pulse administration of GIP[19] and has been suggested to represent a generalized defect of β-cell function rather than a specific defect of GIP action.[19,20] Ultimately, however, such GIP analogues are still subject to renal clearance, which detracts from their therapeutic potency.[21]

One conceivable approach to avoid rapid renal filtration and clearance of GIP from the body might involve the design and synthesis of fatty acid peptide derivatives of GIP. The usefulness of this approach has been successfully exploited using insulin acylated with several fatty acid constituents at the ϵ-amino group of Lys^{B29}.[22] This fatty acid/insulin complex is thought to bind to serum albumin, thereby preventing kidney filtration and prolonging its biological actions. Additionally, a fatty acid derivative of GIP's sister incretin hormone, GLP-1, NN2211 (Liraglutide), has been shown to have a prolonged pharmacodynamic profile making it suitable for once daily subcutaneous administration.[23,24] Unfortunately, a recent study showed that a sizable proportion of patients treated with Liraglutide exhibited gastrointestinal side effects,[23] due likely to a prolonged inhibitory effect of Liraglutide on gastric emptying.[24] Similar side effects have been noted with other long-acting GLP-1 mimetics in humans, including Exenatide and Albugon.[25,26] In contrast, GIP does not affect gastric emptying, even in humans,[27] thereby removing such a barrier from the potential use of GIP analogues in the treatment of type 2 diabetes.

We have recently reported the antidiabetic properties of two N-terminally modified palmitate derivatives of GIP.[28−30] The present study was designed to investigate the metabolic stability and biological activity of two novel solely fatty acid derivatized analogues of GIP, GIP(Lys[16]PAL) and GIP(Lys[37]PAL). The GIP peptide analogues have a C-16 palmitate group conjugated to the ϵ-amino group of Lys side chains at positions 16 or 37

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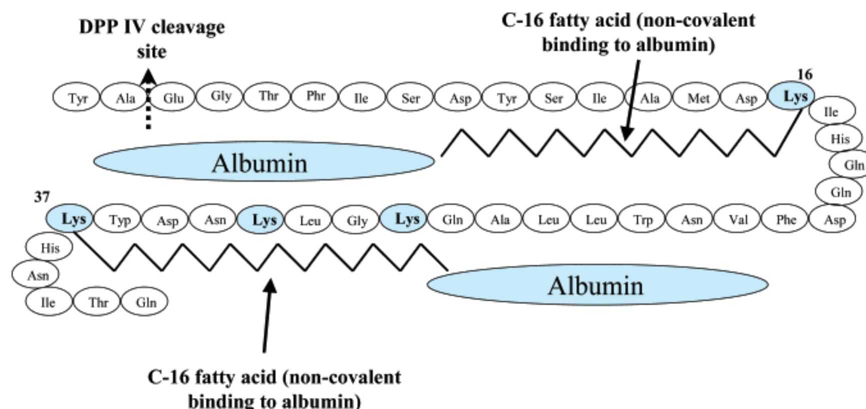

Figure 1. Primary structure of GIP showing site of cleavage by DPP IV, the four constituent lysine residues, and the palmitate acylation positions in GIP(Lys^{16}PAL) and GIP(Lys^{37}PAL).

Table 1. Structural Characterization of GIP and Fatty Acid Derivatized GIP Analogues by MALDI-TOF Mass Spectrometry[a]

peptide	experimental Mr	theoretical Mr	difference
native GIP	4983.6	4982.4	1.2
GIP(Lys^{16}PAL)	5221.3	5220.7	0.6
GIP(Lys^{37}PAL)	5221.6	5220.7	0.9
GIP(Lys^{37}PAL)-albumin	71444.6	-	5224.2
albumin	66220.4	-	

[a] Peptide samples were mixed with matrix (α-cyano-4-hydroxycinnamamic acid), and the m/z ratio versus relative peak intensity was resolved using a Voyager-DE BioSpectrometry workstation. Albumin-bound fatty acid derivatized GIP was prepared by overnight incubation of peptide with bovine serum albumin.

(Figure 1). The stability of both analogues to DPP IV degradation, as well as their biological activities in vitro were examined and compared to the native GIP(1-42). Furthermore, the in vivo antidiabetic potential of these analogues was assessed using ob/ob mice, a commonly employed animal model of type 2 diabetes displaying both β-cell dysfunction and insulin resistance.

Results

Structural Identification of GIP Peptides by MALDI-TOF Mass Spectrometry. Following solid-phase Fmoc peptide synthesis and HPLC purification of GIP or fatty acid derivatized GIP analogues, their molecular masses were determined using MALDI-TOF mass spectrometry. As shown in Table 1, the experimental mass-to-charge (m/z) ratios for protonated GIP, GIP(Lys^{16}PAL), and GIP(Lys^{37}PAL) corresponded closely to the theoretical masses. This confirms successful peptide synthesis. Table 1 also shows that in vitro incubation of GIP(Lys^{37}PAL) with albumin gave a peptide product of 71444.6 Da, representing a GIP derivatized fatty acid albumin complex (see Figure 1).

Degradation of Native GIP and GIP Analogues by DPP IV. Table 2 shows that native GIP was rapidly degraded by DPP IV with only 52 ± 3% of the peptide remaining intact after 2 h incubation. After 8 h, GIP was completely degraded to GIP(3-42). On the other hand, GIP(Lys^{16}PAL) and GIP-(Lys^{37}PAL) remained fully intact after prolonged incubations of up to 24 h.

Stimulation of cAMP. Both GIP(Lys^{16}PAL) and GIP-(Lys^{37}PAL) stimulated cAMP production in a concentration-dependent manner in GIP receptor transfected fibroblasts (Figure 2). The calculated EC_{50} values for GIP, GIP(Lys^{16}PAL), and GIP(Lys^{37}PAL) were 18.2, 2.9, and 5.4 nmol, respectively, indicating both GIP analogues were slightly more potent than native GIP.

Table 2. Percentage of Intact Peptide Remaining after Incubation with DPP IV[a]

peptide	% intact peptide remaining after time (h) 0	2	8	24
native GIP	100	52 ± 3	0	0
GIP(Lys^{16}PAL)	100	100	100	100
GIP(Lys^{37}PAL)	100	100	100	100

[a] Values represent the percentage of intact peptide remaining relative to the major degradation product GIP(3-42) following incubation with DPP IV as determined from HPLC peak area data. The reactions were performed in triplicate and the means ± SEM values calculated.

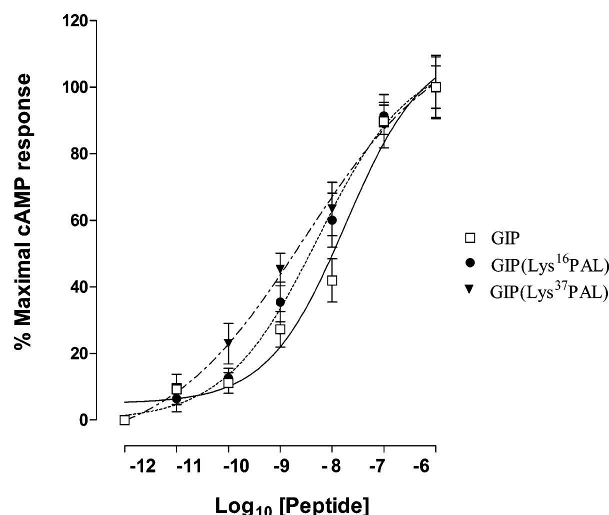

Figure 2. Intracellular cAMP production by GIP, GIP(Lys^{16}PAL), and GIP(Lys^{37}PAL) as determined by column chromatography, in CHL cells stably expressing the human GIP receptor. Each experiment was performed in triplicate ($n = 3$), and the results are expressed (means ± SEM) as a percentage of maximum GIP response.

Stimulation of in Vitro Insulin Secretion. Both GIP-(Lys^{16}PAL) and GIP(Lys^{37}PAL) enhanced insulin release in a concentration-dependent manner similar to native GIP (Figure 3A). Over the range of concentrations tested (10^{-13} to 10^{-6} M), insulin secretion was stimulated by 1.1- to 2.5-fold ($P < 0.05$ to $P < 0.001$) compared to 5.6 mM glucose control. There was no significant difference in potency between the three peptides (Figure 3A). A similar pattern of responsiveness was observed at higher albumin concentrations, but with some diminution of the insulin-releasing activity of GIP(Lys^{37}PAL) at 6% (w/v) BSA. An almost identical effect was observed with pre-incubation of GIP(Lys^{37}PAL) for 2 h in the presence of 1% BSA, suggesting that the effect reflects increased albumin binding.

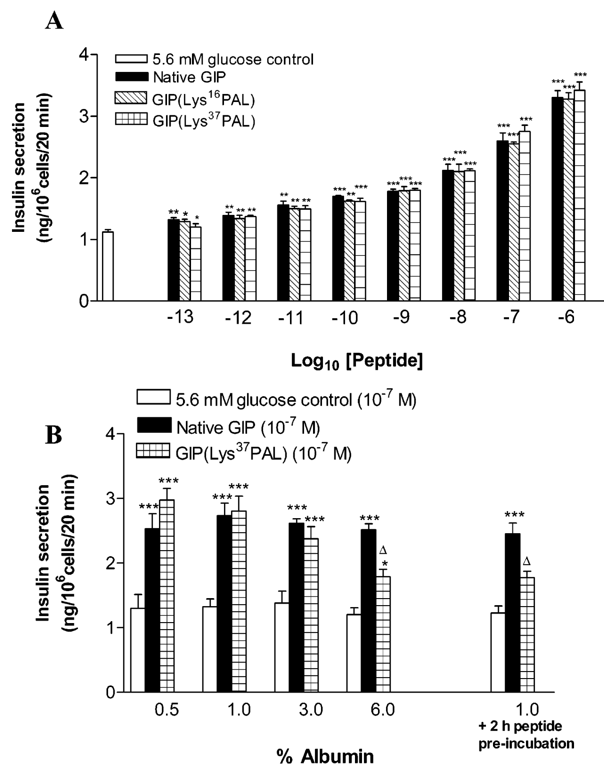

Figure 3. Insulin-releasing activity of GIP, GIP(Lys[16]PAL), and GIP-(Lys[37]PAL) in the clonal pancreatic β-cell line, BRIN-BD11. (A) After a preincubation (40 min), the effects of various concentrations of peptide were tested on insulin release during a 20 min incubation in the presence of 0.5% (w/v) BSA. Values are means ± SEM for eight separate observations. Secretory output in the presence of the various GIP peptides was consistently different ($P < 0.05$ to <0.01) to control (5.6 mM glucose alone). There were no significant differences between the peptide groups. (B) After a preincubation (40 min), the effects of various concentrations of BSA were tested on insulin releasing characteristics of native GIP and GIP(Lys[37]PAL). The effects of 2 h preincubation with peptide are also shown. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to glucose alone and $^{\Delta}P < 0.05$ compared to native GIP at same BSA concentration.

Antihyperglycemic and Insulin Releasing Activity in ob/ob Mice. The relative glucose-lowering abilities of GIP, GIP(Lys[16]PAL), or GIP(Lys[37]PAL) (25 nmol kg^{-1} body weight) in ob/ob mice are shown in Figure 4. Injection of glucose alone resulted in a rapid and marked increase in plasma glucose which continued to rise until 60 min. Native GIP had a tendency to reduce glucose at the later time points monitored, but neither this nor the overall AUC glucose excursion achieved significance (Figure 4A,B). In contrast, GIP(Lys[16]PAL) and GIP-(Lys[37]PAL) significantly reduced plasma glucose concentrations from 15 to 60 min and AUC values when compared to glucose alone. GIP(Lys[37]PAL) further revealed a significant reduction in the AUC response compared to native GIP. As shown in Figure 5, these effects were linked to corresponding changes in plasma insulin concentrations. GIP caused a significantly greater ($P < 0.05$) insulin release than glucose alone. However, most notably GIP(Lys[16]PAL) and GIP(Lys[37]PAL) exhibited substantially greater and more protracted insulin responses than native GIP. Thus plasma insulin from 30 to 60 min and AUC values were significantly increased (Figure 5A,B). The overall enhancement of insulin release by GIP(Lys[16]PAL) and GIP-(Lys[37]PAL) over native GIP was 2.4- and 2.7-fold ($P < 0.05$ to $P < 0.01$), respectively.

Dose-Dependent Metabolic Action in ob/ob Mice. On the basis of results obtained, GIP(Lys[37]PAL) was chosen for more detailed studies. Figure 6 shows the plasma glucose and insulin

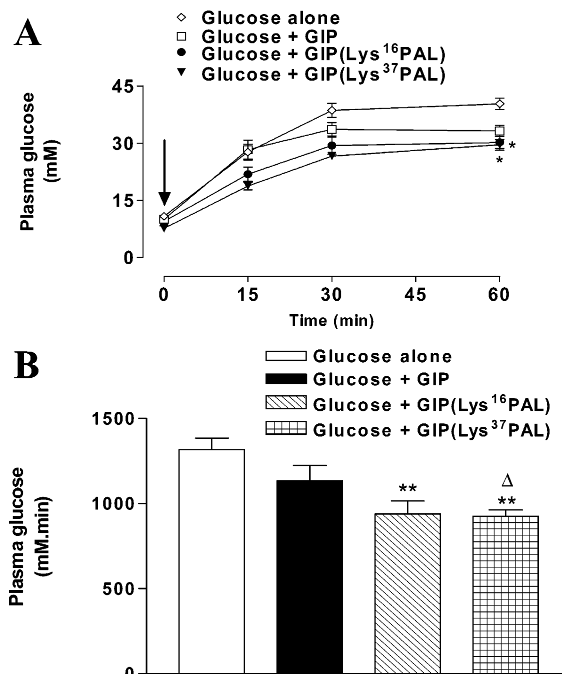

Figure 4. Glucose lowering effects of GIP, GIP(Lys[16]PAL), and GIP-(Lys[37]PAL) in 18 h fasted (ob/ob) mice. (A) Plasma glucose concentrations were measured prior to and after intraperitoneal administration of glucose alone (18 mmol kg^{-1} body weight) as a control or in combination with GIP or fatty acid derivatized analogues (25 nmol kg^{-1} body weight). (B) The incremental area under the glucose curve (AUC) between 0 and 60 min. Values represent means ± SEM for eight mice. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to glucose alone. $^{\Delta}P < 0.05$ compared to native GIP.

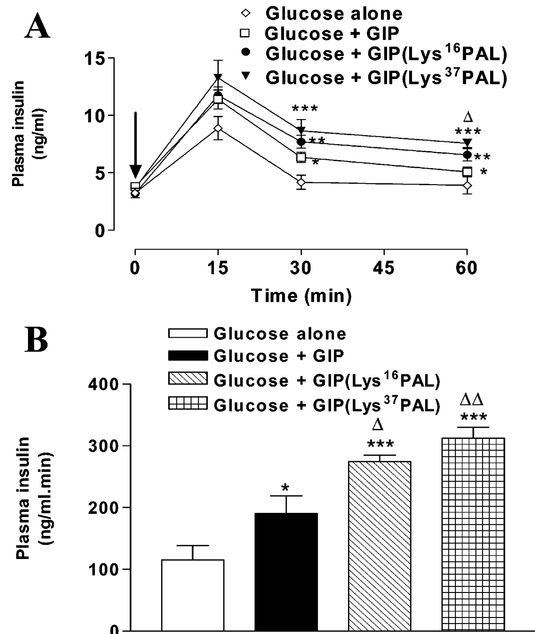

Figure 5. Insulin releasing activity of GIP, GIP(Lys[16]PAL), and GIP-(Lys[37]PAL) in 18 h fasted (ob/ob) mice. (A) Plasma insulin concentrations were measured prior to and after intraperitoneal administration of glucose alone (18 mmol kg^{-1} body weight) as a control or in combination with GIP or fatty acid derivatized analogues (25 nmol kg^{-1} body weight). (B) The incremental area under the insulin curve (AUC) between 0 and 60 min. Values represent means ± SEM for eight mice. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to glucose alone. $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$ compared to native GIP.

AUC (0−60 min) responses when administered together with glucose to ob/ob mice. Maximal effects of GIP and GIP-

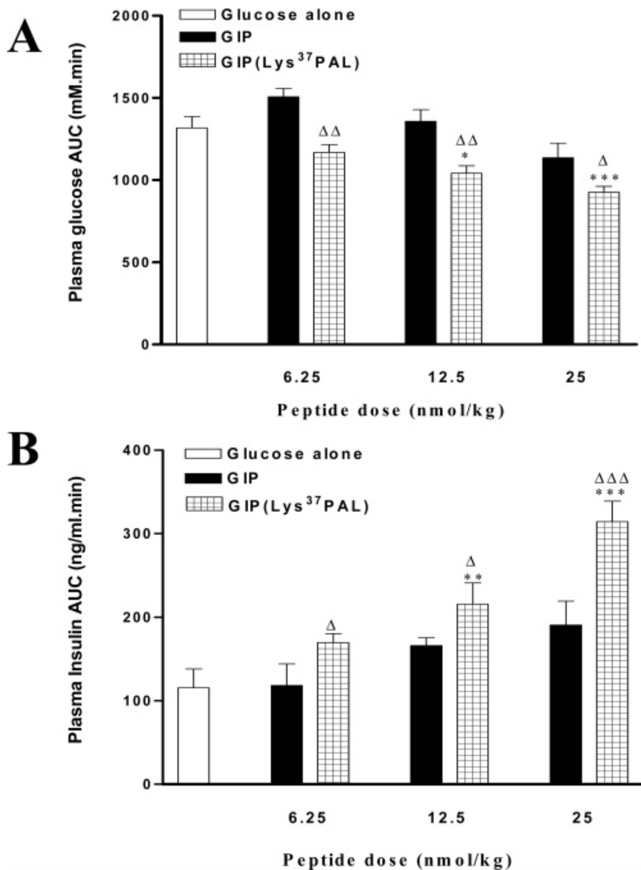

Figure 6. Dose-dependent effects of GIP and GIP(Lys[37]PAL) in 18 h fasted (ob/ob) mice. The incremental area under the curve (AUC) for glucose (A) and insulin (B) between 0 and 60 min after intraperitoneal administration of glucose alone (18 mmol kg^{-1} body weight) or in combination with GIP or GIP(Lys[37]PAL) (each at 6.25, 12.5 and 25 nmol kg^{-1} body weight). Values represent means ± SEM for eight mice. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to glucose alone. $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$, $^{\Delta\Delta\Delta}P < 0.01$ compared to native GIP at the same concentration.

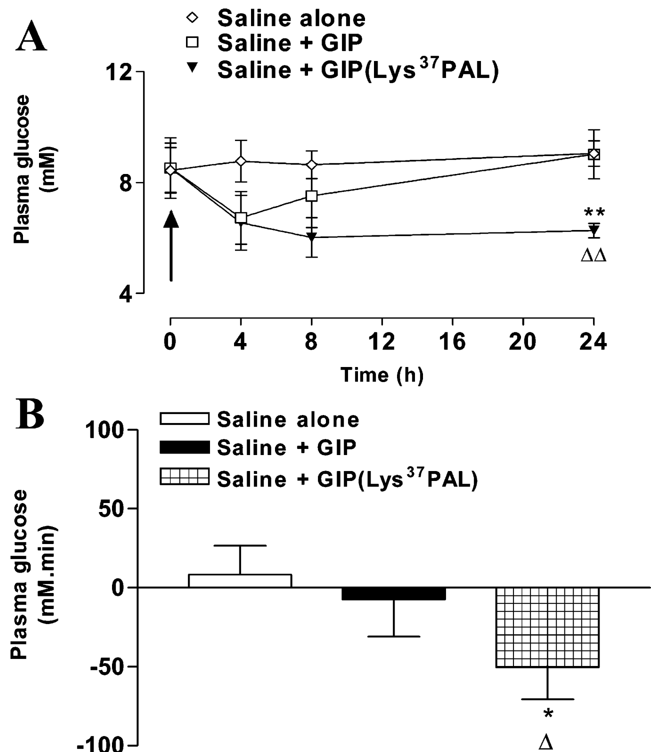

Figure 7. Prolonged glucose lowering effects of GIP and GIP-(Lys[37]PAL) in ob/ob mice. (A) The 24 h plasma glucose concentration profile after intraperitoneal administration of saline alone (0.9% w/v NaCl) as a control, GIP, or GIP(Lys[37]PAL) (12.5 nmol kg^{-1} body weight). (B) The incremental area under the glucose curve (AUC) between 0 and 24 h. Values represent means ± SEM for eight mice. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to saline alone. $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$ compared to native GIP.

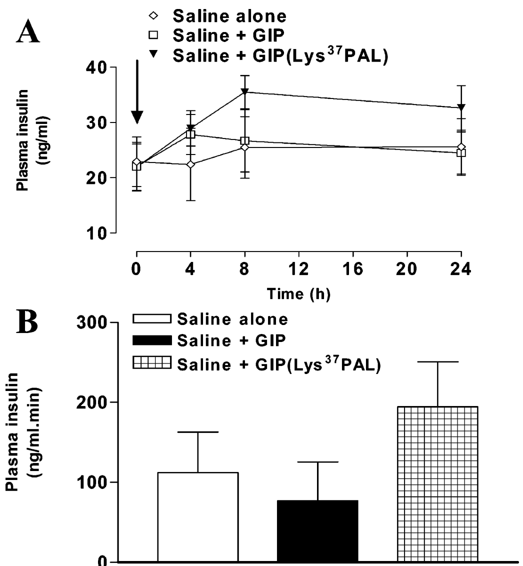

Figure 8. Prolonged insulinotropic effects of GIP and GIP(Lys[37]PAL) in ob/ob mice. (A) The 24 h plasma insulin concentration profile after intraperitoneal administration of saline alone (0.9% w/v NaCl) as a control, GIP, or GIP(Lys[37]PAL) (12.5 nmol kg^{-1} body weight). (B) The incremental area under the insulin curve (AUC) between 0 and 24 h. Values represent means ± SEM for eight mice.

(Lys[37]PAL) were seen at 25 nmol kg^{-1} body weight. GIP-(Lys[37]PAL) evoked significantly reduced glycemic excursions at doses of 12.5 or 25 nmol kg^{-1}, whereas the effects of GIP failed to reach significance throughout the dosage range. A similar pattern was evident from the corresponding insulin AUC values compared to glucose control (Figure 6B). GIP-(Lys[37]PAL) induced significantly raised insulin responses at doses of 12.5 nmol kg^{-1} and above. The derivatized peptide was also significantly more effective than native GIP at all doses tested.

Longer-Term Actions in ob/ob Mice. A dose of 12.5 nmol kg^{-1} was chosen to evaluate the longer-term duration of action of a single dose of native or fatty acid derivatized GIP in nonfasted ob/ob mice. As shown in Figure 7, GIP had a transient, but not significant, effect on plasma glucose. In contrast, GIP(Lys[37]PAL) induced a significant ($P < 0.01$) and sustained decrease of glycemia. Glucose concentrations at 24 h and AUC values were significantly less ($P < 0.05$ to $P < 0.01$) than native GIP or saline treated controls. Insulin concentrations had a tendency to be higher in the GIP(Lys[37]PAL) group from 4 to 24 h, but values were not significantly different from either groups of controls (Figure 8). Food intake was similar in the three groups over the 24 h period.

The effects of once daily injection of ob/ob mice for 14 days with GIP(Lys[37]PAL) (12.5 nmol kg^{-1} per day) are shown in Figure 9. Plasma glucose and glucose tolerance were significantly improved at day 14 compared with saline treated controls ($P < 0.05 - P < 0.01$). Nonfasting insulin concentrations were similar in the two groups, but insulin response to glucose was significantly greater following GIP(Lys[37]PAL) treatment as indicated by AUC measures ($P < 0.05$).

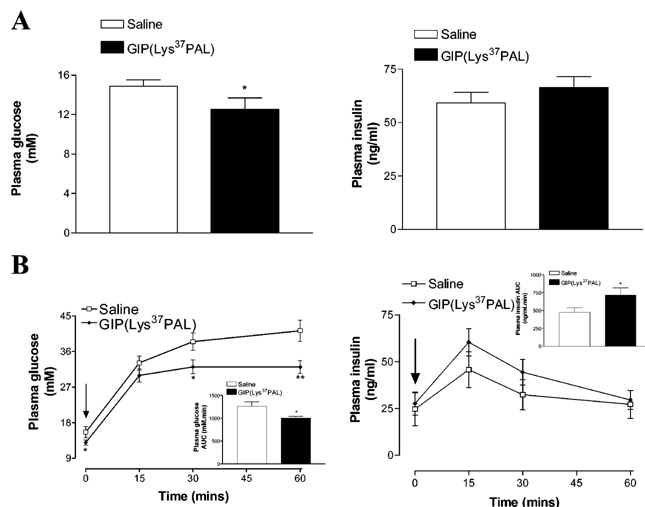

Figure 9. Effects of daily GIP(Lys^{37}PAL) administration in ob/ob mice on (A) nonfasting plasma glucose and insulin concentrations and (B) glucose tolerance and plasma insulin response to glucose. Tests were conducted after 14 daily injections of either GIP(Lys^{37}PAL) (12.5 nmol kg^{-1} body weight per day) or saline vehicle (control). Glucose (18 mmol kg^{-1} body weight) was administered by intraperitoneal injection at the time indicated by the arrow. Plasma glucose and insulin AUC values for 0−60 min postinjection are shown. Values are mean ± SEM for eight mice. *P < 0.05, **P < 0.01 compared to saline control.

Discussion

The multi-action antihyperglycemic and insulinotropic effects of GIP and GLP-1 bestow a major advantage on these native peptides as potential antidiabetic agents compared with present use of single-action nonendogenous drugs.[4] Consequently, there is an abundance of ongoing research concentrating on the administration of GIP or, particularly, GLP-1 for the treatment of type 2 diabetes.[31,32] However, both hormones have a significantly compromised therapeutic potential due to their degradation by DPP IV[6] and rapid elimination from the body.[21]

Recently several approaches have been implemented to design stable, long-acting analogues of GLP-1.[25,26] Currently, the DPP IV resistant GLP-1 derivatives, Liraglutide,[24] Albugon,[33] and CJC-1131,[34] and the naturally occurring GLP-1 receptor agonist exendin-4(1-39)[35] are undergoing clinical trials in type 2 diabetes.[36] Although largely overlooked by all but two major groups, structural analogues of the sister incretin, GIP, may have similar or, due to lack of effects on gastric emptying,[27] enhanced antidiabetic potential compared with GLP-1.[2,13,31] Thus the glucose lowering actions of GIP do not involve inhibition of gastric emptying which appears to be the cause of nausea in a sizable number of individuals treated with GLP-1 agonists.[25,26] A large number of amino-terminally modified GIP analogues exhibiting DPP IV resistance and enhanced bioactivity have now been described, including N-terminally protected palmitate substituted GIP.[2,13,28−31] This study takes a further important step in optimizing the molecular design of GIP analogues by specifically evaluating the lone effects of introducing an acylated palmitate side chain at Lys^{16} or Lys^{37} of native GIP. Such an approach was first adopted for insulin to extend bioactivity by promoting albumin binding and decreasing peptide clearance.[22]

Successful synthesis of fatty acid derivatized GIP analogues and ability to bind serum albumin were confirmed by MALDI-TOF mass spectrometry. Surprisingly, both GIP(Lys^{16}PAL) and GIP(Lys^{37}PAL) displayed resistance to DPP IV, which rapidly cleaved native GIP to the inactive metabolite GIP(3-42), as reported previously.[8] Thus, whereas in vitro degradation of native GIP was substantial by 2 h and complete within 8 h, both fatty acid derivatized analogues remained fully intact after exposure to DPP IV for up to 24 h. This implies that acylation of GIP with a C-16 palmitate group at position 16 or 37 masks the potential cleavage site for DPP IV and that N-terminal protection is not necessary for enzyme resistance.[28−30] This was unexpected given that the three-dimensional structure of GIP amounts to gentle helical turns around a strong backbone as revealed by computer-assisted secondary structure analysis.[37,38] However, other studies have shown that distant regions of GIP affect binding to the active site of DPP IV.[39] Although unconfirmed by mass spectroscopy, another feasible explanation for the unanticipated resistance of both GIP analogues to DPP IV may be the possibility of peptide self-association due to their fatty acid side chains. The possibility also exists that these hydrophobic GIP molecules exhibit inhibitory actions unrelated to normal mechanisms of DPP IV resistance.

Structural modification affecting the DPP IV cleavage site on the amino terminus of GIP is highly influential for bioactivity, potentially resulting in analogues with unchanged, increased, decreased, or even antagonistic potency.[2] Consistent with earlier studies,[9−11] native GIP prominently stimulated cAMP production and insulin secretion in GIP receptor transfected CHL cells and BRIN-BD11 cells in vitro. Both GIP(Lys^{16}PAL) and GIP-(Lys^{37}PAL) exhibited similar dose-dependent effects to native GIP, being reminiscent of the actions of acetylated or pyroglutamyl GIP(Lys^{37}PAL).[28,30] This indicates that the fatty acid derivatized DPP IV resistant GIP molecules retained full affinity for the GIP receptor and ability to activate signal transduction pathways culminating in stimulation of adenylate cyclase and insulin secretion. At very high albumin concentrations insulin-releasing potency of GIP(Lys^{37}PAL) was diminished, presumably reflecting much lower levels of free peptide due to greater albumin binding. Thus, it is apparent from these observations that the inadvertent introduction of DPP IV resistance, by attachment of a palmitate group at either Lys^{16} or Lys^{37} of GIP, is not accompanied by compromised receptor activation and therefore represents a significant and unexpected attribute in terms of likely in vivo bioactivity.

Consistent with this view, GIP(Lys^{16}PAL) and GIP(Lys^{37}PAL) displayed significantly enhanced antihyperglycemic and insulin releasing activity when administered with glucose to the commonly employed ob/ob mouse model.[40] Thus, despite the particularly severe glucose intolerance and insulin resistance exhibited by this mutant, individual plasma glucose and insulin concentrations together with AUC measures were significantly improved compared with native GIP. The insulin response to GIP(Lys^{16}PAL), and especially GIP(Lys^{37}PAL), were particularly prominent during the latter stages of the test. This is consistent with the ability of GIP analogues to overcome any diabetes-associated pancreatic β-cell defects.[20] It also suggests that the two fatty acid derivatized analogues have a protracted in vivo half-life compared to native GIP and in vivo bioactivity similar to N-terminally modified palmitate GIP analogues.[28,30] This is likely due to their receptor binding activity, inherent resistance to DPP IV degradation, and also their affinity to bind to serum albumin, thus preventing kidney catabolism and subsequent removal from the body.

In support of protracted biological activity, single injection of the more potent agonist, GIP(Lys^{37}PAL), resulted in a notable decline of plasma glucose that was sustained for at least 24 h. This was associated with a trend toward elevated insulin, but concentrations were not significantly raised at any time point. This presumably reflects the glucose-dependent insulinotropic actions of GIP, although involvement of the established extra-

pancreatic actions of GIP cannot be totally discounted.[3] In contrast, similar injection of native GIP to nonfasted ob/ob mice had little metabolic consequence even at 4 h, reflecting its rapid degradation by the ubiquitous enzyme DPP IV.[6]

Direct comparison of the efficacy and potency of native GIP and GIP(Lys^{37}PAL) was achieved by dose−response studies involving injection of peptide together with glucose in ob/ob mice. Over a dosage range of 6.25−25 nmol kg^{-1}, native GIP lacked appreciable effects on overall AUC glucose and insulin responses, reflecting the compromised β-cell function and insulin resistance of the ob/ob syndrome at the age studied.[40] In contrast, GIP(Lys^{37}PAL) stimulated insulin release at the lowest dose tested and was consistently more potent than native GIP. Since in vitro potency of the two peptides was similar, this suggests that the benefits of acylation are related to enhancement of biological potency and half-life, plus possible involvement of extrapancreatic actions such as stimulation of glucose uptake,[41] inhibition of hepatic glucose production,[42] and enhancement of insulin-dependent inhibition of glycogenolysis.[43] Comparison with previous studies[29,30] also suggests that GIP(Lys^{37}PAL) has in vivo biological activity at least as good as corresponding acetylated and pyroglutamyl analogues, denoting that acylation of GIP with a C-16 palmitate group at position 37 masks the potential cleavage site for DPP IV and that N-terminal modification may not be obligatory to confer enzyme resistance. Consistent with antidiabetic efficacy, daily injection of ob/ob mice with GIP(Lys^{37}PAL) at 12.5 nmol kg^{-1} body weight per day decreased nonfasting glucose, improved glucose tolerance, and enhanced the plasma insulin response to glucose.

In conclusion, this study has demonstrated that simple acylation of GIP at positions Lys^{16} or Lys^{37} with palmitate prolongs the biological actions and antihyperglycemic activity of GIP. This is presumably achieved through a combination of unexpected disruption of DPP IV mediated peptide degradation and retardation of renal clearance due to albumin binding. The ability of fatty acid derivatized GIP to reproduce the attributes of more complex N-terminally modified analogues, improve glycemic control, and retain antidiabetic effects 24 h after a single injection make these attractive agents for future clinical evaluation as possible once daily treatment of type 2 diabetes.

Experimental Section

Animals. Obese diabetic (ob/ob) mice 14- to 18-week-old were obtained from the Aston colony. The genetic background and characteristics of the colony used have been outlined in detail elsewhere.[40] The animals were housed individually in an air-conditioned room at 22 ± 2 °C with a 12 h light:12 h dark cycle (08:00−20:00 h). All experiments were commenced at 10:00 h. Drinking water and a standard rodent maintenance diet were freely available. All animal experiments were carried out in accordance with the U.K. Animals (Scientific Procedures) Act 1986. No adverse effects were observed following administration of any of the peptides.

Synthesis and Purification of GIP and Related Analogues. GIP was sequentially synthesized on an Applied Biosystems (Foster City, CA) automated peptide synthesizer (Model 432 A) using standard solid-phase Fmoc peptide chemistry as described previously.[11] The two novel fatty acid derivatized GIP analogues, GIP(Lys^{16}PAL) and GIP(Lys^{37}PAL), were synthesized in the same way as native GIP but with the exception that the normal Fmoc-protected lysine residue at either position 16 or 37 was exchanged for an Fmoc-protected Lys(palmitoyl)-OH residue. GIP has four potential acylation sites at Lys^{16}, Lys^{30}, Lys^{33}, and Lys^{37}. The lysine residue nearest the N-terminal and the lysine residue nearest the C-terminal were selected for palmitate acylation (Figure 1). The synthetic peptides were judged pure (>95%) by two diverse reversed-phase HPLC systems, namely Waters Millenium 2010 chromatography system (Software version 2.1.5) and a Thermo Separation Products automated Workstation, using a model P200 pump, an AS3000 autosampler, and a UV2000 tuneable absorbance detector (Thermo Separation Products, CA). Peptides were subsequently characterized using matrix-assisted laser desorption ionization−time-of-flight (MALDI-TOF) mass spectrometry as described previously.[44] This technique was also used to confirm formation of GIP-fatty acid-albumin complex on exposure of GIP analogues to bovine serum albumin in vitro.

DPP IV Degradation Studies. Degradation of GIP and fatty acid derivatized GIP analogues were performed using HPLC analysis as described previously.[11] Briefly, GIP or GIP analogues were incubated in vitro at 37 °C in 50 mM triethanolamine-HCl (pH 7.8, final peptide concentration 2 mM) with purified porcine dipeptidyl peptidase IV (5 mU) for 0, 2, 8, and 24 h. The reactions were subsequently terminated by addition of 10% (v/v) TFA/water, and intact GIP was separated from the major degradation product GIP(3-42) by HPLC using a Vydac C-4 column (4.6 × 250 mm; The Separations Group, Hesparia, CA). Absorbance was monitored at 206 nm using a SpectraSystem UV 2000 detector (Thermoquest Ltd., Manchester, U.K.), and the peaks were collected manually prior to MALDI-TOF mass spectrometry. HPLC peak area data were used to calculate the percentage intact peptide remaining at the various time points during the incubation.

In Vitro Biological Activity. Intracellular cAMP production was measured using previously described GIP-receptor transfected Chinese hamster lung (CHL) fibroblasts.[45] CHL cells were seeded into 12-multiwell plates (Nünc, Roskilde, Denmark) at a density of 10^5 cells per well and allowed to grow for 48 h before being loaded with tritiated adenine (2 μCi). The cells were then incubated at 37 °C for 6 h in 1 mL of DMEM, supplemented with 0.5% (w/v) BSA, and subsequently washed twice with HBS (Hanks buffered saline buffer). The cells were then exposed to varying concentrations of GIP/GIP analogues (10^{-13} to 10^{-6} M) in HBS buffer (estimated albumin concentration 76.5 μM) in the presence of 1 mM 3-isobutyl-1-methylxanthine (IBMX) for 15 min at 37 °C. The medium was subsequently removed, and the cells were lysed with 1 mL of 5% trichloroacetic acid containing 0.1 mM unlabeled cAMP and 0.1 mM unlabeled ATP. The intracellular tritiated cAMP was then separated on Dowex and alumina exchange resins (Life Science Research, Larne, U.K.) as described previously.[11]

Acute insulin-release studies were carried out using clonal pancreatic BRIN-BD11 cells, whose origin, characteristics, and secretory responsiveness have been outlined in detail elsewhere.[46] BRIN-BD11 cells were seeded into 24-multiwell plates at a density of 10^5 cells per well and allowed to attach overnight at 37 °C. Acute tests for insulin release were preceded by 40 min preincubation at 37 °C in 1.0 mL of Krebs Ringer bicarbonate buffer (pH 7.4) supplemented with 0.5% (w/v) BSA and 1.1 mM glucose (estimated albumin concentration 76.5 μM). Test incubations were performed in the presence of 5.6 mM glucose with a range of concentrations (10^{-13} to 10^{-6} M) of GIP or fatty acid derivatized GIP analogues. After 20 min incubation, the buffer was removed from each well, and aliquots (200 μL) were stored at −20 °C for measurement of insulin. The incubation of 0.5% (w/v) BSA in the in vitro incubation buffers is standard practice to minimize loss of peptide (tested or secreted) onto polystyrene tubes and pipet tips. In a separate series of experiments, the insulinotropic effects of native GIP and GIP(Lys^{37}PAL) were examined in the presence of higher concentrations of BSA and following an extended (2 h) peptide incubation in 1% BSA.

In Vivo Biological Activity. Metabolic and dose−response effects of GIP and fatty acid derivatized GIP analogues (at 6.25, 12.5, and 25 nmol kg^{-1} body weight) following a simultaneous intraperitoneal glucose administration (18 mmol kg^{-1} body weight) were examined in 18 h fasted mice. In a separate experiment, nonfasted ob/ob mice received a one-off injection (10:00 h) of saline vehicle (0.9% (w/v), NaCl), native GIP, or fatty acid derivatized GIP (12.5 nmol kg^{-1} body weight). Food was freely available throughout the following 24 h. In a further experimental series,

groups of ob/ob mice received once daily intraperitoneal injections (17:00 h) of either saline vehicle (0.9%, w/v, NaCl) or GIP-(Lys^{37}PAL) at 12.5 nmol kg^{-1} body weight per day. After 14 days, nonfasting glucose, insulin, and intraperitoneal glucose tolerance (18 mmol kg^{-1} body weight) were determined. All test solutions were administered by intraperitoneal injection in a final volume of 5 mL kg^{-1} body weight, and blood was collected from the cut tip of the tail vein of conscious mice into chilled fluoride/heparin glucose microcentrifuge tubes (Sarstedt, Nümbrecht, Germany) immediately prior to injection and at the times indicated on the figures. Plasma was separated using a Beckman microcentrifuge (Beckman Instruments, U.K.; for 30 s at 13 000*g*) and stored at −20 °C prior to glucose and insulin determinations. All acute tests were commenced at 10:00 h.

Plasma Glucose and Insulin Assays. Plasma glucose was assayed by an automated glucose oxidase procedure using a Beckman Glucose Analyzer II.[47] Plasma insulin was assayed by dextran-charcoal RIA as described previously.[40] Incremental areas under plasma glucose and insulin curves (AUC) were calculated using a computer-generated program employing the trapezoidal rule with baseline subtraction.[48]

Analyses. Results are expressed as means ± SEM and data compared using the unpaired Student's *t*-test. Where appropriate, data were compared using repeated measures ANOVA or one-way ANOVA, followed by the Student−Newman−Keuls post hoc test. Groups of data from both were considered to be significantly different if $P < 0.05$.

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Supporting Information Available: Purity of target compounds and spectroscopic data. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Brown, J. C. Enteroinsular axis. In *Gut Peptides: Biochemistry and Physiology*; Dockray, G. J., Walsh, J. H., Eds.; Raven Press: New York, 1994; pp 765−784.

(2) Gault, V. A.; Flatt, P. R.; O'Harte, F. P. M. Glucose-dependent insulinotropic polypeptide analogues and their therapeutic potential for the treatment of obesity-diabetes. *Biochem. Biophys. Res. Commun.* **2003**, *308*, 207−213.

(3) Meier, J. J.; Nauck, M. A.; Schmidt, W. E.; Gallwitz, B. Gastric inhibitory polypeptide: the neglected incretin revisited. *Regul. Pept.* **2002**, *107*, 1−13.

(4) Bailey, C. J.; Flatt, P. R. Development of antidiabetic drugs. In *Drugs, diet and disease: mechanistic approaches to diabetes*; Ioannides, C., Flatt, P. R., Eds.; Ellis Horwood: London, 1995; pp 279−326.

(5) Mentlein, R. Dipeptidyl-peptidase IV (CD26) − role in the inactivation of regulatory peptides. *Regul. Pept.* **1999**, *85*, 9−24.

(6) Kieffer, T. J.; McIntosh, C. H. S.; Pederson, R. A. Degradation of glucose-dependent insulinotropic polypeptide and truncated glucagon-like peptide 1 in vitro and in vivo by dipeptidyl peptidase IV. *Endocrinology* **1995**, *136*, 3585−3596.

(7) Schmidt, W. E.; Siegel, E. G.; Kümmel, H.; Gallwitz, B.; Creutzfeldt, W. Commercially available preparations of porcine glucose-dependent insulinotropic polypeptide (GIP) contain a biologically inactive GIP-fragment and cholecystokinin-33/-39. *Endocrinology* **1987**, *120*, 835−837.

(8) Gault, V. A.; Parker, J. C.; Harriott, P.; Flatt, P. R.; O'Harte, F. P. M. Evidence that the major degradation product of glucose-dependent insulinotropic polypeptide (GIP), GIP(3-42), is a GIP receptor antagonist *in vivo*. *J. Endocrinol.* **2002**, *175*, 525−553.

(9) O'Harte, F. P. M.; Mooney, M. H.; Kelly, C. M. N.; Flatt, P. R. Improved glycaemic control in obese diabetic *ob/ob* mice using N-terminally modified gastric inhibitory polypeptide. *J. Endocrinol.* **2000**, *165*, 639−648.

(10) O'Harte, F. P. M.; Gault, V. A.; Parker, J. C.; Harriott, P.; Mooney, M. H.; Bailey, C. J.; Flatt, P. R. Improved stability, insulin-releasing activity and antidiabetic potential of two novel N-terminal analogues of glucose-dependent insulinotropic polypeptide: *N*-acetyl-GIP and pGlu-GIP. *Diabetologia* **2002**, *45*, 1281−1291.

(11) Gault, V. A.; Flatt, P. R.; Bailey, C. J.; Harriott, P.; Greer, B.; Mooney, M. H.; O'Harte, F. P. M. Enhanced cyclic AMP generation and insulin-releasing potency of two novel N-terminal Tyr^1-modified enzyme resistant forms of GIP, is associated with significant antihyperglycaemic activity in spontaneous obesity-diabetes. *Biochem. J.* **2002**, *367*, 913−920.

(12) Gault, V. A.; Flatt, P. R.; Harriott, P.; Mooney, M. H.; Bailey, C. J.; O'Harte, F. P. M. Improved biological activity of Gly^2- and Ser^2-substituted analogues of glucose-dependent insulinotropic polypeptide. *J. Endocrinol.* **2003**, *176*, 133−141.

(13) Hinke, S. A.; Gelling, R. W.; Pederson, R. A.; Manhart, S.; Nian, C.; Demuth, H. U.; McIntosh, C. H. Dipeptidyl peptidase IV-resistant [d-Ala^2]glucose-dependent insulinotropic polypeptide (GIP) improves glucose tolerance in normal and obese diabetic rats. *Diabetes* **2002**, *51*, 656−661.

(14) Lindsay, J. R.; Au, S. T. B.; Kelly, C. M. N.; O'Harte, F. P. M.; Flatt, P. R. A novel amino-terminally glycated analogue of glucose-dependent insulinotropic polypeptide (GIP), with a prolonged insulinotropic activity in type 2 diabetes mellitus. *Diabetes* **2002**, *51*, 1394 (Abstract).

(15) Elahi, D.; McAloon-Dyke, M.; Kukagawa, N. K.; Meneilly, G. S.; Slater, A. L.; Minaker, K. L.; Habener, J. F.; Andersen, D. K. The insulinotropic actions of glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (7-37) in normal and diabetic subjects. *Regul. Pept.* **1994**, *51*, 63−74.

(16) Nauck, M. A.; Bartels, E.; Ørskov, C.; Ebert, R.; Creutzfeldt, W. Additive insulinotropic effects of exogenous synthetic human gastric inhibitory polypeptide and glucagon-like peptide-1-(7-36) amide infused at near physiological insulinotropic hormone and glucose concentrations. *J. Clin. Endocrinol. Metab.* **1993**, *76*, 912−917.

(17) Vilsboll, T.; Krarup, T.; Madsbad, S.; Holst, J. J. Defective amplification of the late phase insulin response to glucose by GIP in obese Type II diabetic patients. *Diabetologia* **2002**, *45*, 1111−1119.

(18) Krarup, T.; Saurbrey, N.; Moody, A. J.; Kuhl, C.; Madsbad, S. Effect of porcine gastric inhibitory polypeptide on beta-cell function in type I and type II diabetes mellitus. *Metabolism* **1987**, *36*, 677−682.

(19) Meier, J. J.; Gallwitz, B.; Kask, B.; Deacon, C. F.; Holst, J. J.; Schmidt, W. E.; Nauck, M. A. Stimulation of insulin secretion by intravenous bolus injection and continuous infusion of gastric inhibitory polypeptide in patients with type 2 diabetes and healthy control subjects. *Diabetes* **2004**, *53*, S220−224.

(20) Meier, J. J.; Nauck, M. A.; Siepmann, N.; Greulich, M.; Holst, J. J.; Deacon, C. F.; Schmidt, W. E.; Gallwitz, B. Similar insulin secretory response to a gastric inhibitory polypeptide bolus injection at euglycemia in first-degree relatives of patients with type 2 diabetes and control subjects. *Metabolism* **2003**, *52*, 1579−1585.

(21) Meier, J. J.; Nauck, M. A.; Kranz, D.; Holst, J. J.; Deacon, C. F.; Gaeckler, D.; Schmidt, W. E.; Gallwitz, B. Secretion, degradation, and elimination of glucagon-like peptide 1 and gastric inhibitory polypeptide in patients with chronic renal insufficiency and healthy control subjects. *Diabetes* **2004**, *53*, 654−662.

(22) Kurtzhals, P.; Havelund, S.; Jonassen, I.; Kiehr, B.; Larsen, U. D.; Ribel, U.; Markussen, J. Albumin binding of insulins acylated with fatty acids: characterisation of the ligand-protein interaction and correlation between binding affinity and timing of the insulin effect in vivo. *Biochem. J.* **1995**, *312*, 725−731.

(23) Agersø, H.; Jensen, L. B.; Elbrønd, B.; Rolan, P.; Zdravkovic, M. The pharmacokinetics, pharmacodynamics, safety and tolerability of NN2211, a new long-acting GLP-1 derivative, in healthy man. *Diabetologia* **2002**, *45*, 195−202.

(24) (a) Ribel, U.; Larsen, M. O.; Rolin, B.; Carr, R. D.; Wilken, M.; Sturis, J.; Westergaard, L.; Deacon, C. F.; Knudsen, L. B. NN2211: a long-acting glucagon-like peptide-1 derivative with antidiabetic effects in glucose-intolerant pigs. *Eur. J. Pharm.* **2002**, *451*, 217−225. (b) Nauck, M. A.; Niedereichholz, U.; Ettler, R.; Holst, J. J.; Ørskov, C.; Ritzel, R.; Schmigel, W. H. Glucagon-like peptide-1 inhibition of gastric-emptying outweighs its insulinotropic effects in healthy humans. *Am. J. Physiol.* **1997**, *273*, E981−E988.

(25) Ahren, B.; Schmitz, O. GLP-1 receptor agonists and DPP-4 inhibitors in the treatment of type 2 diabetes. *Horm. Metab. Res.* **2004**, *36*, 867−76.

(26) Nauck, M. A.; Meier, J. J. Glucagon-like peptide 1 and its derivatives in the treatment of diabetes. *Regul. Pept.* **2005**, *128*, 135−148.

(27) Meier, J. J.; Goetze, O.; Anstipp, J.; Hagemann, D.; Holst, J. J.; Schmidt, W. E.; Gallwitz, B.; Nauck, M. A. Gastric inhibitory polypeptide (GIP) does not inhibit gastric emptying in man. *Am. J. Physiol. Endocrinol. Metab.* **2003**, *286*, E621−E625.

(28) Irwin, N.; Green, B. D.; Gault, V. A.; Greer, B.; Harriott, P.; Bailey, C. J.; Flatt, P. R.; O'Harte, F. P. M. Antidiabetic potential of two novel fatty acid derivatised, *N*-terminally modified analogues of glucose-dependent insulinotropic polypeptide (GIP): *N*-AcGIP(LysPAL^{16}) and *N*-AcGIP(LysPAL^{37}). *Biol. Chem.* **2005**, *386*, 679−687.

(29) Irwin, N.; Green, B. D.; Mooney, M. H.; Greer, B.; Harriott, P.; Bailey, C. J.; Gault, V. A.; O'Harte, F. P. M.; Flatt, P. R. A novel, long-acting agonist of GIP suitable for once daily administration in type 2 diabetes. *J. Pharm. Exp. Ther.* **2005**, *314*, 1187−1194.

(30) Irwin, N.; Green, B. D.; Gault, V. A.; Greer, B.; Harriott, P.; Bailey, C. J.; Flatt, P. R.; O'Harte, F. P. M. Degradation, insulin secretion and antihyperglycaemic actions of two palmitate-derivitised *N*-terminal pyroglutamyl analogues of glucose-dependent insulinotropic polypeptide (GIP). *J. Med. Chem.* **2005**, *48*, 1244−1250.

(31) Gault, V. A.; O'Harte, F. P. M.; Flatt, P. R. Glucose-dependent insulinotropic polypeptide (GIP): anti-diabetic and anti-obesity potential? *Neuropeptides* **2003**, *37*, 253−263.

(32) Deacon, C. F. Therapeutic strategies based on glucagon-like peptide 1. *Diabetes* **2004**, *53*, 2181−2189.

(33) Baggio, L. L.; Huang, Q.; Brown, T. J.; Drucker, D. J. A recombinant human glucagon-like peptide (GLP)-1-albumin protein (albugon) mimics peptidergic activation of GLP-1 receptor-dependent pathways coupled with satiety, gastrointestinal motility, and glucose homeostasis. *Diabetes* **2004**, *53*, 2492−2500.

(34) Kim, J. G.; Baggio, L. L.; Bridon, D. P.; Castaigne, J. P.; Robitaille, M. F.; Jette, L.; Benquet, C.; Drucker, D. J. Development and characterisation of a glucagon-like peptide 1-albumin conjugate: the ability to activate the glucagon-like peptide 1 receptor in vivo. *Diabetes* **2003**, *52*, 751−759.

(35) Göke, R.; Fehmann, H. C.; Linn, T.; Schmidt, H.; Krause, M.; Eng, J.; Göke, B. Exendin-4 is a high potency agonist and truncated exendin-(9−39)-amide an antagonist at the glucagon-like peptide 1-(7-36)-amide receptor of insulin-secreting beta-cells. *J. Biol. Chem.* **1993**, *268*, 19650−19655.

(36) Meier, J. J.; Gallwitz, B.; Nauck, M. A. Glucagon-like peptide 1 and gastric inhibitory polypeptide: potential applications in type 2 diabetes mellitus. *BioDrugs* **2003**, *17*, 93−102.

(37) Alana, I.; Hewage, C. M.; Malthouse, J. P.; Parker, J. C.; Gault, V. A.; O'Harte, F. P. M. NMR structure of the glucose-dependent insulinotropic polypeptide fragment, GIP(1−30)amide. *Biochem. Biophys. Res. Commun.* **2004**, *325*, 281−286.

(38) Hinke, S. A.; Manhart, S.; Pamir, N.; Demuth, H.; Gelling, R.; Pederson, R. A.; McIntosh, C. H. Identification of a bioactive domain in the amino-terminus of glucose-dependent insulinotropic polypeptide (GIP). *Biochim. Biophys. Acta.* **2001**, *1547*, 143−155.

(39) Kuhn-Wache, K.; Hoffmann, T.; Manhart, S.; Brandt, W.; Demuth, H. U. The specificity of DP IV for natural substrates is peptide structure determined. *Adv. Exp. Med. Biol.* **2003**, *524*, 57−63.

(40) Bailey, C. J.; Flatt, P. R. Influence of genetic background and age on the expression of the obese hyperglycaemic syndrome in Aston *ob/ob* mice. *Internat. J. Obes.* **1982**, *6*, 11−21.

(41) O'Harte, F. P. M.; Gray, A. M.; Flatt, P. R. Gastric inhibitory polypeptide and effects of glycation on glucose transport and metabolism in isolated mouse abdominal muscle. *J. Endocrinol.* **1998**, *156*, 237−243.

(42) Hartmann, H.; Ebert, R.; Creutzfeldt, W. Insulin-dependent inhibition of hepatic glycogenolysis by gastric inhibitory polypeptide (GIP) in perfused rat liver. *Diabetologia* **1986**, *29*, 112−114.

(43) Elahi, D.; Meneilly, G. S.; Minaker, K. L.; Rowe, J. W.; Anderson, D. K. Regulation of hepatic glucose uptake by gastric inhibitory polypeptide in man. *Proceedings of Sixth International Conference on Gastrointestinal Hormones*, Vancouver, 1986, p 18.

(44) Gault, V. A.; Irwin, N.; Harriott, P.; Flatt, P. R.; O'Harte, F. P. M. DPP IV resistance and insulin releasing activity of a novel di-substituted analogue of glucose-dependent insulinotropic polypeptide, (Ser^{2}-Asp^{13})GIP. *Cell Biol. Internat.* **2002**, *27*, 41−46.

(45) Gremlich, S.; Porret, A.; Hani, E. H.; Cherif, D.; Vionnet, N.; Froguel, P.; Thorens, B. Cloning, functional expression, and chromosomal localisation of the human pancreatic islet glucose-dependent insulinotropic polypeptide receptor. *Diabetes* **1995**, *44*, 1202−1208.

(46) McClenaghan, N. H.; Barnett, C. R.; Ah-Sing, E.; Abdel-Wahab, Y. H. A.; O'Harte, F. P. M.; Yoon, T.-W.; Swanston-Flatt, S. K.; Flatt, P. R. Characterization of a novel glucose-responsive insulin-secreting cell line, BRIN−BD11, produced by electrofusion. *Diabetes* **1996**, *45*, 1132−1140.

(47) Stevens, J. F. Determination of glucose by an automatic analyser. *Clin. Chem. Acta* **1971**, *32*, 199−201.

(48) Burington, R. S. *Handbook of Mathematical Tables and Formulae*; McGraw-Hill: New York, 1973.

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