

[Ser²]- and [Ser(P)²]Incretin Analogs

COMPARISON OF DIPEPTIDYL PEPTIDASE IV RESISTANCE AND BIOLOGICAL ACTIVITIES *IN VITRO* AND *IN VIVO**

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Glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP; also known as gastric inhibitory polypeptide) are incretin hormones that reduce postprandial glycemic excursions via enhancing insulin release but are rapidly inactivated by enzymatic N-terminal truncation. As such, efforts have been made to improve their plasma stability by synthetic modification or by inhibition of the responsible protease, dipeptidyl peptidase (DP) IV. Here we report a parallel comparison of synthetic GIP and GLP-1 with their Ser²- and Ser(P)²-substituted analogs, examining receptor binding and activation, metabolic stability, and biological effects *in vivo*. Both incretins and their Ser²-substituted analogs showed similar EC₅₀s (0.16–0.52 nM) and IC₅₀s (4.3–8.1 nM) at their respective cloned receptors. Although both phosphoserine 2-modified (Ser(PO₃H₂); Ser(P)) peptides were able to stimulate maximal cAMP production and fully displace receptor-bound tracer, they showed significantly right-shifted concentration-response curves and binding affinities. Ser²-substituted analogs were moderately resistant to DP IV cleavage, whereas [Ser(P)²]GIP and [Ser(P)²]GLP-1 showed complete resistance to purified DP IV. It was shown that the Ser(P) forms were dephosphorylated in serum and thus *in vivo* act as precursor forms of Ser²-substituted analogs. When injected subcutaneously into conscious Wistar rats, all peptides reduced glycemic excursions (rank potency: [Ser(P)²]incretins ≥ [Ser²]incretins > native hormones). Insulin determinations indicated that the reductions in postprandial glycemia were at least in part insulin-mediated. Thus it has been shown that despite having low *in vitro* bioactivity using receptor-transfected cells, *in vivo* potency of [Ser(P)²]incretins was comparable with or greater than that of native or [Ser²]peptides. Hence, Ser(P)²-modified incretins present as novel glucose-lowering agents.

Incretins are gut-derived hormones released by nutrients that potentiate insulin release under elevated glycemic conditions (1).

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To date, GIP¹ (2) and GLP-1 (3) are considered to be the major, if not sole, incretins. They have received considerable recent attention as potential therapeutic agents for treatment of diabetes, not only because of their insulin-stimulating ability but also because of their general role in promoting the "healthy" β -cell phenotype (*i.e.* proinsulin biosynthesis, hexose transporter and glucokinase expression, neogenesis/proliferation, cytoprotection/antiapoptotic effects, and glucose responsiveness).

Early studies on type 2 diabetes mellitus (T2DM) indicated a diminished incretin effect in these patients (4). Subsequent work has suggested that the impaired enteroinsular axis in T2DM is a consequence of a secretory defect of GLP-1 and a resistance to the activity of GIP on the β -cell; in any case, the efficacy of exogenous infusion of either incretin is moderately reduced in T2DM relative to healthy control patients (5). N-terminal cleavage of GIP and GLP-1 to GIP_{3–42} and GLP-1_{9–36} by purified dipeptidyl peptidase (DP) IV and serum has been demonstrated (6–8), and several studies have suggested that these truncated peptides are unable to activate their cloned receptors (9, 10). Metabolically stable peptide analogs of GLP-1 and GIP with N-terminal modifications were first reported by Buckley and Lundquist (11) and Gelling *et al.* (12), respectively, and subsequent reports have highlighted the therapeutic potential of enzyme-resistant incretins in experimental diabetic models (for review, see Refs. 13–15).

The present study aimed to achieve two goals: (i) parallel comparison of enzyme-resistant GLP-1- and GIP-based insulinotropic analogs and (ii) examination of the mechanism responsible for the enhanced biological potency of phosphoserine 2-substituted incretins *in vivo* when they showed poor potency at their cloned transfected receptors *in vitro*. We have reported previously the synthesis and DP IV resistance of [Ser²]GIP (16), and others have reported that of [Ser²]GLP-1 (17–19); however, it is not possible to directly compare studies using different approaches to examine enzyme resistance and efficacy *in vitro* and *in vivo*. In an earlier study, we noted that [Ser(P)²]glucagon was completely resistant to purified DP IV but was time dependently dephosphorylated in serum and then degraded in a similar fashion to the native hormone (20). Hence, we hypothesized that Ser(P)²-substituted incretins could act as novel precursor or prodrug forms of moderately resistant [Ser²]incretins and thus add an extra level of protec-

¹ The abbreviations used are: GIP, glucose-dependent insulinotropic polypeptide or gastric inhibitory polypeptide; GLP-1, glucagon-like peptide-1; T2DM, type 2 diabetes mellitus; DP IV, dipeptidyl peptidase IV or CD26; HPLC, high pressure liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; CHO, Chinese hamster ovary; GIPR, GIP receptor-transfected; GLP-1R, GLP-1 receptor-transfected; bw, body weight; OGTT, oral glucose tolerance test.

tion against degradation. A parallel study of GIP- and GLP-1-derived peptides was designed, examining ligand binding and cyclic AMP stimulation on the cloned transfected GIP and GLP-1 receptors, and peptides were bioassayed in the rat, monitoring glucose-lowering and insulin-stimulating effects. MALDI-TOF mass spectrometry was used to monitor degradation by purified DP IV and rat serum, allowing direct identification of the peptide metabolites. This study has been presented in abstract form (21).

EXPERIMENTAL PROCEDURES

Peptide Synthesis—Peptides were synthesized according to published methods (20) using a Symphony automatic peptide synthesizer (Rainin Instrument Co., Woburn, MA) and a modified solid phase Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) protocol. Crude peptides were purified by preparative HPLC and subjected to analytical HPLC and MALDI-TOF mass spectrometry to confirm identity and purity. Predicted and measured masses of GIP₁₋₄₂, [Ser²]GIP_{1-30NH₂}, [Ser(P)²]GIP_{1-30NH₂}, GLP-1_{7-36NH₂}, [Ser²]GLP-1_{7-36NH₂}, and [Ser(P)²]GLP-1_{7-36NH₂} are presented in Table I. GIP₁₋₄₂ and GIP_{1-30NH₂} have identical binding affinities and the ability to activate the GIP receptor and stimulate insulin release *in vivo* (22); in these studies, GIP₁₋₄₂ was used for control purposes.

Cell Culture and Transfection—CHO-K1 cells were propagated and transfected as reported previously (23). Cells were stably transfected by the CaPO₄ co-precipitation method with either the GIP or GLP-1 receptor cDNAs in the eukaryotic expression vector pcDNA3 (Invitrogen); these established stable cell lines have been well characterized (23). For the purposes of this report, cells transfected with the GIP receptor are designated GIPR cells, and those transfected with the GLP-1 receptor are designated GLP-1R cells. Cells were grown in Dulbecco's modified Eagle's medium/F12 medium (Invitrogen) supplemented with 10% newborn calf serum (Cansera, Rexdale, Canada), antibiotics, and G418 selection agent (Invitrogen). Cells were harvested with trypsin/EDTA (Invitrogen) and seeded (50,000 cells/well) into 24-well plates (Falcon, BD Biosciences) such that when the experiment was performed 48 h later, cells were ~90–95% confluent (2–5 × 10⁵ cells/well).

Competitive Binding Studies—Radioligand binding displacement curves were generated essentially as described previously (9). Monocomponent HPLC-purified ¹²⁵I-GIP and ¹²⁵I-GLP-1 were prepared by the chloramine T method using established protocols (8, 9). Cells were washed twice with ice-cold buffer (as defined below but without aprotinin). Binding studies were performed by incubating cells with tracer (50,000 cpm) with or without unlabelled peptides (concentrations shown in the figures) in Dulbecco's modified Eagle's medium/F12 buffer containing 15 mM HEPES (pH 7.4), 0.1% bovine serum albumin (Sigma), and 1% Trasylol (aprotinin; Bayer, Etobicoke, Canada); binding studies reached steady state over a period of 12–16 h at 4 °C. Cells were washed, and cell-associated radioactivity was counted after solubilization. Nonspecific binding was determined by inclusion of excess unlabelled GIP or GLP-1 (1 μM).

Cyclic AMP Stimulation—Receptor activation by GIP and GLP-1 analogs in CHO-K1 cells transfected with their appropriate receptors was according to published methodologies (23). Briefly, cells were washed twice in warm buffer (as described above, without aprotinin) and preincubated for 60 min. Cells were stimulated with peptides in the same buffer with 0.5 mM isobutylmethylxanthine (Research Biochemicals International, Natick, MA) and 1% Trasylol. Stimulation of cells continued for 30 min at 37 °C prior to lysis in ice-cold ethanol, removal of cell debris, and drying *in vacuo*. Intracellular cyclic AMP content was measured by radioimmunoassay according to the manufacturer's specifications (Biomedical Technologies Inc., Stoughton, MA).

Enzymology—Degradation of peptides was determined as described previously using MALDI-TOF mass spectrometry. Peptides were incubated with purified pig kidney DP IV (Probiobdrug, Halle, Germany) as per published methods (7, 20), and molecular peaks of intact and truncated peptides were identified on mass spectrograms. Incubation of peptides in serum was similar to previously published studies (7, 20);

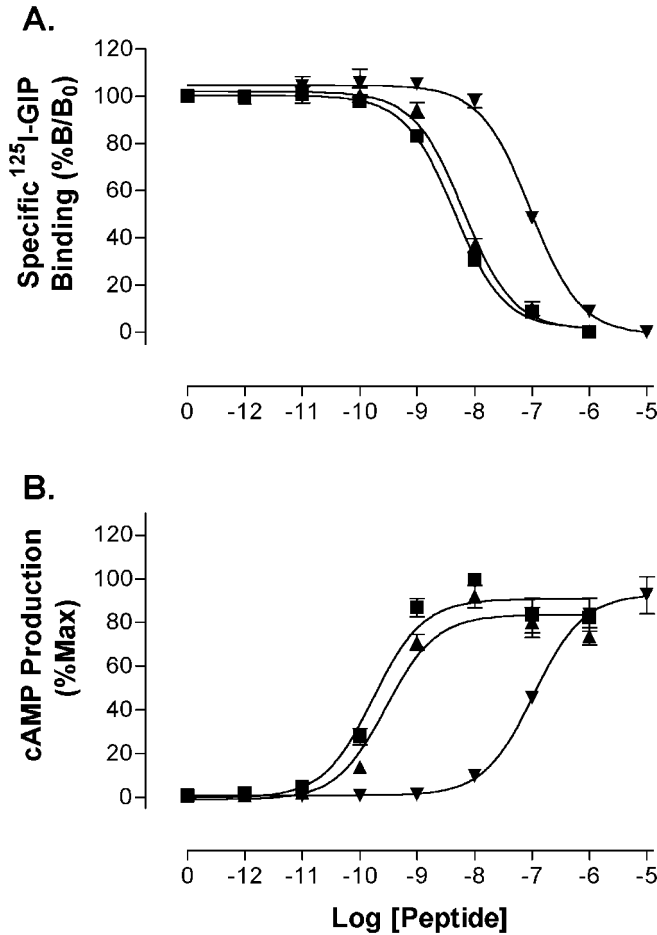


FIG. 1. Binding and cAMP stimulation in wild-type GIPR cells by GIP₁₋₄₂, [Ser²]GIP_{1-30NH₂}, and [Ser(P)²]GIP_{1-30NH₂}. A, competitive binding inhibition of ¹²⁵I-GIP₁₋₄₂. B, cyclic AMP stimulation in wild-type GIPR cells. Each data point represents the mean ± S.E. of ≥4 independent experiments. Refer to Table I for numerical IC₅₀ and EC₅₀ parameters and statistical analysis. ■, GIP₁₋₄₂; ▲, [Ser²]GIP_{1-30NH₂}; ▼, [Ser(P)²]GIP_{1-30NH₂}.

TABLE I

Measured and expected masses of synthetic peptides, receptor binding, and activation statistics from data in Figs. 1 and 2

Biological activity and receptor binding affinity were measured by testing on CHO-K1 cells transfected with either the GIP or GLP-1 receptor. Data represent mean ± S.E. (n = 4), and * indicates p < 0.05. See text for specific methods.

Peptide	Mass		Receptor binding		Receptor activation	
	Expected	Measured	IC ₅₀	Maximum displacement %	EC ₅₀	Maximum cAMP %
GIP ₁₋₄₂	4984.7	4983.6	4.49 ± 0.55 nM	100 ^a	245 ± 63 pM	100 ^b
[Ser ²]GIP _{1-30NH₂}	3567.1	3568.0	6.40 ± 0.73 nM	99.7 ± 2.3	289 ± 42 pM	91.8 ± 5.1
[Ser(P) ²]GIP _{1-30NH₂}	3647.1	3646.9	93.1 ± 8.6 nM*	100.1 ± 0.6	106 ± 7 nM*	92.5 ± 8.4
GLP-1 _{7-36NH₂}	3297.7	3298.4	4.26 ± 1.19 nM	100 ^a	155 ± 42 pM	100 ^b
[Ser ²]GLP-1 _{7-36NH₂}	3313.9	3314.3	8.08 ± 3.11 nM	97.7 ± 2.3	509 ± 119 pM	97.3 ± 7.1
[Ser(P) ²]GLP-1 _{7-36NH₂}	3393.7	3394.3	1.21 ± 0.43 μM*	97.3 ± 1.2	513 ± 107 nM*	122.9 ± 13.1

^a 1 μM GIP or GLP-1 displaced 100% of radioligand by definition.

^b Maximal responses to GIP and GLP-1 were used for normalization.

however, conditions were slightly modified. Peptides (25 μ M) were incubated in 33.3% rat serum (Wistar; provided by Dr. E. J. Freyre, Karlsburg, Germany) buffered with 5 mM Tris-HCl (pH 7.6). Samples were incubated at 37 °C with gentle shaking for the indicated times. The reaction was stopped by addition of 0.1% trifluoroacetic acid, and samples were desalted using ZipTip pipette tips (Millipore, Schwalbach, Germany) and prepared in matrix for mass spectrometry as described previously.

Bioassay—Male Wistar rats (250–350 g; University of British Columbia Animal Care Facility, Vancouver, Canada) were group-housed with free access to food and water and with a 12-h light/dark cycle. Animals were food-deprived overnight prior to experimentation but still had water *ad libitum*. Animals received a glucose challenge (1 g/kg from a 40% solution) by oral gavage and an interscapular subcutaneous injection of peptide (8 or 80 nmol/kg in phosphate-buffered saline) immediately following glucose administration. Whole blood glucose was monitored at intervals throughout the experiment using a handheld glucometer (SureStep[®]; LifeScan Canada Ltd., Burnaby, Canada), and blood samples were obtained by tail vein collection in heparinized capillary tubes for insulin determinations. Plasma was separated by centrifugation (12,000 \times g, 20 min, 4 °C). This previously published protocol (9) was approved by the University of British Columbia Committee on Animal Care and followed the guidelines of the Canadian Council on Animal Care.

Hormone Radioimmunoassay—Measurement of plasma insulin was performed as recently described (9) with no modifications. Incretin radioimmunoassays were performed in control saline-injected animals and rodents receiving native GIP or GLP-1 injections. Thus it was possible to measure incretin release in response to oral glucose (saline-injected control animals) and the circulating hormone concentrations following oral glucose with concomitant peptide injection (*i.e.* the sum of endogenous and exogenous hormone). GIP was measured as described previously using an antibody that recognizes both GIP_{1–42} and GIP_{3–42} (9). Measurement of immunoreactive GLP-1 was accomplished using a commercially available radioimmunoassay kit (Linco Research Inc., St. Charles, MO) that employed an antibody recognizing C-terminally amidated forms of GLP-1 independent of whether the N terminus was intact (*i.e.* GLP-1_{7–36NH₂}, GLP-1_{9–36NH₂}, and GLP-1_{1–36NH₂}).

Analytical Methods—Data are presented as means \pm S.E. Receptor binding and cAMP data were analyzed using the nonlinear regression analysis program Prism (version 3.02; GraphPad, San Diego, CA). Significance was established by analysis of variance followed by the Newman-Keuls multiple comparison test, where appropriate. $p < 0.05$ was considered significant; F tests confirmed variances were equal. Integrated glucose and insulin responses were calculated by the trapezoidal method, using the algorithm included in the Prism software package, with basal levels as base line.

RESULTS

GIP Receptor Binding and Activation—Initial studies were designed to compare activities of peptide analogs *in vitro*. Using CHO-K1 cells transfected with the GIP or GLP-1 receptor, it was possible to compare Ser²- and Ser(P)²-substituted incretin analogs with respect to receptor binding affinity and ability to activate the adenylyl cyclase/cyclic AMP cascade. Native GIP was able to displace 50% of ¹²⁵I-GIP binding to GIPR cells at a concentration of \sim 4.5 nM (Fig. 1A and Table I). The IC₅₀ of [Ser²]GIP was slightly higher (1.4-fold; $p > 0.05$), whereas the affinity of [Ser(P)²]GIP was significantly reduced 21-fold ($p < 0.05$; Fig. 1A and Table I). All three peptides were able to fully displace specific ¹²⁵I-GIP binding. When examining GIP receptor activation by these peptides, a similar pattern was observed. GIP and [Ser²]GIP displayed EC₅₀ values ranging between 245 and 289 pM ($p > 0.05$), but the concentration-response curve for [Ser(P)²]GIP was significantly right-shifted (433-fold; $p < 0.05$; Fig. 1B and Table I). However, the GIP analogs were able to produce maximal cyclic AMP stimulation in GIPR cells similar to that of native hormone (Fig. 1B and Table I).

GLP-1 Receptor Binding and Activation—Receptor binding of GLP-1 and analogs to the transfected GLP-1 receptor is shown in Fig. 2A, and binding statistics are shown in Table I. The binding affinity of GLP-1 for the GLP-1 receptor was similar to that of GIP for the GIP receptor, having an IC₅₀ on

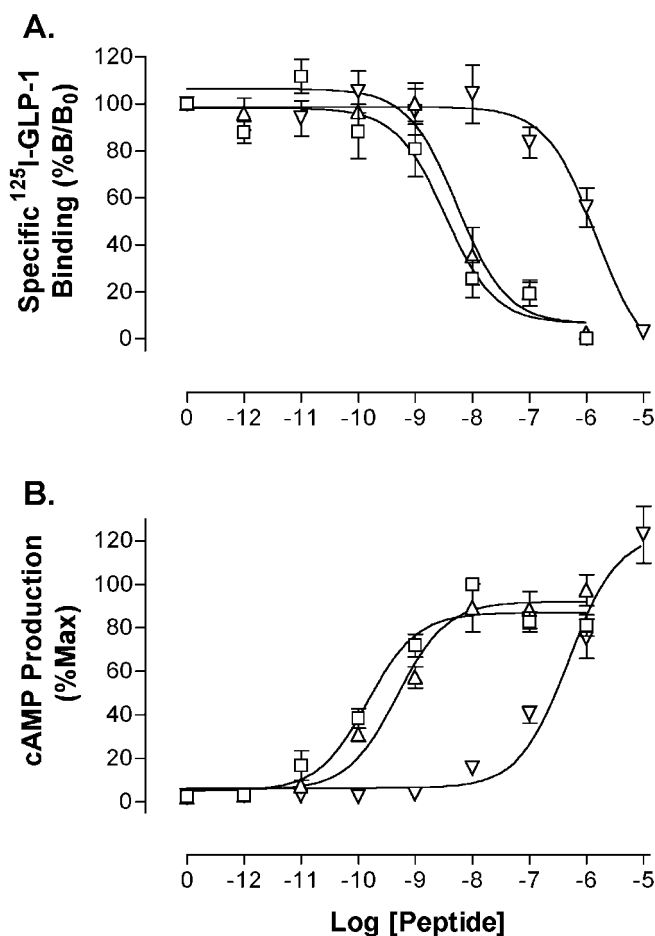


FIG. 2. Binding and cAMP stimulation in wild-type GLP-1R cells by GLP-1_{7–36NH₂}, [Ser²]GLP-1_{7–36NH₂}, and [Ser(P)²]GLP-1_{7–36NH₂}. A, competitive binding inhibition of ¹²⁵I-GLP-1_{7–36NH₂}. B, cyclic AMP stimulation in wild-type GLP-1R cells. Each data point represents the mean \pm S.E. of ≥ 4 independent experiments. Refer to Table I for numerical IC₅₀ and EC₅₀ parameters and statistical analysis. □, GLP-1_{7–36NH₂}; △, [Ser²]GLP-1_{7–36NH₂}; ▽, [Ser(P)²]GLP-1_{7–36NH₂}.

the order of \sim 4.3 nM. The concentration of [Ser²]GLP-1 required to displace 50% of ¹²⁵I-GLP-1 binding was \sim 8.1 nM, or 1.9-fold lower affinity than native hormone ($p > 0.05$; Fig. 2A and Table I). As was the case with [Ser(P)²]GIP peptide, [Ser(P)²]GLP-1 showed significantly lower affinity for its respective receptor, with an IC₅₀ value right-shifted by 284-fold ($p < 0.05$). GLP-1, [Ser²]GLP-1, and [Ser(P)²]GLP-1 were all able to fully compete with ¹²⁵I-GLP-1 binding to the GLP-1 receptor (Fig. 2A and Table I). The cyclic AMP EC₅₀ values parallel results for binding affinity, with rank potency being as follows: GLP-1 $>$ [Ser²]GLP-1 $>$ [Ser(P)²]GLP-1. Although the potency of [Ser²]GLP-1 was found to be 3.3-fold lower than native GLP-1, statistical significance was not reached ($p > 0.05$); however, the EC₅₀ value of [Ser(P)²]GLP-1 was significantly reduced by three orders of magnitude ($p < 0.05$; Fig. 2B and Table I).

Enzyme Resistance of GIP and GLP-1 Analogs—*In vitro* assessment of peptide degradation was monitored by mass spectrometry (Figs. 3, 4, 5, and 6). Degradation kinetic data have been reported previously (7, 16, 21), and thus only representative spectra are shown here. GIP and GLP-1 were rapidly degraded by purified DP IV, with the majority of intact peptide lost by 20 min. Ser²-substituted incretin analogs were moderately resistant to pig DP IV but still N-terminally cleaved by the enzyme over a longer time course. Although [Ser²]GLP-1 appeared to be more resistant to deg-

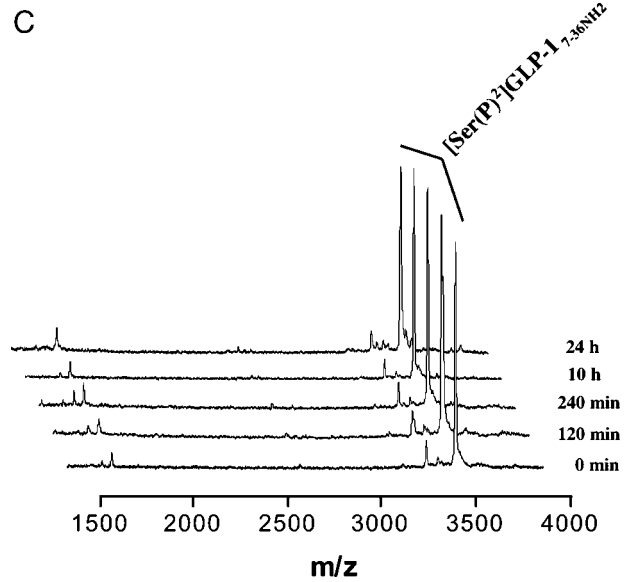
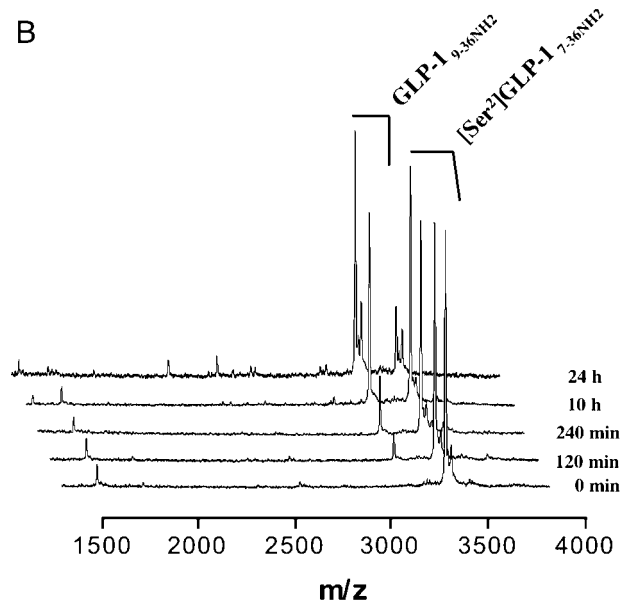
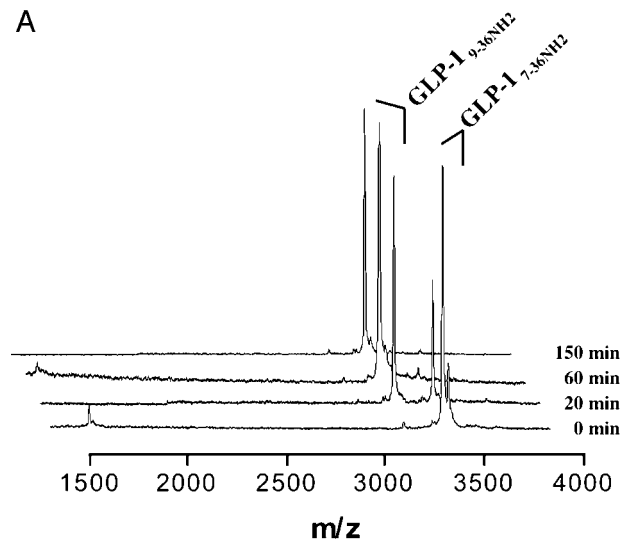
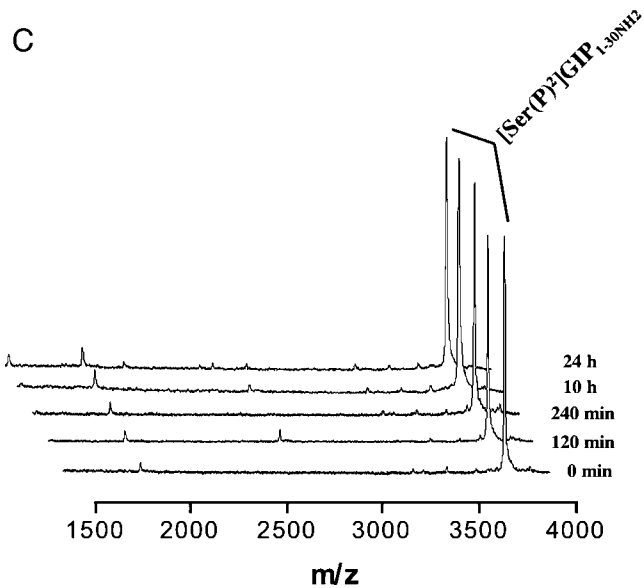
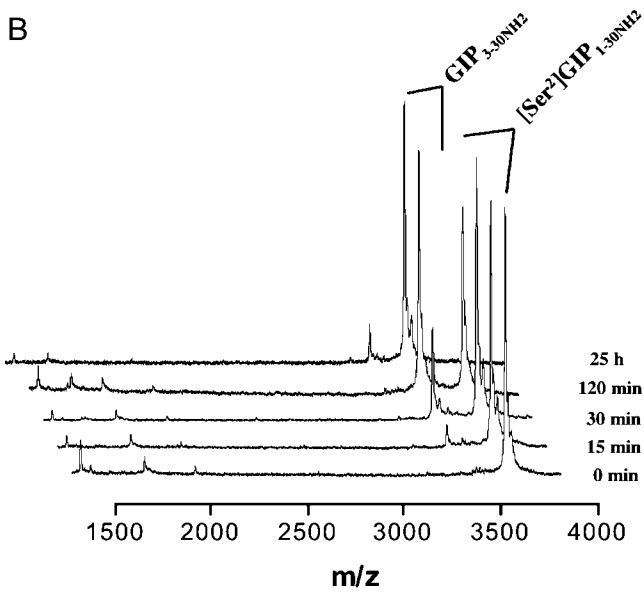
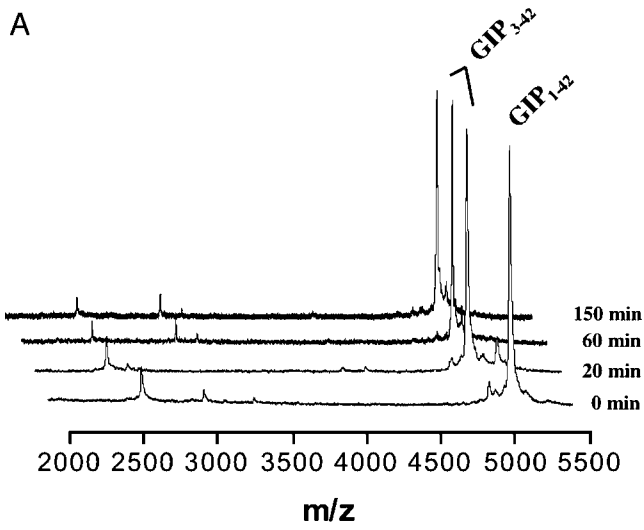


FIG. 3. MALDI-TOF mass spectrometry of synthetic GIP and N-terminally modified analogs incubated with purified porcine dipeptidyl peptidase IV. A, native GIP₁₋₄₂. B, [Ser²]GIP_{1-30NH₂}. C, [Ser(P²)]GIP_{1-30NH₂}. Data are representative spectra from repeated measurements.

FIG. 4. MALDI-TOF mass spectrometry of GLP-1 and N-terminally modified analogs incubated with purified porcine dipeptidyl peptidase IV. A, native GLP-1_{7-36NH₂}. B, [Ser²]GLP-1_{7-36NH₂}. C, [Ser(P²)]GLP-1_{7-36NH₂}. Data are representative spectra from repeated measurements.

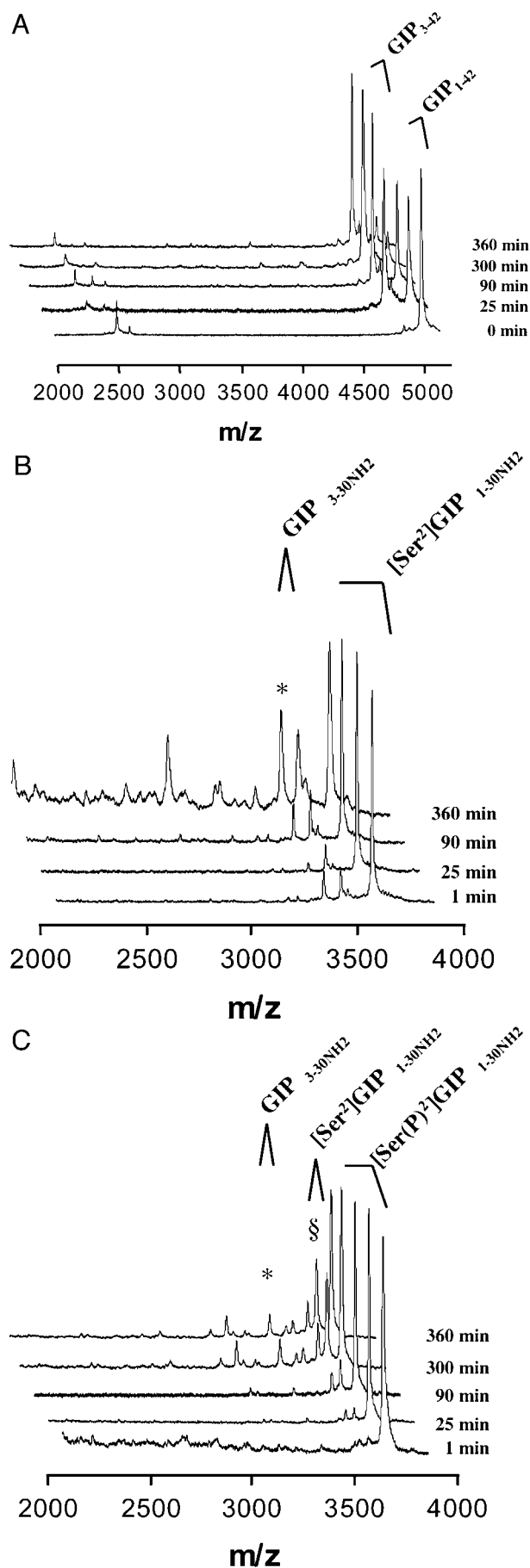


FIG. 5. MALDI-TOF mass spectrometry of synthetic GIP and N-terminally modified analogs incubated with 33.3% buffered

radiation than [Ser²]GIP, when compared with the kinetics of degradation of respective native hormones, this was not the case (21). Comparing $t_{1/2}$ values (calculation of $t_{1/2}$ times from exponential decay curves allows comparison of slowly degraded substrates (20)), [Ser²]GLP-1 was degraded 19 \times more slowly than unmodified GLP-1, whereas [Ser²]GIP was degraded 54 \times more slowly than native GIP. Ser(P)²-substituted peptides showed no significant degradation by purified enzyme over 24 h (Figs. 3C and 4C).

In buffered rat serum, degradation of peptides followed a similar pattern, with the exception of [Ser(P)²]incretin analogs. [Ser²]GIP and [Ser²]GLP-1 were moderately more resistant to DP IV-like degradation by serum than Ala²-containing native peptides (Figs. 5 and 6). In contrast to the complete resistance of [Ser(P)²]peptides to purified DP IV, these compounds were time dependently dephosphorylated in serum and then degraded in a similar fashion to Ser²-substituted peptides (Figs. 5C and 6C). This additional dephosphorylation step in serum slowed conversion of these compounds to inactive N-terminally cleaved peptides and thus potentially could allow a greater proportion of biologically active hormone analog to reach its target tissues. Minor peaks observed during serum incubation are consistent with the previous identification of other GIP and GLP-1 metabolites resulting from non-DP IV-like degradation; however, these contribute little to the overall metabolism of incretins by serum (7).

Bioassay of Incretin Analogs—Initial experiments were performed with conscious Wistar rats (312 ± 20 g of bw, fasting glycemia = 4.3 ± 0.1 mM, fasting insulin = 72.2 ± 7.5 pM, $n = 54$; fasting GIP = 897 ± 49 pg/ml, fasting GLP-1 = 13.4 ± 1.2 pg/ml; $n \geq 9$). Subcutaneous injection of 8 nmol/kg of bw GIP₁₋₄₂ or GLP-1_{7-36NH₂} with a concurrent oral glucose tolerance test (OGTT) significantly reduced the glycemic profile relative to the saline control, and this was associated with significantly increased “early phase” circulating insulin levels ($p < 0.05$; Fig. 7). GIP and GLP-1 reduced the integrated glycemic excursion by similar degrees ($p > 0.05$): 27.7 and 33.9%, respectively ($n = 6$; Table II). Measurement of hormone levels by radioimmunoassay indicated that this dose of peptide resulted in a 9-fold greater peak GIP level (control: 1.60 ± 0.10 ng/ml, treated: 14.3 ± 0.4 ng/ml) and a 2-fold greater peak GLP-1 level (control: 24.0 ± 5.7 pg/ml, treated: 48.4 ± 6.0 pg/ml) during the OGTT. The difference in the values for GIP and GLP-1, despite identical dosages, likely reflects the differing pharmacokinetics of the two hormones. Given that their insulinotropic potency was similar, it can be inferred that the mode of action of GIP and GLP-1 is rapid, as is their inactivation by N-terminal dipeptide cleavage, such that peptide pharmacokinetics do not noticeably affect their biological activity in this assay system. Although not statistically significant, subcutaneous injection of [Ser²]GIP_{1-30NH₂} or [Ser²]GLP-1 resulted in a slightly more pronounced reduction in the glycemic profile and an enhanced insulin time course during an oral glucose tolerance test relative to native peptides ($p < 0.05$ versus saline injection; Fig. 7 and Table II). Equivalent doses (8 nmol/kg) of Ser(P)²-substituted incretin analogs reduced the integrated blood glucose profile between 31 and 44% but only slightly increased the immunoreactive insulin profile, likely due to blunted insulin responses at the first time point sampled (3.5 min). Increasing the dosage of phosphoserine 2-substituted incretin analogs to 80 nmol/kg replaced this deficit, allowing

rat serum. A, native GIP₁₋₄₂. B, [Ser²]GIP_{1-30NH₂}. C, [Ser(P)²]GIP_{1-30NH₂}. Data are representative spectra from repeated measurements. In spectra where other minor metabolites were observed, * indicates GIP_{3-30NH₂}, and § denotes [Ser²]GIP_{1-30NH₂}.

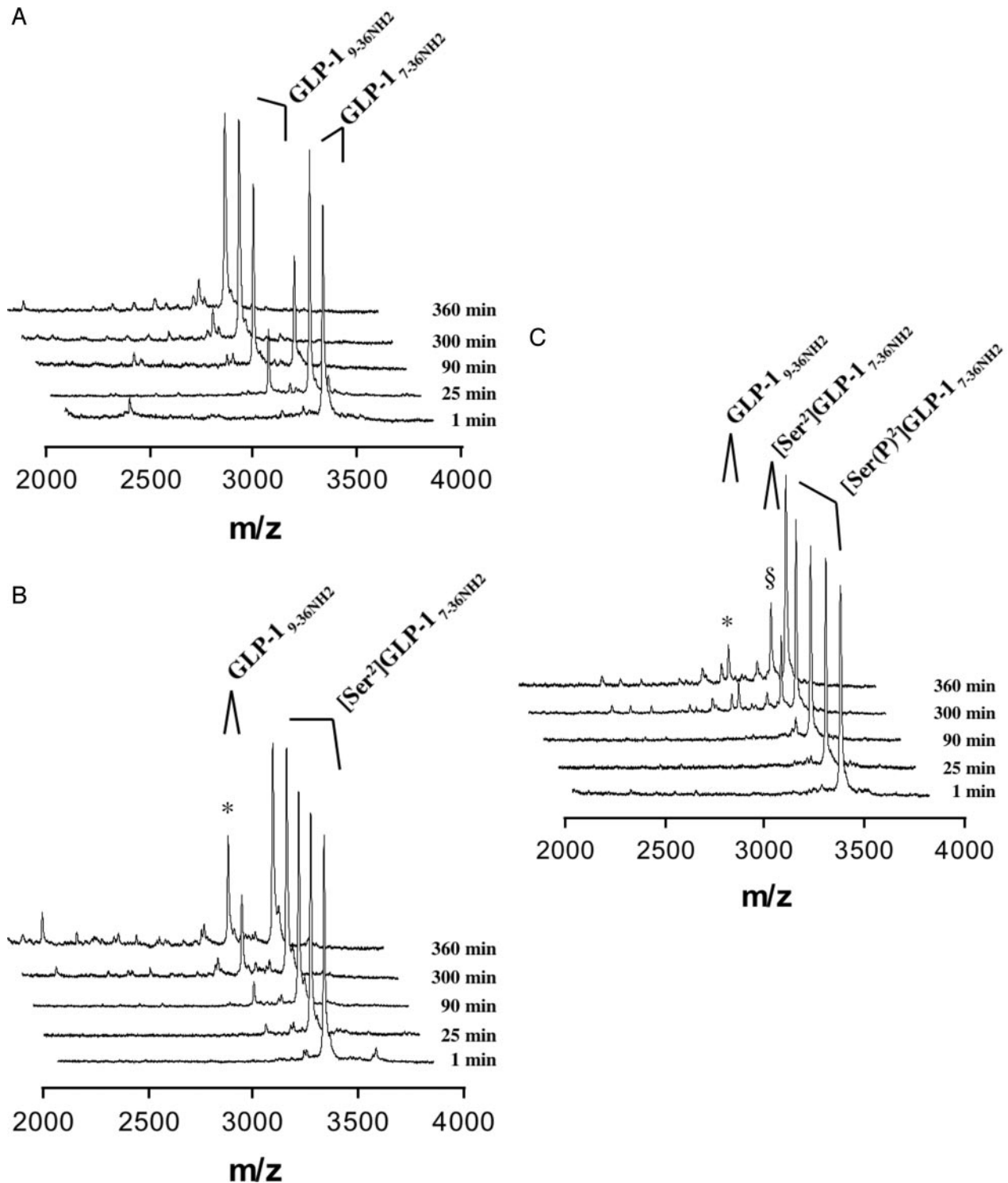


FIG. 6. MALDI-TOF mass spectrometry of GLP-1 and N-terminally modified analogs incubated with 33.3% buffered rat serum. A, native GLP-1_{7-36NH₂}. B, [Ser²]GLP-1_{7-36NH₂}. C, [Ser(P)²]GLP-1_{7-36NH₂}. Data are representative spectra from repeated measurements. In spectra where other minor metabolites were observed, * identifies GLP-1_{9-36NH₂}, and § marks [Ser²]GLP-1_{7-36NH₂}.

greater reduction in the glycaemic profile and significantly enhanced insulin responses (Fig. 8 and Table II).

DISCUSSION

The first suggestion of N-terminal enzymatic degradation of GIP₁₋₄₂ was made upon the isolation of GIP₃₋₄₂ from hog mucosal extracts (24), and it was shown that this peptide was inactive in the perfused stomach (examining somatostatin release), perfused pancreas, and isolated islets (insulin release) (25, 26). Subsequently, N-terminally cleaved GLP-

1₉₋₃₇ was identified on incubation of GLP-1₇₋₃₇ in serum (11), and two groups, Mentlein *et al.* (6) and Kieffer *et al.* (8), independently showed degradation of GIP and GLP-1 by DP IV *in vitro* and *in vivo*, resulting in peptides truncated by two amino acids from their N terminus. Clinical relevance of these findings was aptly demonstrated using specific N-terminally directed radioimmunoassays in humans; importantly, evidence suggested DP IV likely does not contribute to the defective enteroinsular axis in T2DM (27, 28). Both

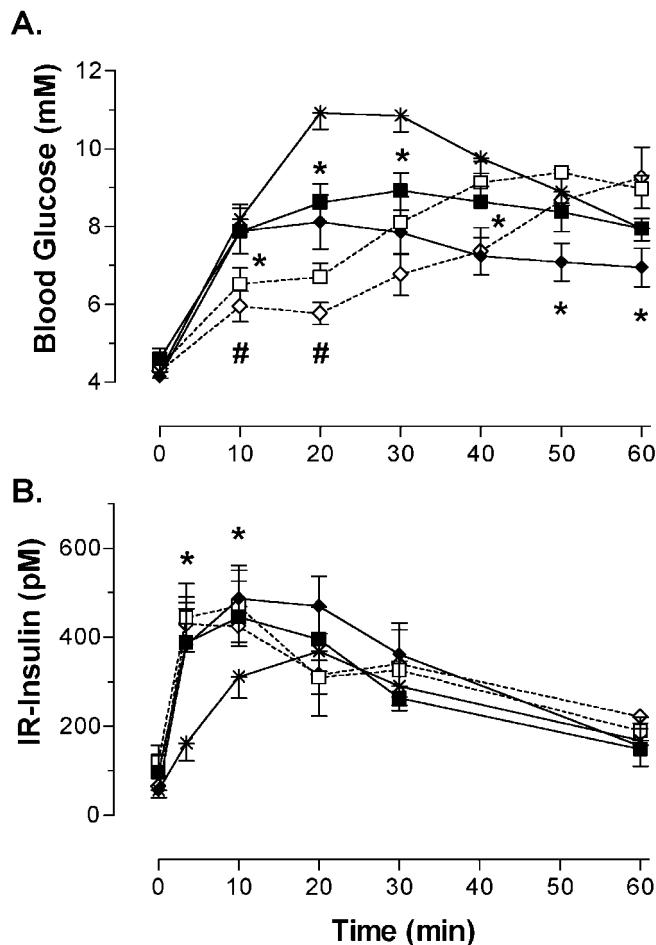


FIG. 7. Bioassay of native and Ser²-substituted incretin analogs in Wistar rats. Bioassay of GIP₁₋₄₂, [Ser²]GIP_{1-30NH₂}, GLP_{1-7-36NH₂}, and [Ser²]GLP_{1-7-36NH₂} was performed in conscious unrestrained male Wistar rats, compared with a saline control. *A*, whole blood glycemia measured from tail vein samples. *B*, immunoreactive (IR) plasma insulin levels from tail vein samples. Peptides (8 nmol/kg of bw in 500 μ l of saline) were injected subcutaneously at time 0 immediately following an OGTT (1 g/kg of bw). Each data point represents the mean \pm S.E. of six animals. * indicates $p < 0.05$ versus saline control, and # indicates $p < 0.05$ between peptides. Integrated responses and statistics can be found in Table II. *, saline; ■, GIP₁₋₄₂; □, GLP_{1-7-36NH₂}; ◆, [Ser²]GIP_{1-30NH₂}; ◇, [Ser²]GLP_{1-7-36NH₂}.

GIP₃₋₄₂ and GLP_{1-9-36NH₂} have been shown to act as competitive antagonists at their receptors; however, given their potency and circulating concentrations, it is unlikely that this results in physiological antagonism (9, 10). Three main approaches have been made to exploit the role of DP IV in regulating incretin bioactivity as potential therapies for diabetes: (i) specific inhibition of DP IV to preserve endogenously released GIP and GLP-1 (29), (ii) inhibition of DP IV to prevent degradation of exogenously administered GIP or GLP-1 (30, 31), and (iii) development of DP IV-resistant GIP and GLP-1 analogs (for review, see Refs. 13–15). Although a number of independent reports of DP IV-resistant analogs for both GIP and GLP-1 have been published, parallel comparison of incretin analogs has yet to be reported.

Here we present parallel data examining both GIP- and GLP-1-derived metabolically stable peptides, and results suggest that despite potential differences in their modes of action, the potencies of these hormones are remarkably similar. Several reports have claimed that Ser²-substituted incretin analogs show enhanced *in vivo* potency due to resistance to N-terminal cleavage (17–19, 32). We noted a moderate DP IV resistance of [Ser²]GIP (16), but our prior work on DP IV

degradation of glucagon (20) showed that native [Ser²]glucagon is also a substrate for DP IV, albeit with slower degradation kinetics than native [Ala²]incretins. Hence, we sought to clarify whether moderate DP IV resistance was enough to confer increased *in vivo* biological activity. Thus we examined in parallel the effects of native GIP, GLP-1, [Ser²]GIP, and [Ser²]GLP-1, as well as two novel phosphoserine 2-modified (Ser(P)²-modified) incretin analogs, *in vitro* on transfected cloned hormone receptors and examined their degradation with purified DP IV or serum using an *in vivo* bioassay.

Both Ser²-substituted incretins showed relatively unchanged receptor binding and activation parameters (Figs. 1 and 2) with little or no reduction in potency ($p > 0.05$). We have shown previously that [Ser²]GIP binding affinity and cAMP stimulation in transfected cells are not significantly different from native GIP (16). Deacon *et al.* (18) reported a significant ~ 11.5 -fold lower affinity of [Ser²]GLP-1 relative to native hormone using baby hamster kidney cells transfected with the GLP-1 receptor, whereas Siegel *et al.* (17) found only a 3-fold reduction in affinity in studies conducted with RINm5F cells. To our knowledge, cAMP responses to [Ser²]GLP-1 have not been reported previously. It was hypothesized that Ser(P)² modification of incretins would provide an extra degree of protection from enzymatic degradation during absorption after subcutaneous injection, as these peptides were also completely resistant to purified DP IV (Figs. 3 and 4) and would likely also be dephosphorylated *in vivo* to the moderately resistant [Ser²]incretin analogs, similar to the time-dependent dephosphorylation of [Ser(P)²]glucagon in serum (20). Dephosphorylation of [Ser(P)²]incretins was confirmed by mass spectrometry of samples incubated in buffered rat serum (Figs. 5 and 6). On subcutaneous injection into rats with concurrent OGTT, [Ser(P)²]incretins produced equivalent or more potent glucose-lowering effects than their non-phosphorylated counterparts at the same dosage (Fig. 8 and Table II), indicating that the peptides must also be dephosphorylated *in vivo*, as the [Ser(P)²]peptides have low potency at their cloned receptors (Figs. 1 and 2). Preliminary studies have indicated that Ser(P)²-modified incretins are able to be dephosphorylated by human alkaline phosphatase,² although the precise phosphatase in serum has yet to be identified. Hence, [Ser(P)²]incretin analogs represent the first generation of “prodrug” forms of long acting metabolically stable peptides.

The overall shape of the glycemic profiles appeared to differ substantially when comparing GIP-based peptides to GLP-1 derivatives, which could be due to different non-insulin-dependent effects of these hormones on glucose homeostasis and gastrointestinal physiology. In addition to stimulating insulin release, incretins may also have effects on gastric motility, glucagon release, glucose uptake and utilization by muscle and adipose tissue, and, although not applicable to the current report, as brain satiety factors (2, 3, 13, 33). GLP-1 is a well characterized inhibitor of gastric emptying, an area of GIP physiology that has been largely unreported. The slowing of gastric emptying lowers postprandial glycemic excursions by reducing delivery of nutrients to their site of absorption (33), which may be the reason why in GLP-1-injected animals glycemia was more potently reduced at early time points (Fig. 7) but then continued to increase at later time points, during which time glycemia of saline and GIP-injected rodents had already begun to decline toward basal. It is an important observation, however, that although GLP-1 may reduce postprandial glycemic excursions via inhibition of gastric emptying in human studies, this function is also thought to result in asso-

² K. Kühn-Wache, unpublished observations.

TABLE II
Integrated glucose and insulin profiles for GIP, GLP-1, Ser²- and Ser(P)²-substituted analogs *in vivo*

Data represent the mean area under the curve ± S.E. of data shown in Figs. 7 and 8 (*n* = 6). Peptides were injected subcutaneously with a concurrent oral glucose load. Blood glucose and insulin were determined from samples obtained from the tail vein. * indicates *p* < 0.05 compared to saline control.

Test	Glucose profile	Early insulin response	Complete insulin profile
	<i>mM</i> × 60 <i>min</i>	<i>nM</i> × 10 <i>min</i>	<i>nM</i> × 60 <i>min</i>
Saline	292 ± 16	1.91 ± 0.35	13.5 ± 2.0
GIP ₁₋₄₂	211 ± 18*	3.35 ± 0.38*	15.7 ± 3.4
[Ser ²]GIP _{1-30NH2}	188 ± 24*	3.31 ± 0.55*	16.9 ± 2.4
[Ser(P) ²]GIP _{1-30NH2}	163 ± 29*	2.21 ± 0.50	15.2 ± 1.2
10 × [Ser(P) ²]GIP _{1-30NH2}	138 ± 30*	3.35 ± 0.75*	19.5 ± 3.9*
GLP-1 _{7-36NH2}	193 ± 26*	2.73 ± 0.60	14.0 ± 2.4
[Ser ²]GLP-1 _{7-36NH2}	156 ± 27*	2.99 ± 0.39*	17.2 ± 4.3
[Ser(P) ²]GLP-1 _{7-36NH2}	202 ± 30*	2.29 ± 0.45	14.6 ± 2.4
10 × [Ser(P) ²]GLP-1 _{7-36NH2}	123 ± 16*	3.65 ± 0.69*	16.7 ± 5.5

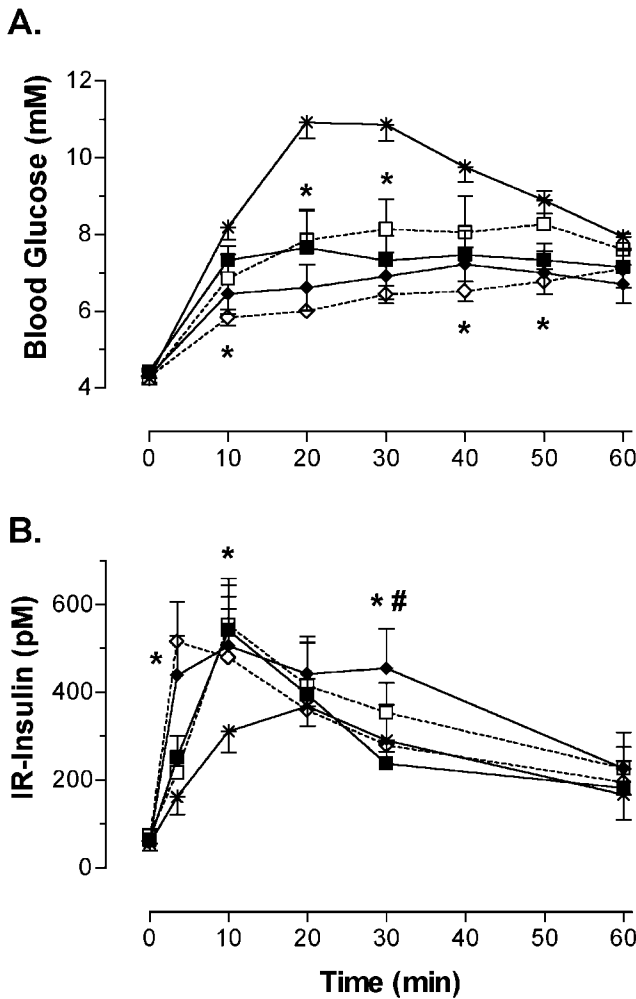


FIG. 8. Bioassay of phosphoserine 2-substituted incretin analogs in Wistar rats. Bioassay of [Ser(P)²]GIP_{1-30NH2} and [Ser(P)²]GLP-1_{7-36NH2} was performed in conscious unrestrained male Wistar rats, compared with a saline control. A, whole blood glycemia measured from tail vein samples. B, immunoreactive (IR) plasma insulin levels from tail vein samples. Peptides (8 or 80 nmol/kg of bw in 500 μl of saline) were injected subcutaneously at time 0 immediately following an OGTT (1 g/kg of bw). Each data point represents the mean ± S.E. of six animals; * indicates *p* < 0.05 versus saline control, and # indicates *p* < 0.05 between peptides. Integrated responses and statistics can be found in Table II. *, saline; ■, [Ser(P)²]GIP_{1-30NH2}; □, [Ser(P)²]GLP-1_{7-36NH2}; ◆, 10 × [Ser(P)²]GIP_{1-30NH2}; ◇, 10 × [Ser(P)²]GLP-1_{7-36NH2}.

ciated nausea and vomiting (13), side effects not likely to be of concern during GIP administration. Regardless, despite the potential differences in the modes of action of GIP and GLP-1,

their overall glucose-lowering action is quite similar in rats (Fig. 7 and Table II), consistent with the equal insulin-releasing potency of the two hormones (34).

Studies have primarily focused on GLP-1 because the therapeutic potential of this hormone has been proven in numerous basic and clinical scientific reports. Although a strong foundation for use of GIP in the treatment of metabolic disease has been demonstrated in laboratory animals, corresponding controlled trials in humans are few. Literature to date suggests that a secretory defect of GLP-1 and receptor/post-receptor defect in GIP signaling contribute to the decreased incretin effect in diabetes (35). Early clinical trials indicated that both exogenous GIP and GLP-1 showed reduced biological activity in type 2 diabetes but that the potency of GLP-1 in diabetics is better preserved compared with GIP (5, 36). Questions have been raised as to the biological potency of early batches of synthetic peptides used in these studies (9, 34); however, another possibility to explain the reduction of GIP activity in T2DM could be a decrease in receptor expression, a deficiency that has been established experimentally in the obese Vancouver diabetic fatty Zucker rat, a model of human T2DM (37). Regardless, in the same animal model, bolus subcutaneous injections of GIP and a DP IV-resistant analog of GIP ([D-Ala²]GIP) were still able to reduce glycemic excursions (9), and similar results have been demonstrated using obese (*ob/ob*) mice administered intraperitoneal injections of other N-terminally modified GIP analogs (15). Thus it seems that both GIP and GLP-1, as well as analogs with improved plasma stability, may have a role in the treatment of human type 2 diabetes mellitus.

The insulinotropic activity of GIP and GLP-1 is enhanced when the hormones are combined (36, 38, 39) or in patients undergoing either sulfonylurea or metformin treatment (40, 41), lending support to combination therapy (DP IV inhibition in animal models with exogenous incretin infusion also enhances the insulinogenic effects of these hormones (30, 31)). Several “sustained release” analogs of GLP-1 have recently been examined in the human studies (13). Typically these are DP IV-resistant GLP-1 analogs modified to bind serum albumin, and on repeated injection, these compounds bioaccumulate and likely improve glycemia via tonic stimulation of the β-cell, obviating the need for repeated or continuous peptide injection. Although the possibility of incretin receptor desensitization/down-regulation has not been directly studied in humans or animals receiving these compounds, their efficacy *in vivo* is apparent from the data available. Given the renewed interest in GIP as a potential therapeutic agent, it is probable that similar formulations based on DP IV-resistant compounds reviewed here modified to bind serum proteins will show similar results to this promising new class of incretin-based therapy.

In summary, Ser²-substituted GIP and GLP-1 analogs were reassessed in parallel with respect to *in vitro* potency at their cloned receptors (binding affinity and cAMP stimulation), their resistance to dipeptidyl peptidase IV degradation, and bioassay in conscious unrestrained Wistar rats. [Ser²]GIP and [Ser²]GLP-1 showed binding affinities and receptor activation potency similar to those of their native parent peptides with only slight enhancement of their antihyperglycemic effects *in vivo*, consistent with their moderate resistance to degradation. In contrast, Ser(P)²-modified incretins were completely resistant to DP IV degradation for over 24 h but displayed significantly reduced *in vitro* potency at their transfected receptors. *In vivo*, these peptides showed equal or greater glucose-lowering activity than native or Ser²-substituted incretins. Mass spectrometry revealed that [Ser(P)²]incretins were dephosphorylated in serum to the moderately stable [Ser²]molecules. It is proposed that this modification provides an extra degree of protection from serum proteases, thus increasing the proportion of biologically active peptides reaching target tissues. Because both GIP- and GLP-1-based analogs showed comparable glucose-lowering effects despite potential differences in mode of action, analogs based on either incretin may have future therapeutic use.

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