Degradation, Insulin Secretion, and Antihyperglycemic Actions of Two Palmitate-Derivitized *N*-Terminal Pyroglutamyl Analogues of Glucose-Dependent Insulinotropic Polypeptide

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Exploitation of glucose-dependent insulinotropic polypeptide (GIP) is hindered by its short biological half-life and rapid renal clearance. To circumvent these problems, two novel acylated *N*-terminally modified GIP analogues, *N*-pGluGIP(LysPAL^{16}) and *N*-pGluGIP(LysPAL^{37}), were evaluated. In contrast to native GIP, both analogues were completely resistant to dipeptidyl peptidase IV degradation. In GIP-receptor transfected fibroblasts, *N*-pGluGIP(LysPAL^{16}) and *N*-pGluGIP(LysPAL^{37}) exhibited enhanced stimulation of cAMP production. Insulinotropic responses in clonal beta-cells were similar to native GIP. When administered together with glucose to ob/ob mice, the glycemic excursions were significantly less for both analogues and insulin responses were greater than native GIP. Extended insulinotropic and antihyperglycemic actions were also evident. These data indicate that palmitate-derivitized analogues of *N*-terminal pyroglutamyl GIP represent a novel class of stable, long-acting, and effective GIP analogues for potential type 2 diabetes therapy.

Introduction

Glucose-dependent insulinotropic polypeptide (GIP) is an incretin hormone secreted from the intestinal K-cells in response to oral nutrient absorption.[1] A potent insulin-releasing hormone of the enteroinsular axis,[2] GIP exerts various beneficial effects on pancreatic beta-cell function[3−5] and exhibits antihyperglycemic extra-pancreatic effects.[6] These actions and the glucose-dependency of insulin secretion which minimizes the risk of hypoglycemia make GIP very attractive as a potential antidiabetic drug.[7] A spate of recent publications has led to a resurgence of interest in GIP-based antidiabetic therapies. Despite earlier claims,[8,9] it now seems that a reduced GIP-induced insulin secretion in patients with type 2 diabetes is more likely due to a general defect of beta-cell function rather than a specific defect of GIP action.[10,11] Furthermore, GIP does not slow gastric emptying in humans, underlining a major difference between GIP and its sister incretin, glucagon-like peptide-1 (GLP-1).[12]

Dipeptidyl peptidase IV (DPP IV) rapidly hydrolyses GIP at the *N*-terminus to GIP(3−42),[13] rendering the peptide noninsulinotropic[14] and antagonistic of its own receptor.[15] This ubiquitous serine protease has been a major hindrance to the clinical development of GIP; however, various strategies to overcome its degradation by DPP IV are proving successful. For example, several analogues of GIP have been tested that are profoundly resistant to DPP IV and able to overcome any reported unresponsiveness of the beta-cell in type 2 diabetes.[16−21] A small number of these analogues, such as *N*-pGluGIP, appear to be highly effective agonists of the GIP receptor.[17,18] Furthermore, administration of DPP IV inhibitors to dogs and anesthetized pigs increases the proportion of intact GIP significantly and the result is improved insulin release and glucose-lowering action.[22,23]

While there are now a number of publications identifying how GIP may be *N*-terminally modified to prevent degradation by DPP IV, the problem of rapid elimination from the bloodstream by renal filtration has not yet been tackled.[24] Thus, although DPP IV resistant analogues of GIP may improve the short half-life in vivo (7 min),[25] these analogues may not be optimal as they are still subject to clearance by the kidneys. The importance of the kidneys in the final elimination of GIP has been underlined by a study comparing half-lives of GIP and its metabolites in healthy control subjects and patients with chronic renal insufficiency.[24] In healthy control subjects, intact GIP had a half-life of less than 5 min and GIP(3−42) less than 23 min, while patients with renal disease displayed half-lives of 7 and 38 min, respectively.[24]

Various strategies are available to extend the half-life of peptide hormones in the circulation. One such strategy involves the derivitization of peptides with fatty acids, otherwise known as acylation. Acylation facilitates binding to serum proteins, thus reducing renal filtration and prolonging biological activity. This has been successfully applied to extend the action of insulin, through acylation of the ϵ-amino group of Lys^{B29}.[26] Acylated compounds of GIP's sister hormone, GLP-1, have been produced.[27,28] GLP-1 compounds such as

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Table 1. Structural Characterization of GIP Peptides by MALDI-TOF Mass Spectrometry[a]

GIP peptide	M_r (Da)		difference (Da)
	theoretical	experimental	
GIP	4982.4	4983.7	1.3
N-pGluGIP(LysPAL[16])	5332.1	5333.1	1.0
N-pGluGIP(LysPAL[37])	5332.1	5334.5	2.4

[a] Peptide samples were mixed with α-cyano-4-hydroxycinnamic acid and applied to the sample plate of a Voyager-DE BioSpectrometry workstation, and the mass-to-charge (*m/z*) ratio vs relative peak intensity was recorded.

NN2211, CJC-1131, LY315902, LY307161, and AC2993 display greatly prolonged pharmacokinetic profiles (8−18 h) in humans.[27] However, some acylated GLP-1 analogues are problematic in terms of bioactivity and bioavailability,[28] while others, such as NN2211, have met with side effects such as nausea and dizziness.[29,30] Given the fact that GLP-1 is highly inhibitive of gastric emptying in humans[31] and GIP is not,[12] it is possible that long-acting analogues of GIP would be more attractive therapeutically.

The development and testing of novel GIP analogues with enhanced metabolic stability and reduced renal filtration can potentially be useful treatment of type 2 diabetes. We have previously demonstrated that *N*-terminal pyroglutamyl GIP (*N*-pGluGIP) is a DPP IV resistant GIP agonist that exhibits potent insulinotropic and antihyperglycemic actions in an animal model of type 2 diabetes.[17] In this study, we have synthesized two novel acylated analogues of *N*-terminally modified *N*-pGluGIP, namely *N*-pGluGIP(LysPAL[16]) and *N*-pGluGIP(LysPAL[37]). Both GIP analogues contain a C-16 palmitate (PAL) group linked to the ε-amino group of lysine (Lys) at positions 16 or 37. Initially, we investigated their resistance to DPP IV degradation, ability to stimulate cAMP production, and in vitro insulinotropic action. We then undertook a series of in vivo metabolic studies to examine the glucose-lowering and insulin-releasing actions of *N*-pGluGIP(LysPAL[16]) and *N*-pGluGIP(LysPAL[37]) in the commonly employed (ob/ob) mouse model of type 2 diabetes.

Results

Structural Identification. Table 1 shows the experimental masses obtained using MALDI-TOF mass spectrometry for purified GIP, *N*-pGluGIP(LysPAL[16]), and *N*-pGluGIP(LysPAL[37]). The molecular masses resolved using the mass-to-charge (*m/z*) ratio were 4983.7 Da for native GIP (theoretical mass 4982.4 Da), 5333.1 Da for *N*-pGluGIP(LysPAL[16]) (theoretical mass 5332.1 Da), and 5334.5 Da for *N*-pGluGIP(LysPAL[37]) (theoretical mass 5332.1 Da). Experimental masses closely correlated to theoretical masses and thus confirmed successful peptide synthesis.

Degradation by DPP IV. Figure 1 shows the progressive degradation by DPP IV of GIP(1−42) to truncated GIP(3−42). Degradation of native GIP was complete by 8 h, with only truncated GIP(3−42) metabolite remaining. The estimated $t_{1/2}$ following exposure to DPP IV was 2.1 h. Contrastingly, acylated GIP analogues *N*-pGluGIP(LysPAL[16]) and *N*-pGluGIP(LysPAL[37]) were fully intact, even following prolonged 24 h incubations (Figure 1).

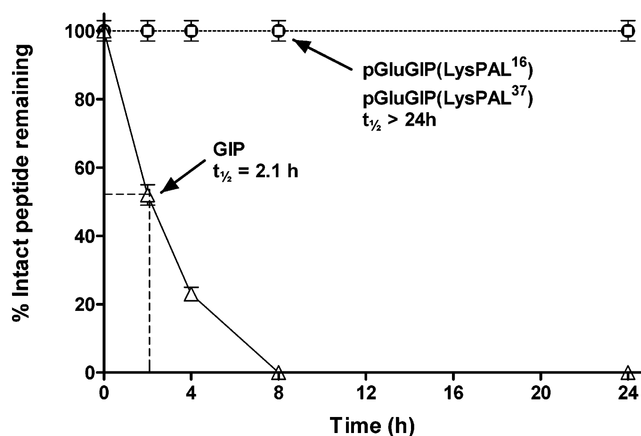

Figure 1. DPP IV degradation profiles for GIP (Δ), *N*-pGluGIP(LysPAL[16]) (□), and *N*-pGluGIP(LysPAL[37]) (●). Resistance of each peptide to degradation by DPP IV (5 mU) was measured following 0, 2, 4, 8, and 24 h incubations. Reaction products were subsequently analyzed by HPLC, and degradation was expressed as a percentage of intact peptide relative to the major degradation fragment, GIP(3−42). Values represent means ± SEM for three separate experiments.

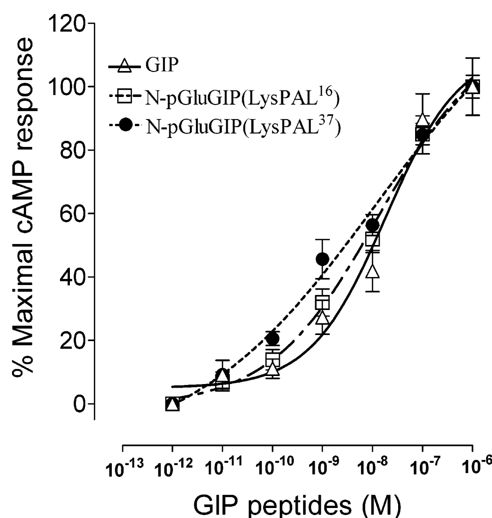

Figure 2. Stimulation of intracellular cAMP production by GIP, *N*-pGluGIP(LysPAL[16]), and *N*-pGluGIP(LysPAL[37]). CHL cells stably transfected with the human GIP receptor were exposed to various peptide concentrations for 20 min. Each experiment was performed in triplicate (n = 3), and values are expressed (means ± SEM) as a percentage maximum GIP response.

Stimulation of in Vitro cAMP Production. The dose-dependent stimulatory effects of GIP and fatty acid derivatized analogues on intracellular cAMP production following incubation with GIP-receptor transfected CHL fibroblasts are shown in Figure 2. The concentration of GIP, *N*-pGluGIP(LysPAL[16]), or *N*-pGluGIP(LysPAL[37]) that produced 50% maximal formation of cAMP (EC_{50}) was approximately 18.2, 3.2, and 2.7 nM, respectively. No significant differences were found between the potency of the three peptides.

Stimulation of in Vitro Insulin Secretion. Figure 3 shows the effect of increasing concentrations of GIP, *N*-pGluGIP(LysPAL[16]), and *N*-pGluGIP(LysPAL[37]) on insulin secretion from clonal BRIN-BD11 cells. All peptides stimulated insulin release by up to 2.5-fold in a concentration-dependent manner. There were no apparent differences in potency in vitro.

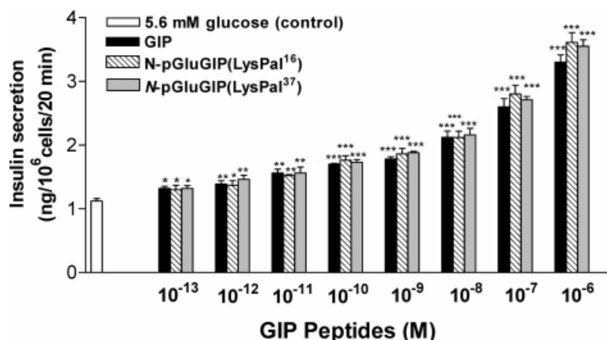

Figure 3. Insulinotopic effects of GIP, N-pGluGIP(LysPAL^{16}), and N-pGluGIP(LysPAL^{37}) in BRIN-BD11 cells. Cells were exposed to various concentrations of GIP peptides for an acute 20 min period in the presence of 5.6 mM glucose. Values represent means ± SEM for eight separate observations. *P < 0.05, **P < 0.01, ***P < 0.001 compared with glucose control.

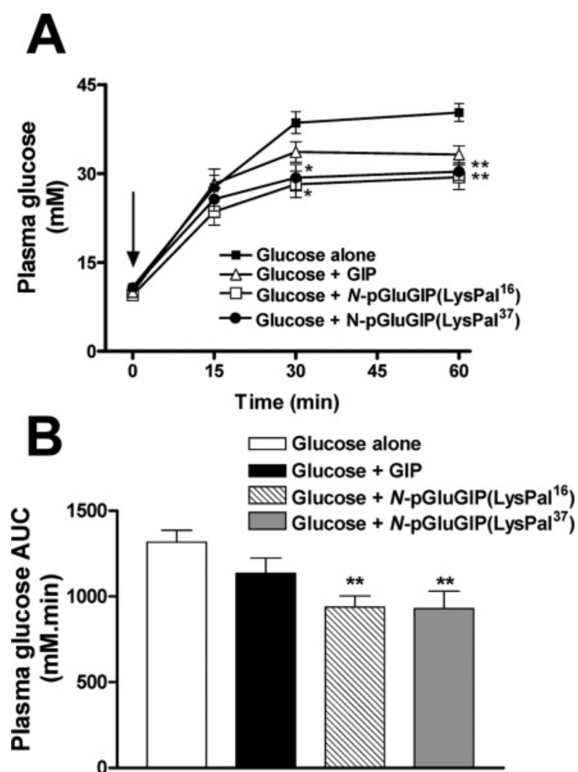

Figure 4. Glucose-lowering effects of GIP, N-pGluGIP-(LysPAL^{16}), and N-pGluGIP(LysPAL^{37}) in 18-h fasted ob/ob mice. (A) Plasma glucose concentrations were measured prior to and after ip administration of glucose alone (18 mmol/kg of body weight), or in combination with GIP, N-pGluGIP(Lys-PAL^{16}), or N-pGluGIP(LysPAL^{37}) (25 nmol/kg). The time of injection is indicated by the arrow (0 min). (B) Plasma glucose area under the curve (AUC) values for 0−60 min postinjection. Values represent means ± SEM for eight mice. *P < 0.05, **P < 0.01 compared to glucose alone.

Antihyperglycemic and Insulin-Releasing Activity in ob/ob Mice. Figure 4A shows the plasma glucose responses to intraperitoneal glucose alone or in combination with GIP, N-pGluGIP(LysPAL^{16}), or N-pGluGIP-(LysPAL^{37}) (25 nmol/kg of body weight). Injection of glucose alone resulted in a rapid and protracted rise in glucose concentration. The glucose excursion following native GIP was not significantly different at the 15, 30, and 60 min time points. In contrast, glucose concentrations following N-pGluGIP(LysPAL^{16}) or N-pGluGIP-(LysPAL^{37}) were significantly lower after 30 min (P < 0.05) and 60 min (P < 0.01). Area under the curve (AUC) analysis (0−60 min, Figure 4B) confirmed the significant glucose-lowering actions of N-pGluGIP(LysPAL^{16}) and N-pGluGIP(LysPAL^{37}) (P < 0.01). As shown in Figure 5A,B, these effects were associated with corresponding changes of insulin release. GIP caused significantly greater insulin release than glucose alone (P < 0.05). However, the insulin-releasing actions of N-pGluGIP(LysPAL^{16}) and N-pGluGIP(LysPAL^{37}) were substantially greater (P < 0.001) and protracted at 60 min. N-pGluGIP(LysPAL^{37}) was the most potent analogue based on overall changes in glucose and insulin concentrations (AUC, Figures 4B and 5B).

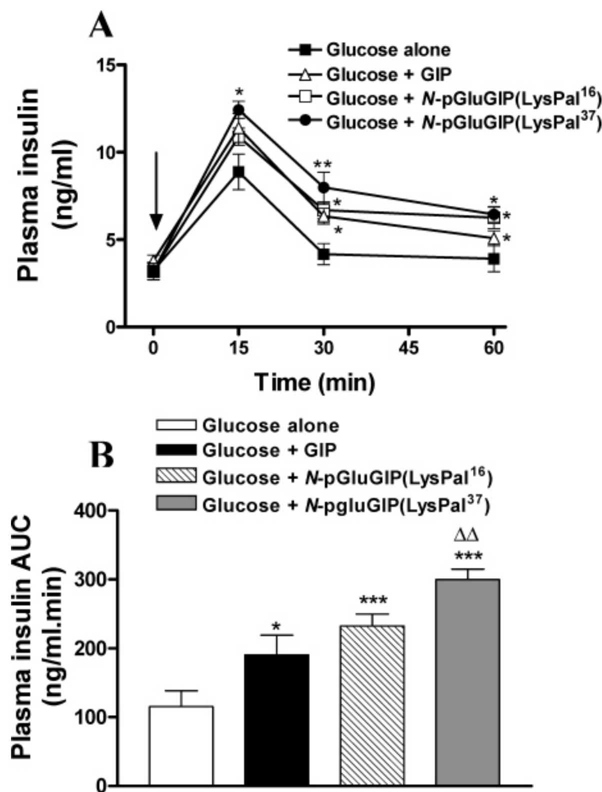

Figure 5. Insulin-releasing effects of GIP, N-pGluGIP-(LysPAL^{16}), or N-pGluGIP(LysPAL^{37}) in 18-h fasted ob/ob mice. (A) Plasma insulin concentrations were measured prior to and after ip administration glucose alone (18 mmol/kg of body weight) or in combination with GIP, N-pGluGIP(LysPAL^{16}), or N-pGluGIP(LysPAL^{37}) (25 nmol/kg). The time of injection is indicated by the arrow (0 min). (B) Plasma insulin area under the curve (AUC) values for 0−60 min postinjection. Values represent means ± SEM for eight mice. *P < 0.05, **P < 0.01, ***P < 0.001 compared to glucose alone. $^{\Delta\Delta}P$ < 0.01 compared to native GIP.

Long-Lasting Antihyperglycemic Effects in ob/ob Mice. N-pGluGIP(LysPAL^{37}) was selected for further evaluation of long-lasting metabolic effects. As shown in Figure 6, the glucose-lowering action of N-pGluGIP-(LysPAL^{37}) was clearly evident when given 4 h before administration of an intraperitoneal glucose load. AUC glucose values were decreased by 21% compared with saline-treated controls (P < 0.05; Figure 6B). In contrast, native GIP lacked significant effects on AUC glucose values even when administered simultaneously with glucose (Figure 4B).

Dose-Dependent Metabolic Effects in ob/ob Mice. Dose-dependent studies were conducted using N-pGlu-

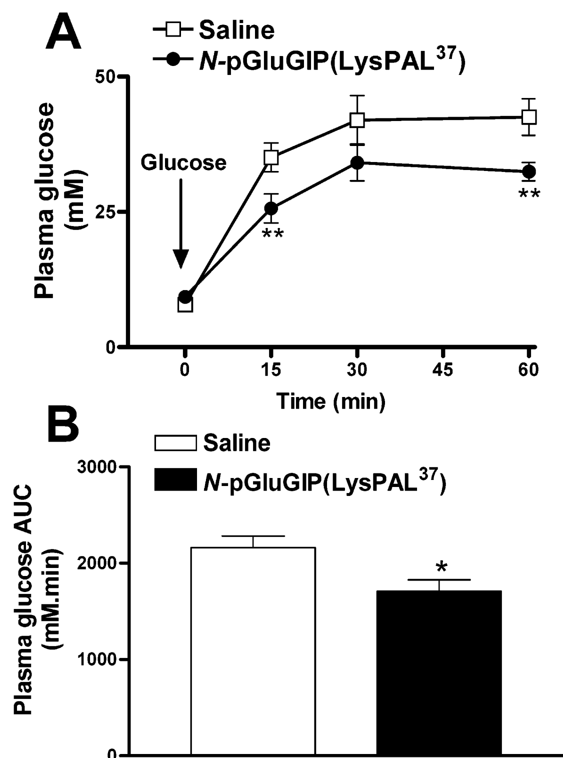

Figure 6. Persistence of glucose-lowering effects of *N*-pGluGIP(LysPAL^{37}) in 18-h fasted ob/ob mice at 4 h after injection. (A) Plasma glucose concentrations were measured prior to and after ip administration of glucose (18 mmol/kg of body weight) in mice injected 4 h previously with *N*-pGluGIP-(LysPAL^{37}) (25 nmol/kg of body weight, ip) or saline. The time of the glucose injection is indicated by the arrow. (B) Plasma glucose area under the curve (AUC) values for 0−60 min postinjection. Values represent means ± SEM for six mice. *P < 0.05, **P < 0.01 compared mice injected 4 h earlier with saline.

GIP(LysPAL^{37}). Figure 7 shows the AUC values (0−60 min) for plasma glucose and insulin following the administration of glucose alone or in combination with either GIP or *N*-pGluGIP(LysPAL^{37}) at 6.25, 12.5, and 25 nmol/kg of body weight. The maximum glucose-lowering effects of GIP and *N*-pGluGIP(LysPAL^{37}) were observed at the 25 nmol/kg dose. However, only *N*-pGluGIP(LysPAL^{37}) caused significant glucose-lowering (1.4-fold; P < 0.05; Figure 7A). Figure 7B shows the equivalent AUC values for plasma insulin. While a 6.25 or 12.5 nmol/kg dose of GIP had no effect on insulin levels, a 25 nmol/kg dose significantly raised overall plasma insulin levels (1.7-fold; P < 0.05). In contrast, either a 12.5 or 25 nmol/kg dose of *N*-pGluGIP(LysPAL^{37}) caused significant increases in circulating insulin of 1.6-fold (P < 0.05) and 2.6-fold (P < 0.001), respectively.

Discussion

In recent years, many different GIP analogues have been synthesized and tested for potential antidiabetic properties.[16−21] Until now, the aim has been to *N*-terminally modify GIP to confer it with resistance to DPP IV. So far, this has been very successful with some extremely potent DPP IV-resistant analogues of GIP being developed. Interestingly, some of these analogues (e.g. *N*-pGluGIP) have enhanced insulinotropic and glucose-lowering activities compared with native GIP.[17]

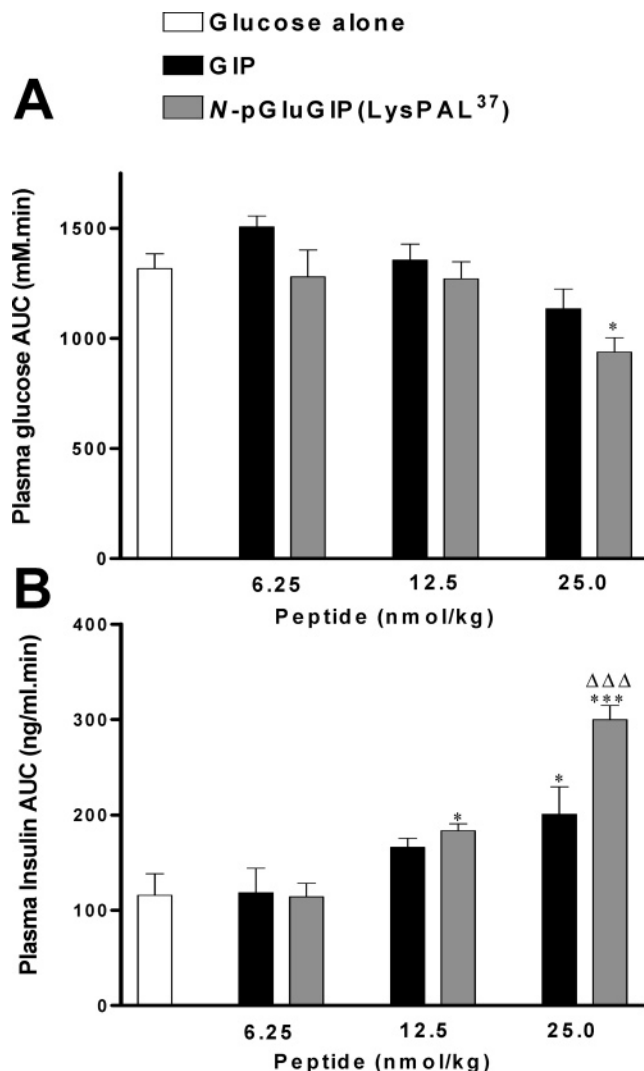

Figure 7. Dose-dependent effects of GIP and *N*-pGluGIP-(LysPal^{37}) on glucose and insulin levels in 18-h fasted ob/ob mice. The incremental area under the curve (AUC; 0−60 min) for (A) glucose and (B) insulin following ip administration of glucose alone (18 mmol/kg of body weight) or with various doses (6.25, 12.5, and 25 nmol/kg) of GIP or *N*-pGluGIP-(LysPal^{37}). Values represent means ± SEM for eight mice. *P < 0.05, ***P < 0.001 compared to glucose alone. $^{\Delta\Delta\Delta}P$ < 0.01 compared to native GIP at same dose.

However, recent reports indicate the importance of the kidney for the final elimination of GIP from the circulation.[24] As a result, it is predicted that further structural modification of GIP to delay removal of the peptide by renal filtration may prolong bioactivity.

Although the *N*-terminally modified analogue, *N*-pGluGIP, displays profound resistance to DPP IV and strong biological activity,[17] it is still subject to renal clearance. Using an approach already established for insulin and other peptides,[26,27] we have acylated this GIP analogue with palmitate to facilitate binding to plasma proteins, disrupt clearance by the kidney, and ultimately prolong biological activity. The ϵ-amino group of lysine residues is particularly well-suited for peptide acylation. GIP has four such sites at Lys^{16}, Lys^{30}, Lys^{33}, and Lys^{37}. A computer-generated three-dimensional molecular model has approximated the structure of GIP to be a gently rotating helical chain.[32] Accordingly, we selected the lysine nearest the *N*-terminus and the

lysine nearest the C-terminus as palmitate acylation sites. Thus, in addition to having an *N*-terminal pyroglutamyl group at Tyr[1], the analogues prepared in this study possessed a 16-carbon palmitate group conjugated to the ϵ-amino group of either Lys[16] or Lys[37].

After confirmation of successful synthesis by MALDI-TOF mass spectrometry, the susceptibility of GIP peptides to DPP IV degradation was examined. In contrast to native GIP, which underwent rapid metabolism by DPP IV to the truncated peptide GIP(3−42), *N*-pGluGIP(LysPAL[16]) and *N*-pGluGIP(LysPAL[37]) were completely resistant to enzymatic degradation. Thus, as expected, fatty acid derivatization did not affect the established DPP IV resistance of *N*-pGluGIP.[17]

Consistent with previous studies,[18,19] native GIP concentration-dependently stimulated cAMP production with an EC_{50} value of 18.2 nM. Corresponding values for *N*-pGluGIP(LysPAL[16]) and *N*-pGluGIP(LysPAL[37]) were approximately 6-fold better than native GIP, but this was not statistically significant. Similarly, native GIP and both fatty acid derivatized pGluGIP analogues induced concentration-dependent stepwise increases of insulin secretion from BRIN-BD11 cells. No significant differences were noted between the potency of the three peptides, indicating that the acylated forms of GIP were at least equally as potent as native GIP in activating the GIP-receptor. This is significant, especially when considering that these analogues will presumably bind appreciably to albumin in the incubation buffers. Thus, the present inability to show significantly greater insulinotropic potency of acylated forms of pGluGIP over native GIP, as noted in previous studies for pGluGIP,[17] may largely reflect lower effective peptide concentrations due to in vitro albumin binding.

In vivo bioactivity of acylated forms of *N*-pGluGIP and native GIP were compared using obese diabetic (ob/ob) mice, a commonly employed animal model of type 2 diabetes.[33] These mice display several characteristics associated with type 2 diabetes; including obesity, insulin resistance, hyperglycemia, hyperinsulinemia, and defective beta-cell function.[33] GIP has been shown previously to have modest antihyperglycemic and insulinotropic activity, when administered to ob/ob mice.[18,19] Although native GIP significantly stimulated insulin release in the present study, this was not accompanied by significant glucose-lowering, reflecting the severe insulin resistance of the ob/ob syndrome at the age tested.[34] However, both *N*-pGluGIP(LysPAL[16]) and *N*-pGluGIP(LysPAL[37]) were highly effective in lowering glucose and raising insulin levels, indicating prolonged action and an ability to overcome the insulin resistance and any apparent desensitization of GIP-receptor action.

Although the fatty acid derivatized analogues appeared equipotent in terms of glucose-lowering activities, *N*-pGluGIP(LysPAL[37]) was substantially more effective and longer acting in stimulating insulin release. The reason behind the increased effectiveness of this C-terminally derivatized peptide to raise plasma insulin is unclear but may include differences in beta-cell stimulation due to differences in effective concentrations as a result of protein binding. Further, lack of reciprocal glucose-lowering effect may suggest that *N*-pGluGIP(LysPAL[16]) and *N*-pGluGIP(LysPAL[37]) have different potencies in stimulating glucose disposal in peripheral tissues. The extent to which this reflects changes in the various non-beta-cell and extrapancreatic actions of GIP merits further investigation.[35] However, it is clear from present dose−response studies that *N*-pGluGIP(LysPAL[37]) was more potent than native GIP, even in acute studies, and more effective over 60 min at doses as low as 12.5 nmol/kg.

Comparison of the present short-term in vivo effects of the two acylated *N*-pGluGIP analogues with previous studies of *N*-pGluGIP [17] suggest quite similar bioactivities of these first- and second-generation analogues. One possible explanation is slower release of fatty acid derivatized GIP into the circulation from the injection site, but further studies are needed to clarify this point. However, such an effect would be more than offset in the longer term by the prolonged biological activity profile of the acylated peptide. Indeed, the persistence of the glucose-lowering actions of *N*-pGluGIP(LysPAL[37]) even at 4 h after a single injection greatly encourages further studies evaluating the longer term benefits of this *N*-terminally protected palmitate-derivatized GIP analogue in type 2 diabetes.

In conclusion, acylation of DPP IV resistant *N*-terminal analogues of GIP, such as *N*-pGluGIP, with palmitate generates bioactive molecules with the prospect of improved long-term potency and duration of action. *N*-pGluGIP(LysPAL[37]) appears to be a particularly promising antihyperglycemic, insulin-releasing analogue of GIP for further evaluation. It is hoped that long-acting GIP candidate molecules such as *N*-pGluGIP(LysPAL[37]) will lead in the future to a once-daily treatment for type 2 diabetes.

Experimental Section

Reagents. High-performance liquid chromatography (HPLC) grade acetonitrile was obtained from Rathburn (Walkersburn, Scotland). Sequencing grade trifluoroacetic acid (TFA), dipeptidylpeptidase IV (DPP IV), isobutylmethylxanthine (IBMX), adenosine 3,5-cyclic monophosphate (cAMP), adenosine 5′-triphosphate (ATP), and α-cyano-4-hydroxycinnamic acid were all purchased from Sigma (Poole, Dorset, UK). Fmoc-protected amino acids were obtained from Calbiochem Novabiochem (Beeston, Nottingham, UK). RPMI 1640 and DMEM tissue culture medium, fetal bovine serum, penicillin, and streptomycin were all purchased from Gibco (Paisley, Strathclyde, Scotland). The chromatography columns used for cAMP assay, Dowex AG 50WX and neutral alumina AG7, were obtained from Bio-Rad (Life Science Research, Alpha Analytical, Larne, N. Ireland). Tritiated adenine (TRK311) was obtained from Amersham Pharmacia Biotech, Bucks, UK. All water used in these experiments was purified using a Milli-Q water purification system (Millipore, Milford, MA). All other chemicals used were of the highest available purity.

Synthesis, Purification, and Characterization of GIP and Related Analogues. GIP, *N*-pGluGIP(LysPAL[16]), and *N*-pGluGIP(LysPAL[37]) were sequentially synthesized on an Applied Biosystems automated peptide synthesizer (Model 432A, Applied Biosystems, Foster City, CA) using standard solid-phase Fmoc peptide chemistry.[36] Peptides were synthesized sequentially, starting with a preloaded Fmoc-Gln-Wang resin. The synthetic peptides were judged pure by reversed-phase HPLC on a Waters Millenium (Milford, MA) 2010 chromatography system (Software version 2.1.5) when purity was in excess of 99%. Peptides were characterized by matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry, as described previously.[37]

Degradation of GIP and Related Analogues by DPP IV. GIP, *N*-pGluGIP(LysPAL[16]), and *N*-pGluGIP(LysPAL[37]) were incubated in vitro at 37 °C with purified DPP IV (5 mU)

for 0, 2, 4, 8 and 24 h in 50 mM triethanolamine-HCl buffer (pH 7.8; final peptide concentration 2 mM). The enzymatic reactions were terminated by the addition of 15 μL 10% (v/v) TFA/water. The reaction products were then applied to a Vydac C-4 column (4.6 × 250 mm; The Separations Group, Hesparia, CA) and the major degradation fragment, GIP(3−42), was separated from intact GIP. The column was equilibrated with 0.12% (v/v) TFA/water at a flow rate of 1.0 mL/min using 0.1% (v/v) TFA in 70% acetonitrile/water, the concentration of acetonitrile in the eluting solvent was raised from 0% to 40% over 10 min, and from 40% to 75% over 35 min. The absorbance was monitored at 206 nm using a SpectraSystem UV 2000 Detector (Thermoquest Ltd., Manchester, UK), and the peaks were collected manually prior to MALDI-TOF mass spectrometry. HPLC peak area data were used to calculate the percent intact peptide remaining during the incubation.

Cells and Culture. Chinese hamster lung (CHL) fibroblasts transfected with the GIP-receptor[38] were cultured using DMEM tissue culture medium containing 10% fetal bovine serum and 1% (v/v) antibiotics (100 U/mL penicillin and 0.1 mg/mL streptomycin). BRIN-BD11 cells, characterized previously,[39] were cultured using RPMI-1640 tissue culture medium containing 10% fetal bovine serum and 1% (v/v) antibiotics (100 U/mL penicillin, 0.1 mg/mL streptomycin). All cells were maintained in sterile tissue culture flasks (Corning Glass Works, UK) at 37 °C in an atmosphere of 5% CO_2 and 95% air using a LEEC incubator (Laboratory Technical Engineering, Nottingham, UK).

Effects of GIP and Related Analogues on Cyclic AMP Production. CHL fibroblasts were seeded into 12-multiwell plates (Nünc, Roskilde, Denmark) at a density of 1.0 × 10^5 cells per well. The cells were allowed to grow in culture for 48 h before being preincubated (16 h at 37 °C) in media supplemented with tritiated adenine (2 μCi). The cells were washed twice with cold HBS buffer. The cells were then exposed for 20 min at 37 °C to varying concentrations (10^{-13}–10^{-6} M) of GIP, *N*-pGluGIP(LysPAL^{16}), or *N*-pGluGIP-(LysPAL^{37}) in HBS buffer (estimated albumin concentration 76.5 μM), in the presence of 1 mM IBMX. The medium was subsequently removed and 1 mL of lysis solution added, containing 0.3 mM unlabeled cAMP and 5 mM unlabeled ATP. The intracellular tritiated cAMP was then separated on Dowex and alumina exchange resins as described previously.[40]

Secretion of Insulin and Related Analogues in Vitro. BRIN-BD11 cells were seeded into 24-multiwell plates at a density of 1.0 × 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 [115 mM NaCl, 4.7 mM KCl, 1.28 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 10 mM $NaHCO_3$, 0.5% (w/v) BSA, pH 7.4] supplemented with 1.1 mM glucose. Test incubations were performed in the presence of 5.6 mM glucose with a range of concentrations (10^{-13}−10^{-6} M) of GIP, *N*-pGluGIP(LysPAL^{16}), and *N*-pGluGIP(LysPAL^{37}). After 20-min incubation, the buffer was removed from each well, and aliquots (200 μL) were used for measurement of insulin.

Effects of GIP and Related Analogues on Glucose-Lowering and Insulin Release in (ob/ob) Mice. The effects of GIP, *N*-pGluGIP(LysPAL^{16}), or *N*-pGluGIP(LysPAL^{37}) on plasma glucose and insulin concentrations were examined in 14−18-week-old obese diabetic (ob/ob) mice. The genetic background and characteristics of the colony used have been outlined in detail elsewhere.[33] The animals were housed individually in an air-conditioned room at 22 ± 2 °C with a 12 h light/12 h darkness cycle. Drinking water and a standard rodent maintenance diet (Trouw Nutrition, Cheshire, UK) were freely available until 18 h before acute tests. In the first series of experiments, mice received an intraperitoneal injection of glucose alone (18 mmol/kg of body weight) or in combination with GIP, *N*-pGluGIP(LysPAL^{16}), or *N*-pGluGIP-(LysPAL^{37}) (each at 25 nmol/kg). In a second series of experiments, glucose (18 mmol/kg) was administered 4 h after *N*-pGluGIP(LysPAL^{37}) (25 nmol/kg) in order to assess longer term duration of action. In a final series of dose-dependent experiments, GIP and the most potent analogue, *N*-pGluGIP-(LysPAL^{37}), were administered at 6.25, 12.5, or 25 nmol/kg in combination with glucose (18 mmol/kg). All test solutions were administered in a final volume of 8 mL/kg of body weight. Blood samples were collected from the cut tip on the tail vein of conscious mice into chilled fluoride/heparin glucose microcentrifuge tubes (Sarstedt, Nümbrecht, Germany) immediately prior to injection and at 15, 30, and 60 min postinjection. Plasma was aliquoted and stored at −20 °C prior to glucose and insulin determinations. No adverse effects were observed following administration of any of the peptides. All animal experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986.

Analyses. Plasma glucose was assayed by an automated glucose oxidase procedure using a Beckman Glucose Analyzer II.[41] Plasma insulin was assayed by dextran-charcoal RIA as described previously.[42] Incremental areas under plasma glucose and insulin curves (AUC) were calculated using a computer-generated program employing the trapezoidal rule[43] with baseline subtraction. 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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