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Characterization of the Cellular and Metabolic Effects of a Novel Enzyme-Resistant Antagonist of Glucose-Dependent Insulinotropic Polypeptide

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A novel N-terminally substituted Pro^3 analogue of glucose-dependent insulinotropic polypeptide (GIP) was synthesized and tested for plasma stability and biological activity both *in vitro* and *in vivo.* Native GIP was rapidly degraded by human plasma with only 39 ± 6% remaining intact after 8 h, whereas (Pro^3)GIP was completely stable even after 24 h. In CHL cells expressing the human GIP receptor, (Pro^3)GIP antagonized the cyclic adenosine monophosphate (cAMP) stimulatory ability of 10^{-7} M native GIP, with an IC_{50} value of 2.6 μM. In the clonal pancreatic beta cell line BRIN-BD11, (Pro^3)GIP over the concentration range 10^{-13} to 10^{-8} M dose dependently inhibited GIP-stimulated (10^{-7} M) insulin release (1.2- to 1.7-fold; $P < 0.05$ to $P < 0.001$). In obese diabetic (*ob*/*ob*) mice, intraperitoneal administration of (Pro^3)GIP (25 nmol/kg body wt) countered the ability of native GIP to stimulate plasma insulin (2.4-fold decrease; $P < 0.001$) and lower the glycemic excursion (1.5-fold decrease; $P < 0.001$) induced by a glucose load (18 mmol/kg body wt). Collectively these data demonstrate that (Pro^3)GIP is a novel and potent enzyme-resistant GIP receptor antagonist capable of blocking the ability of native GIP to increase cAMP, stimulate insulin secretion, and improve glucose homeostasis in a commonly employed animal model of type 2 diabetes. © 2002 Elsevier Science (USA)

Key Words: **dipeptidylpeptidase IV (DPP IV); gastric inhibitory polypeptide; GIP analogue; GIP receptor antagonist; glucose-dependent insulinotropic polypeptide; insulin secretion; obese diabetic (*ob*/*ob*) mice.**

Glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1-(7–36) amide (tGLP-1) are two major insulin-releasing hormones, or incretins, secreted from endocrine cells in the intestinal tract in response to feeding (1, 2). Recent years have witnessed an upsurge in the interest of tGLP-1 in being the more important physiological incretin hormone with greater insulinotropic activity (3, 4). However, several recent observations, including studies in GIP and GLP-1 receptor knockout mice, suggest an underestimation of the role of GIP in the enteroinsular axis (5–8).

Due to cross-reactivity with inactive tGLP-1 metabolites, the measurement of physiological concentrations of tGLP-1 would appear to have been significantly overestimated (6). While tGLP-1 appears to be more potent than GIP on an equimolar basis, the rise in immunoreactive GIP levels following oral glucose is greater in magnitude than the increment in plasma immunoreactive tGLP-1 after a similar glucose load (5, 9). Thus when infused at concentrations that will produce plasma levels comparable to the values usually found after oral glucose in human subjects, GIP seems to be more insulinotropic than tGLP-1 (9). Recently, using a putative tGLP-1 receptor antagonist, exendin(9–39) amide, it was demonstrated that postprandial insulin release was reduced by 48% illustrating that tGLP-1 contributes significantly to meal-induced insulin secretion (10). However, further studies have shown that exendin(9–39) amide interferes with GIP receptor binding and reduces GIP-stimulated cyclic adenosine monophosphate (cAMP) production (11, 12). Therefore, it seems likely that the antagonistic actions of exendin(9–39) amide are not merely confined to the tGLP-1 receptor.

Further evidence for the importance of GIP as an important physiological incretin has come from studies using GIP(7–30) amide, as an inhibitor of GIP-induced insulin secretion (13, 14). Circulating insulin concentrations were significantly reduced (52%) using this antagonist, when administered before enteral glucose infusion in rats (14). In the present paper we describe a novel enzyme-resistant GIP-receptor antagonist sub-

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stituted at Glu^3 by a proline residue. Antagonistic effects of (Pro^3)GIP have been characterized at both the cellular level *in vitro* and in a commonly employed animal model of type 2 diabetes, suggesting that this analogue will be useful for future studies on the physiological roles of GIP.

MATERIALS AND METHODS

Chemicals. 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). Dipeptidylpeptidase IV (DPP IV), forskolin (FSK), isobutylmethylxanthine (IBMX), adenosine 3′:5′-cyclic monophosphate (cAMP), adenosine 5′-triphosphate (ATP) were all purchased from Sigma (Poole, Dorset, UK). Glucagon-like peptide-1-(7–36) amide (tGLP-1) was purchased from the American Peptide Co. (Sunnyvale, CA). Fmoc-protected amino acids were 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, UK). The chromatography columns used for cAMP assay, Dowex AG 50 WX and neutral alumina AG7 were obtained from Bio-Rad (Alpha Analytical, Larne, N. Ireland). 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 purity available.

Peptide synthesis. GIP and (Pro^3)GIP were sequentially synthesized on an Applied Biosystems automated peptide synthesizer (Model 432 A) using standard solid-phase Fmoc procedure, starting from an Fmoc-Gln-Wang resin. Following cleavage from the resin by trifluoroacetic acid:water, thianisole, ethanediol (90/2.5/5/2.5, a total volume of 20 ml/g resin), the resin was removed by filtration and the filtrate volume 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, the procedure being repeated five times. The resulting pellets were then dried *in vacuo* and judged pure by reversed-phase HPLC on a Waters Millenium 2010 chromatography system (Software version 2.1.5) and the peptides subsequently characterized using electrospray ionization–mass spectrometry (ESI–MS).

Peptide stability. Native GIP and (Pro^3)GIP were incubated *in vitro* at 37°C in 50 mM triethanolamine–HCl (pH 7.8; final peptide concentration 2 mM) with pooled fasted human plasma (10 μl) for 0, 8, and 24 h. The enzymatic reactions were stopped by the addition of 10 μl of 10% (v/v) TFA/water. The terminated reaction products were then applied to a Vydac C-18 column (4.6 × 250 mm) and the major degradation fragment GIP(3–42) separated from intact native 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 form 0 to 28% over 10 min, and from 28 to 42% over 30 min. The absorbance was monitored at 206 nm using a Spectrasystem UV 2000 detector (Thermoquest Limited, Manchester, UK) and peaks were collected manually prior to ESI–MS analysis.

Cell culture. Chinese hamster lung (CHL cells; ATCC CCL39) fibroblasts stably transfected with the human GIP receptor (11) were cultured in DMEM tissue culture medium containing 10% (v/v) fetal bovine serum, 1% (v/v) antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin). BRIN-BD11 cells were cultured in sterile tissue culture flasks (Corning, Glass Works, UK) using RPMI 1640 tissue culture medium containing 10% (v/v) fetal calf serum, 1% (v/v) antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin) and 11.1 mM glucose as described previously (15). The cells were maintained at 37°C in an atmosphere of 5% CO_2 and 95% air using a LEEC incubator (Laboratory Technical Engineering, Nottingham, UK).

cAMP determination. CHL cells were seeded into 12-multiwell plates (Nunc, Roskilde, Denmark) at a density of 1.0×10^5 cells per well. The cells were then allowed to grow for 48 h before being loaded at 37°C for 5–6 h with 2 μCi of tritiated adenine (TRK311, Amersham, Buckinghamshire, UK) in 1 ml DMEM supplemented with 0.5% fetal bovine serum. The cells were then washed twice with HBS buffer (130 mM NaCl, 20 mM Hepes, pH 7.4, 0.9 mM $NaHPO_4$, 0.8 mM $MgSO_4$, 5.4 mM KCl, 1.8 mM $CaCl_2$, 25 mM glucose, and 25 μM phenol red). The cells were then exposed for 10 min at 37°C to FSK (10 μM) or varying concentrations (10^{-12} to 10^{-6} M) of (Pro^3)GIP in the presence of 10^{-7} M GIP in HBS buffer supplemented with 1 mM IBMX. The medium was subsequently removed and the cells lysed with 1 ml of 5% trichloroacetic acid (TCA) 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 as described previously (16).

Insulin secretion. BRIN-BD11 cells were harvested with the aid of trypsin/EDTA (Gibco), seeded into 24-multiwell plates (Nunc, Roskilde, Denmark) 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 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) bovine serum albumin, pH 7.4) supplemented with 1.1 mM glucose. Test incubations were performed ($n = 8$) in the presence of 5.6 mM glucose over a range of concentrations (10^{-13} to 10^{-8} mol/L) of native GIP and (Pro^3)GIP in the presence or absence of 10^{-7} M GIP. After 20 min incubation, the buffer was removed and used for measurement of insulin by radioimmunoassay (17).

In vivo biological activity. Plasma glucose and insulin responses were evaluated using 14- to 18-week old obese diabetic (*ob*/*ob*) mice (18) following intraperitoneal (ip) injection of native GIP, (Pro^3)GIP (25 nmol/kg body wt) or saline [0.9% (w/v) NaCl; control] immediately following the combined injection of GIP (25 nmol/kg body wt) with glucose (18 mmol/kg body wt). All test solutions were administered in a final volume of 8 ml/kg body wt. Blood samples were collected from the cut tip of the tail of conscious mice into chilled fluoride/heparin microcentrifuge tubes (Sarstedt, Numbrecht, Germany) immediately prior to injection and at 15, 30, and 60 min postinjection. Blood samples were immediately centrifuged using a Beckman microcentrifuge (Beckman Instruments, UK) for 30 s at 13,000*g*. Plasma glucose was assayed by an automated glucose oxidase procedure using a Beckman Glucose Analyzer II (19) and plasma insulin was determined by RIA (17). Incremental areas under plasma glucose and insulin curves (AUC) were calculated using a computer generated program (CAREA) employing the trapezoidal rule (20) with baseline subtraction.

Statistical analysis. Results are expressed as means ± SEM. Data were compared using Student's *t* test or ANOVA, followed by the Student–Newman–Keuls post hoc test, as appropriate. Groups of data were considered to be significantly different if $P < 0.05$.

RESULTS AND DISCUSSION

Glucose-dependent insulinotropic polypeptide (GIP) together with the structurally related peptide, glucagon-like peptide-1-(7–36) amide (tGLP-1) are considered to be the two most important incretin hormones of the enteroinsular axis (1, 21). Previous attempts have been made to acutely disturb GIP action *in vivo* to assess its contribution to the enteroinsular axis. Both the GIP fragment, GIP(7–30)amide, and the exendin fragment, exendin(9–39)amide have been shown to disrupt the insulin response to oral nutrients in ro-

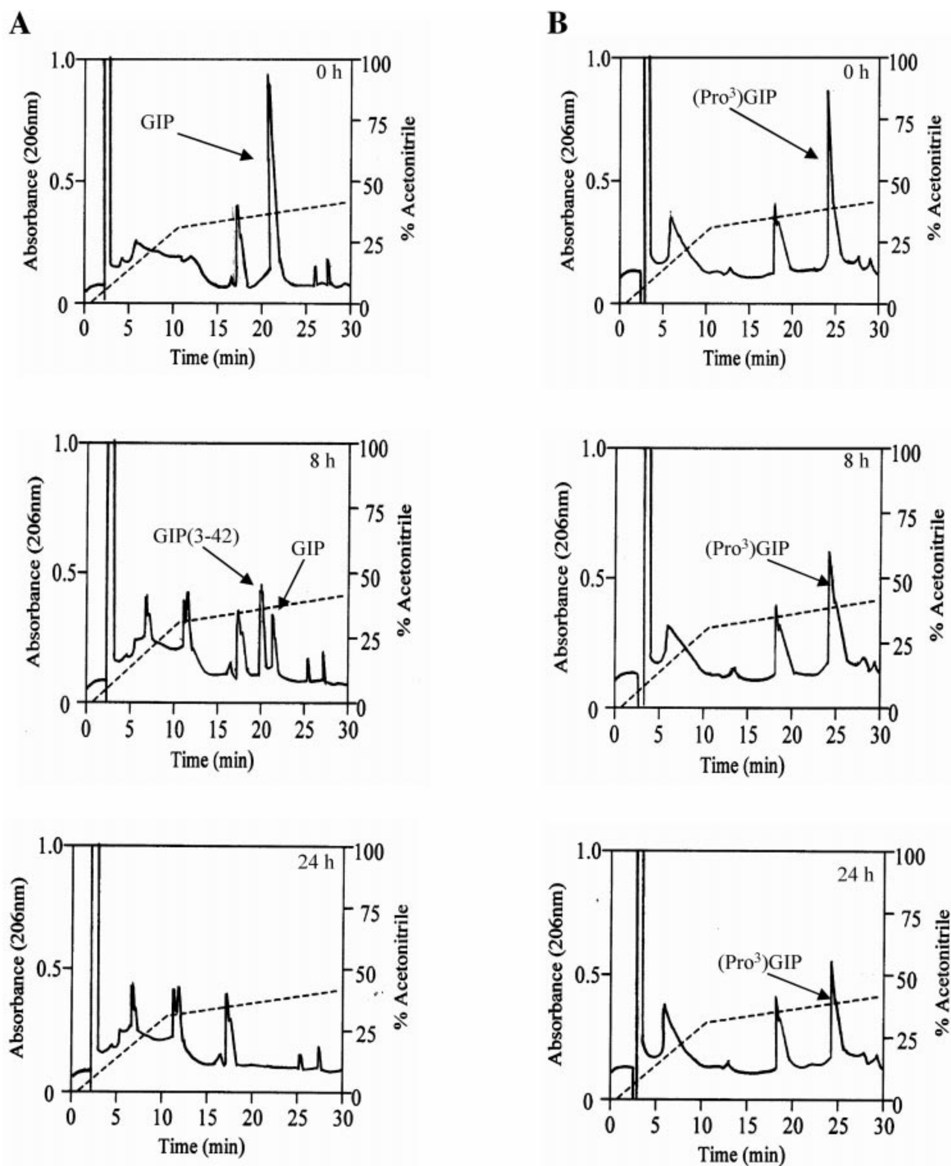

FIG. 1. Representative HPLC profiles obtained after the incubation of native GIP (A) and (Pro[3])GIP (B) with human plasma for 0, 8, and 24 h. Reaction products were separated on a Vydac C-18 column using linear gradients from 0 to 28% acetonitrile over 10 min and from 28 to 42% over 30 min. HPLC peaks corresponding to intact peptides and the major degradation fragment GIP(3-42) are indicated.

dents (10, 13, 14, 22). However, there are several limitations of these peptide antagonists for *in vivo* work, some of which include short biological half-life, nonspecificity at high doses (11, 12), and the possibility that degradation products may have additional unknown biological properties. In the present paper we describe the properties of a novel DPP IV-resistant GIP receptor antagonist, which promises to be very useful for evaluating GIP action and the role of this incretin hormone *in vivo.*

As shown in Fig. 1A, native GIP was rapidly cleaved by human plasma (39 ± 6% intact), giving a major degradation product by 8 h, which upon ESI-MS analysis corresponded to GIP(3–42). This is consistent with previous findings in which DPP IV has been shown to be the major enzyme responsible for GIP metabolism *in vivo* (23–25). In contrast, Fig. 1B illustrates that (Pro[3])GIP remained fully intact after incubation under the same conditions with human plasma for up to 24 h as no degradation fragment was present. This demonstrates that substituting the N-terminal Glu[3] in GIP with a proline residue renders the peptide resistant to DPP IV degradation.

Previous studies have shown that GIP dose-dependently stimulates cAMP production in CHL cells stably expressing the human GIP receptor (11). In comparison, however, (Pro[3])GIP only very weakly stimulated cAMP production when applied to transfected fibro-

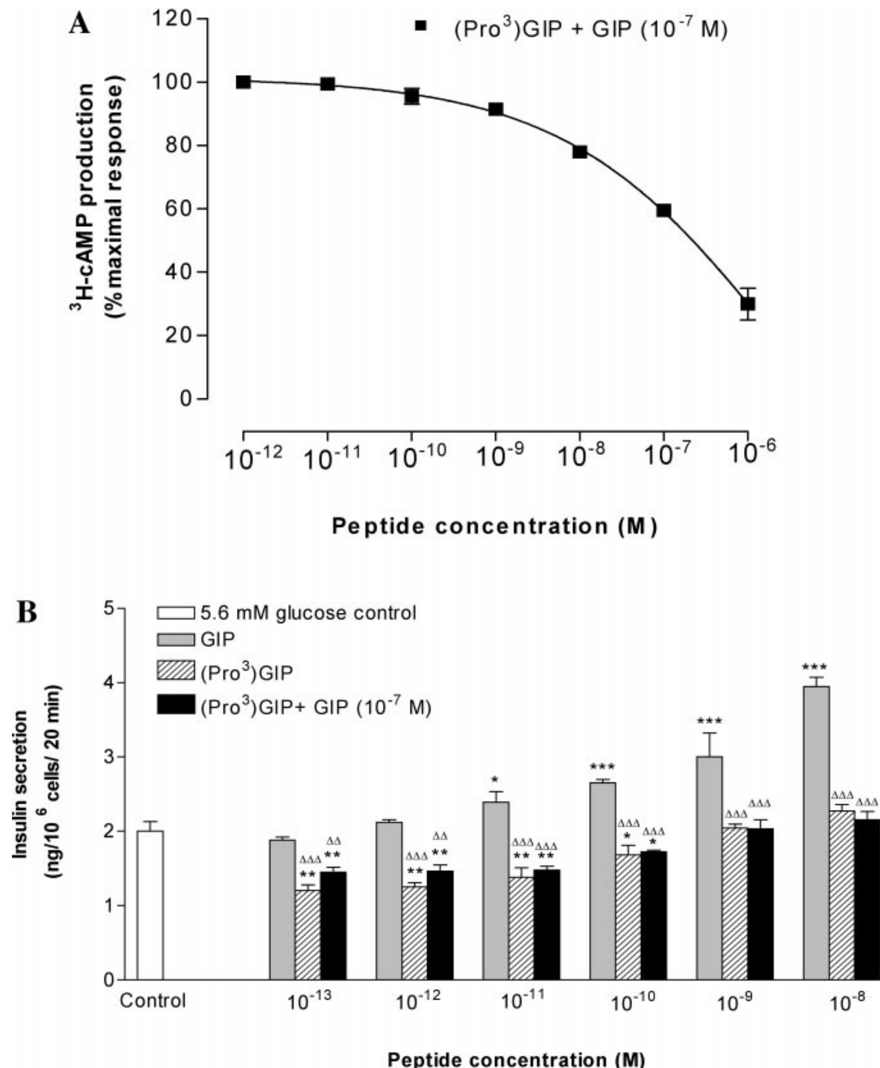

FIG. 2. Antagonistic effects of (Pro^3)GIP on GIP-stimulated cAMP production in CHL cells (A) and insulin secretion in BRIN-BD11 (B). (A) GIP receptor transfected CHL cells were incubated with 10^{-12} to 10^{-6} M (Pro^3)GIP in the presence of native GIP (10^{-7} M). (Pro^3)GIP inhibited GIP-induced cAMP formation with an IC_{50} of 2.6 μM. Data are expressed as percentages of maximal cAMP response. Values are means ± SEM of three observations. (B) Insulin-releasing activity of native GIP and (Pro^3)GIP (in the absence and presence of 10^{-7} M native GIP) were tested during a 20-min incubation period. Values are means ± SEM for eight observations. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to control. $^{\Delta\Delta}P < 0.01$, $^{\Delta\Delta\Delta}P < 0.001$ compared with native GIP at the same concentration.

blasts, the maximal stimulatory value compared to native GIP (100%) being 9 ± 2.1% ($P < 0.001$) over the concentration range 10^{-12} to 10^{-6} M (data not shown). In contrast, as demonstrated in Fig. 2A, (Pro^3)GIP inhibited cAMP formation of cells exposed to 10^{-7} M native GIP, with an IC_{50} for this inhibitory effect of 2.6 μM. This clearly indicates that (Pro^3)GIP effectively served as a potent antagonist of GIP-stimulated cAMP production.

In glucose-responsive BRIN-BD11 cells, as shown in Fig. 2B, native GIP (10^{-11} to 10^{-8} M) dose dependently stimulated insulin secretion (1.2- to 1.8-fold; $P < 0.05$ to $P < 0.001$) compared with control incubations. This observation agrees with a previous observation that GIP serves as an effective insulin secretagogue in this clonal cell line (26). (Pro^3)GIP, on the other hand, was significantly less potent at stimulating insulin secretion (1.5- to 1.8-fold; $P < 0.01$) compared with native GIP, consistent with cAMP data. (Pro^3)GIP dose-dependently inhibited (1.2- to 1.7-fold; $P < 0.01$ to $P < 0.001$) insulin release induced by 10^{-7} M GIP. At 10^{-8} M especially, (Pro^3)GIP exhibited a 1.8-fold decrease in insulinotropic activity compared with the native peptide. It is clear from these data that (Pro^3)GIP effectively antagonized both GIP-stimulated cAMP production and insulin secretion. The analogue (10^{-12} to 10^{-6} M) had no significant effects on 10^{-7} M GLP-1-stimulated insulin secretion from BRIN-BD11 cells, indicating true specificity for GIP among the incretin hormones (data not shown).

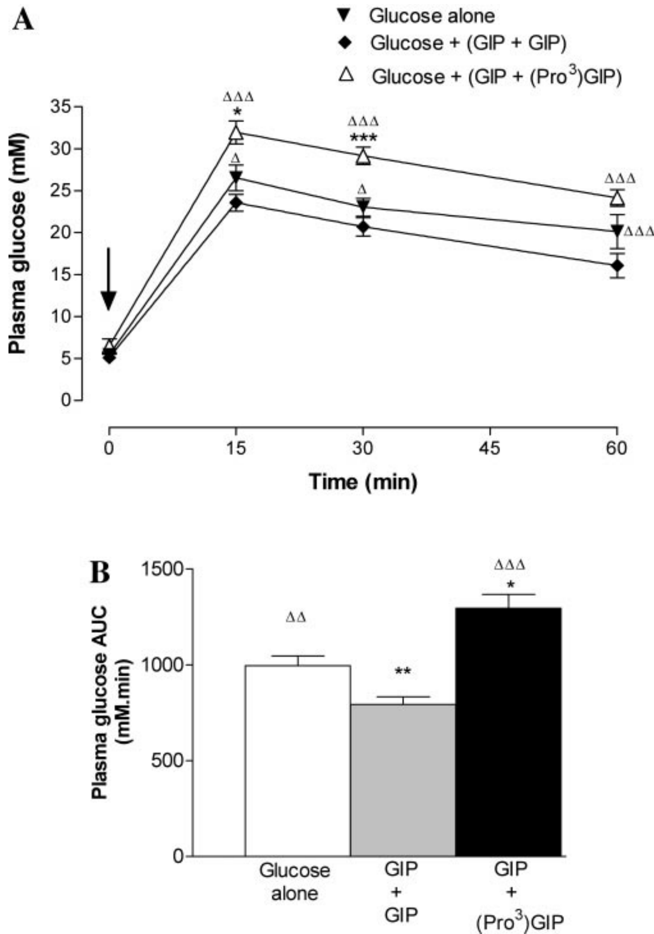

FIG. 3. Effects of native GIP and (Pro^3)GIP on plasma glucose homeostasis in obese diabetic (*ob*/*ob*) mice. (A) Plasma glucose concentrations after intraperitoneal administration of glucose alone (18 mmol/kg) or in combination with either native GIP (2 × 25 nmol/kg) or native GIP (25 nmol/kg) plus (Pro^3)GIP (25 nmol/kg). The time of injection is indicated by the arrow (0 min). (B) Plasma glucose AUC values for 0–60 min postinjection. Values are means ± SEM for eight mice. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with glucose alone. $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$, $^{\Delta\Delta\Delta}P < 0.001$ compared with native GIP.

GIP has been shown previously to display insulinotropic and antihyperglycemic activity in a commonly employed animal model of type 2 diabetes (27, 28). In the present study, native GIP significantly decreased the glycemic excursion induced by intraperitoneal glucose (Fig. 3A), which corroborates previous findings. Administration of (Pro^3)GIP completely blocked the action of native GIP, and plasma glucose concentrations were elevated ($P < 0.05$ to $P < 0.001$) compared with control (glucose alone) and GIP-only treated groups throughout the entire 60 min test (Fig. 3A). In particular, the plasma glucose concentration at 60 min postinjection was 1.7-fold higher ($P < 0.001$) following administration of (Pro^3)GIP. Moreover, the area under the curve (AUC) for plasma glucose concentrations was significantly raised (1.5-fold; $P < 0.001$), following administration of (Pro^3)GIP compared with the GIP-only treated group (Fig. 3B). Consistent with these observations, plasma insulin concentrations of obese diabetic (*ob*/*ob*) mice treated with (Pro^3)GIP were significantly lowered (1.6- to 2.2-fold; $P < 0.001$) at 15–60 min postinjection compared with the GIP-only treated group (Fig. 4A). Furthermore, the overall plasma insulin responses, estimated as AUC were significantly lower (2.4-fold; $P < 0.001$) following administration of (Pro^3)GIP compared with the GIP only-treated group (Fig. 4B). Interestingly, the glycemic and insulin-releasing responses to the combination of (Pro^3)GIP plus GIP were less favorable than to glucose alone. This could be due to blockade of the effects of high concentrations of endogenous GIP (27) including extrapancreatic effects and actions on islet non-beta cells (29, 30).

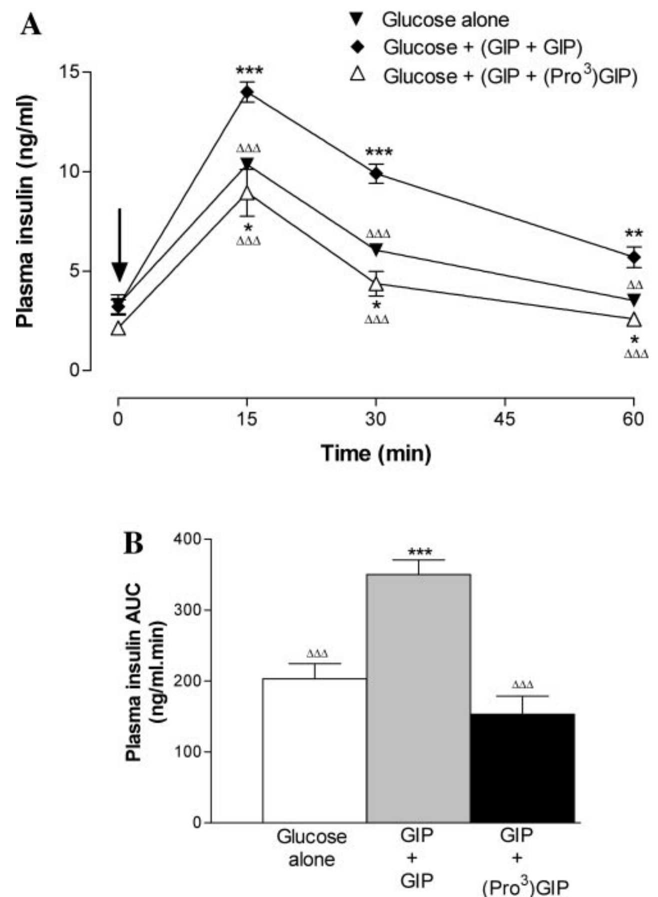

FIG. 4. Effects of native GIP and (Pro^3)GIP on plasma insulin responses in obese diabetic (*ob*/*ob*) mice. (A) Plasma insulin concentrations after intraperitoneal administration of glucose alone (18 mmol/kg) or in combination with either native GIP (2 × 25 nmol/kg) or native GIP (25 nmol/kg) plus (Pro^3)GIP (25 nmol/kg). The time of injection is indicated by the arrow (0 min). (B) Plasma insulin AUC values for 0–60 min postinjection. Values are means ± SEM for eight mice. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with glucose alone. $^{\Delta\Delta}P < 0.01$, $^{\Delta\Delta\Delta}P < 0.001$ compared with native GIP.

In conclusion, this study indicates that substitution of Glu^3 in GIP with proline produces a novel DPP IV-resistant GIP receptor antagonist. (Pro^3)GIP inhibits GIP-induced cAMP generation and insulin secretion with high sensitivity and specificity *in vitro.* (Pro^3)GIP also effectively countered the insulin-releasing and antihyperglycemic actions of native GIP in obese diabetic (*ob*/*ob*) mice *in vivo.* This novel (Pro^3)GIP receptor antagonist may prove useful in elucidating the full spectrum of physiological functions of GIP and help understanding of the relative contribution of GIP to the enteroinsular axis in humans.

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