A Novel Glucagon-like Peptide-1 (GLP-1)/Glucagon Hybrid Peptide with Triple-acting Agonist Activity at Glucose-dependent Insulinoetric Polypeptide, GLP-1, and Glucagon Receptors and Therapeutic Potential in High Fat-fed Mice*

Received for publication, August 23, 2013, and in revised form, October 24, 2013 Published, JBC Papers in Press, October 28, 2013, DOI 10.1074/jbc.M113.512046

Victor A. Gault, Vikas K. Bhat, Nigel Irwin, and Peter R. Flatt
From the SAAD Centre for Pharmacy and Diabetes, School of Biomedical Sciences, University of Ulster, Coleraine, BT52 1SA Northern Ireland, United Kingdom

**Background:** Glucagon-like peptide-1 (GLP-1), glucose-dependent insulinoetric polypeptide (GIP), and glucagon have important gluco-regulatory actions.

**Results:** Fusion of amino acid sequences of GLP-1, GIP, and glucagon produces hybrid peptides with triple-acting agonist activity.

**Conclusion:** Hybrid peptides possess beneficial biological actions equivalent, or superior to, activation of single receptors.

**Significance:** Multitargeting peptides offer a new class of therapeutics for obesity and diabetes.

Glucagon-like peptide-1 (GLP-1), glucose-dependent insulinoetric polypeptide (GIP), and glucagon bind to related members of the same receptor superfamily and exert important effects on glucose homeostasis, insulin secretion, and energy regulation. The present study assessed the biological actions and therapeutic utility of novel GIP/glucagon/GLP-1 hybrid peptides. Nine novel peptides were synthesized and exhibited complete DPP-IV resistance and enhanced in vitro insulin secretion. The most promising peptide, [dA2]GLP-1/GcG, stimulated cAMP production in GIP, GLP-1, and glucagon receptor-transfected cells. Acute administration of [dA2]GLP-1/GcG in combination with glucose significantly lowered plasma glucose and increased plasma insulin in normal and obese diabetic (ob/ob) mice. Furthermore, [dA2]GLP-1/GcG elicited a protracted glucose-lowering and insulinoetric effect in high fat-fed mice. Twice daily administration of [dA2]GLP-1/GcG for 21 days decreased body weight and nonfasting plasma glucose and increased circulating plasma insulin concentrations in high fat-fed mice. Furthermore, [dA2]GLP-1/GcG significantly improved glucose tolerance and insulin sensitivity by day 21. Interestingly, locomotor activity was increased in [dA2]GLP-1/GcG mice, without appreciable changes in aspects of metabolic rate. Studies in knock-out mice confirmed the biological action of [dA2]GLP-1/GcG via multiple targets including GIP, GLP-1, and glucagon receptors. The data suggest significant promise for novel triple-acting hybrid peptides as therapeutic options for obesity and diabetes.

Peripheral signals that control glucose homeostasis and energy regulation are carefully balanced and encompass a number of factors, including a variety of peptide hormones (1). The major focus on gut hormone-based therapies over the past decades has concentrated on single molecules that target one specific pathway (2). Although specific glucagon-like peptide-1 (GLP-1)2 mimetics are used clinically for type 2 diabetes, the glycemic control and weight reductions achieved with certain types of gastric bypass surgery is markedly superior (3). Recognition is growing that these beneficial effects reflect changes in circulating levels of multiple peptide hormones that trigger a broad spectrum of pathways involved in glucose regulation and energy balance (4). Therefore, combining the activity of two or more regulatory hormones, with complementary biological actions, offers a favorable approach for the treatment of obesity and diabetes. In this context, GLP-1, glucose-dependent insulinoetric polypeptide (GIP), and glucagon possesses a number of biological effects that would suggest significant combined therapeutic effectiveness (2).

Together GIP and GLP-1 account for almost all of the well established physiological incretin effect and have powerful insulin-releasing and gluco-regulatory properties (5). Moreover, both peptides appear to have important pancreatic beta-cell protective actions and additional extra-pancreatic glucose lowering effects that further promote therapeutic applicability for diabetes (6). On the other hand, glucagon is classically regarded as an important hormone in maintaining normal glucose concentrations through enhanced hepatic glucose production (7). However, recent evidence now suggests that glucagon can be exploited therapeutically as a satiety factor, which also increases energy expenditure and body weight loss (8). Fur-
GLP-1/Glucagon Hybrid Peptide

TABLE 1

| Name Shortened name | Amino acid sequence |
|---------------------|---------------------|
| [d-Ala²]-GIP-1 [G1⁷-30]amide | Y-αA²-E-G-T-I-S-D-Y-S-I-A-M-D-K-I-H-Q-D-F-V-N-W-L-L-A-Q-K-NH₂ |
| [d-Ala²]-GIP-1 | H-αA²-E-G-T-I-S-D-Y-S-I-A-M-D-K-I-H-Q-D-F-V-N-W-L-L-A-Q-K-NH₂ |
| [d-Ala²]-GLP-1 | H-αA²-E-G-T-I-S-D-Y-S-I-A-M-D-K-I-H-Q-D-F-V-N-W-L-L-A-Q-K-NH₂ |
| [d-Ala²]-GIP-1-GLP-1 | H-αA²-E-G-T-I-S-D-Y-S-I-A-M-D-K-I-H-Q-D-F-V-N-W-L-L-A-Q-K-NH₂ |
| [d-Ala²]-GLP-1-GIP-1 | Y-αA²-E-G-T-I-S-D-Y-S-I-A-M-D-K-I-H-Q-D-F-V-N-W-L-L-A-Q-K-NH₂ |
| [d-Ala²]-GIP-Glucagon-GLP-1 | Y-αA²-E-G-T-I-S-D-Y-S-I-A-M-D-K-I-H-Q-D-F-V-N-W-L-L-A-Q-K-NH₂ |
| [d-Ala²]-GIP-GLP-1-Glucagon | Y-αA²-E-G-T-I-S-D-Y-S-I-A-M-D-K-I-H-Q-D-F-V-N-W-L-L-A-Q-K-NH₂ |

Thermore, transgenic mice overexpressing the glucagon receptor (R) in pancreatic beta-cells demonstrate increased insulin secretion and pancreatic beta-cell mass, with protection against impaired glucose tolerance following high fat feeding (9). Thus, it follows that design of a single hybrid peptide, capable of simultaneous activation of GLP-1, GIP, and glucagon Rs, would have substantially enhanced therapeutic promise for obesity and diabetes.

To generate just such a compound, we have constructed nine novel GLP-1/GIP/glucagon hybrid peptides. These hybrid peptides have been created through fusion of the key amino acid sequences of GLP-1, GIP, and glucagon known to be important for biological activity (see Table 1). Importantly, because all three peptides are substrates for dipetidyl peptidase IV (10, 11), hybrid peptides with GIP or GLP-1-like N termini have substitution of the naturally occurring alanine L-isomer residue for a D-isomer, whereas peptides with a glucagon-like N terminus have substitution of alanine for serine at position 2. These specific modifications are known to impart DPP IV resistance and improve biological activity of respective parent peptides (11–13). We initially examined DPP IV resistance, in vitro insulin secretion, and in vivo glucose-lowering and insulino-notropic actions of all hybrid peptides. The acute anti-diabetic effects of the most efficacious hybrids were then evaluated in obese diabetic (ob/ob) mice. The most effective peptide, [dAla²]-GLP-1-glucagon-GLP-1 ([dAla²]GLP/GcG), was progressed to a twice daily injection regime in high fat-fed mice to examine effects of chronic treatment on body weight, food intake, energy expenditure, nonfasting glucose and insulin, glucose tolerance, insulin sensitivity, locomotor activity, and aspects of metabolic rate. Finally, to elucidate potential mechanism of action, cAMP production capabilities of [dAla²]GLP/GcG were examined in cells transfected with either the GLP-1, GIP, or glucagon R, and in vivo gluco-regulatory and insulin-releasing activity was assessed in GIP, GLP-1, and double incretin R knock-out mice.

EXPERIMENTAL PROCEDURES

Peptides—Table 1 displays the amino acid sequence of the nine hybrid peptides used in this study, which were based on the structures of GLP-1, GIP, and glucagon. In addition, native GLP-1, GIP, and glucagon, along with [dAla²]GIP, [dSer²]glucagon, and [dAla²]GLP-1 were used as control peptides. All peptides were purchased from GL Biochem Ltd. (Shanghai, China; greater than 90% purity). In addition to quality control data supplied with peptide purchased, all peptides were characterized in-house using MALDI-TOF MS, as described previously (14).

DPP-IV Degradation Assay—Peptides were incubated at 37 °C in 50 mmol/liter TEA-HCl (pH 7.8; Sigma-Aldrich) with purified porcine DPP-IV (5 milliunits; Sigma-Aldrich) for 0, 2, 4, and 8 h. Degradation profiles were obtained using RP-HPLC analysis as described previously (14), and the HPLC peak area data were used to calculate the percentage of intact peptide remaining at time points during the incubation.

In Vitro Insulin Secretion—Effects of peptides on in vitro insulin secretion were examined using BRIN-BD11 cells whose characteristics have been reported previously (15). Briefly, BRIN-BD11 cells were seeded (150,000 cells/well) into 24-well plates (Nunc, Roskilde, Denmark) and allowed to attach overnight at 37 °C. Following 40 min of preincubation (1.1 mmol/liter glucose; 37 °C), cells were incubated (20 min; 37 °C) in the presence of 5.6 and 16.7 mmol/liter glucose with a range of peptide concentrations (10⁻¹²–10⁻⁶ mol/liter). After 20 min of incubation, buffer was removed from each well, and aliquots (200 μl) were stored at −20 °C prior to determination of insulin by radioimmunoassay (16).

In Vitro cAMP Production—Effects of [DA²]GLP-1/GcG, GLP-1, GIP, and glucagon on cAMP production were assessed in Chinese hamster lung cells transfected with either the human GIP- or GLP-1-R, as well as human embryonic kidney (HEK292) cells transfected with the human glucagon R (17). Cells were seeded (200,000 cells/well) into 96-well plates (Nunc) and washed with Hanks’ balanced salt solution buffer before incubation with test peptides (10⁻⁶–10⁻¹² mol/liter) in the presence of 200 μmol/liter 3-isobutyl-1-methylxanthine for 20 min at 37 °C. After incubation, medium was removed, and the cells were lysed before measurement of cAMP using Parameter cAMP assay (R&D Systems, Abingdon, UK) according to the manufacturer’s instructions.

Animals—Acute animal studies were carried out in male National Institutes of Health Swiss mice (Harlan Ltd., Black-thorne, UK; 12–14 weeks old), obese (ob/ob) mice (derived from the colony originally maintained at Aston University (18); 14–16 weeks old), and also C57BL/6 mice with genetic deletion of either the GIP- or GLP-1 R and both incretin Rs (the background and generation of GIP, GLP-1, and double incretin R knock-out mice has been previously described (19)). Longer term experiments were performed in National Institutes of Health Swiss mice previously fed a high fat diet for 140 days...
RESULTS

**DPP-IV Stability**—As expected, [dAla^2]GIP, [dAla^2]GLP-1, and [dSer^2]glucagon were resistant to DPP-IV action, whereas native peptides were degraded with estimated half-lives of 2–4 h (Table 2). In addition, all novel hybrid peptides were completely stable to the actions of DPP-IV up to and including 8-h incubations (Table 2).

**In Vitro Insulin Secretion**—Table 2 displays the effects of all test peptides on insulin secretion at 5.6 and 16.7 mmol/liter glucose in BRIN-BD11 cells. At both glucose concentrations, all peptides (10^-6 mol/liter), with the exception of native glucagon at 16.7 mmol/liter glucose, significantly (p < 0.05 vs. p < 0.001) increased insulin secretion compared with respective glucose controls. All hybrid peptides displayed similar insulin secretory potencies (Table 2). Thus, none of the hybrid peptides displayed absolute superior insulinotropic actions when compared with the native or control peptides (Table 2).

**Acute Glucose-lowering and Insulinotropic Actions of Hybrid Peptides in Normal and ob/ob Mice**—Native GIP and glucagon failed to elicit any significant insulin-releasing or glucose-modulating actions in normal mice at the dose employed when compared with glucose alone controls (Table 2). In contrast, GLP-1 significantly reduced (p < 0.05) overall 0–60 min AUC plasma glucose values and increased (p < 0.01) the overall insulin secretory response when compared with controls (Table 2). All three positive control peptides, [dAla^2]GIP, [dAla^2]GLP-1, and [dSer^2]glucagon, induced a significantly (p < 0.05) enhanced insulinotropic response when compared with glucose alone, but only [dAla^2]GLP-1-treated mice had a significantly (p < 0.05) reduced glycemic excursion in normal mice (Table 2). Among the novel hybrid peptides, only [dSer^2]GcG/GIP, [dAla^2]GLP-1/GcG, and [dAla^2]GIP/GLP-1 significantly (p < 0.05 vs. p < 0.01) decreased the overall plasma glycemic excursion and potentiated (p < 0.01) glucose-induced insulin release compared with controls (Table 2). Consequently, the insulin-releasing and glucose-lowering capabilities of these three novel hybrid peptides were examined in ob/ob mice (Fig. 1). [dSer^2]GcG/GIP, [dAla^2]GLP-1/GcG, and [dAla^2]GIP/GLP-1 did not display overall superiority when compared with native or positive control peptides (Table 2). In ob/ob mice, [dSer^2]GcG/GIP exhibited only a mildly enhanced insulin secretory action when compared with glucose alone control (Fig. 1, a and b), whereas [dAla^2]GIP/GLP-1 had no obvious beneficial effects (Fig. 1, e and f). However, [dAla^2]GLP-1/GcG retained substantial and significant (p < 0.05 to p < 0.01) glucose-lowering and insulin-releasing actions in ob/ob mice (Fig. 1, c and d).

**Persistent Glucose-lowering and Insulinotropic Actions of [dAla^2]GLP-1/GcG in High Fat-fed Mice**—When administered 4 (Fig. 2, a and b) or 8 h (Fig. 2, c and d) prior to a glucose load, [dAla^2]GLP-1/GcG significantly reduced individual post-injection and overall 0–60 min AUC glucose values in high-fat-fed mice when compared with injection of native GLP-1 (Fig. 2, a and c). In agreement, post-injection and overall glucose-induced insulin concentrations were markedly (p < 0.05 vs. p < 0.001) elevated by [dAla^2]GLP-1/GcG administration 4 or 8 h prior to a glucose challenge when compared with native GLP-1 (Fig. 2, b and d).
GLP-1/Glucagon Hybrid Peptide

TABLE 2
DPP-IV stability, in vitro insulin secretory activity, and in vivo glucose lowering and insulin releasing actions of native, control, and novel hybrid peptides

Resistance of peptides to degradation by DPP-IV (5 millunits) was measured (n = 3) following 0, 2, 4, and 8 h of incubation. Reaction products were subsequently analyzed by HPLC. For in vitro insulin secretory studies, peptides (10^-6 m) were incubated with BRIN-BD11 cells in the presence of 5.6 or 16.7 mM glucose (20 min; n = 8), and insulin release was measured by radioimmunoassay and presented as a percentage of respective control. For in vivo studies, plasma glucose and insulin concentrations were measured immediately prior to and 15, 30, and 60 min after intraperitoneal administration of glucose alone (18 mmol/kg body weight; n = 8) or in combination with respective peptides (each at 25 nmol/kg body weight) in 18-h fasted NIH Swiss normal mice. The data are expressed as means ± S.E.

| Peptide            | In vitro DPP-IV half-life (h) | Maximal in vitro insulin secretion | AUC                                                                 |
|--------------------|-------------------------------|----------------------------------|----------------------------------------------------------------------|
|                    |                               | 5.6 mM glucose                   | 16.7 mM glucose                                                      |                                                                 |
|                    |                               | %                               | %                                                                   | % glucose alone         |
| Native GIP         | 2.2 ± 0.0                     | 245.7 ± 25.6*                    | 167.0 ± 9.0*                                                        | 81.43 ± 14.9           |
| Native GLP-1       | 4.4 ± 0.3                     | 328.0 ± 39.5*                    | 210.6 ± 19.8*                                                       | 68.75 ± 1.3            |
| Native glucagon    | 2.0 ± 0.2                     | 171.9 ± 21.3*                    | 131.2 ± 13.8                                                       | 88.42 ± 12.2           |
| [oA2]GIP          | > 8                           | 375.1 ± 25.8*                    | 225.5 ± 24.9*                                                       | 72.53 ± 9.0            |
| [oA2]GcG          | > 8                           | 361.3 ± 29.7*                    | 191.4 ± 7.9                                                        | 76.17 ± 4.6            |
| [oA2]GLP-1        | > 8                           | 198.1 ± 11.9*                    | 160.6 ± 14.3                                                       | 64.73 ± 6.8            |
| [oA2]GcG/GIP      | > 8                           | 297.2 ± 31.6*                    | 171.2 ± 16.3                                                       | 66.32 ± 2.2            |
| [sA2]GcG/Glucagon | > 8                           | 319.6 ± 38.9*                    | 168.9 ± 12.3                                                       | 84.26 ± 3.5            |
| [oA2]GIP/GcG      | > 8                           | 217.1 ± 10.5*                    | 145.6 ± 9.0                                                        | 89.78 ± 3.4            |
| [oA2]GIP/Glucagon | > 8                           | 238.3 ± 19.7*                    | 162.9 ± 10.5                                                       | 90.62 ± 4.8            |
| [oA2]GcG/GP       | > 8                           | 249.9 ± 25.7*                    | 185.2 ± 3.9                                                        | 59.58 ± 3.6            |
| [sA2]GcG/Glucagon | > 8                           | 211.4 ± 17.3*                    | 127.5 ± 5.0                                                        | 87.69 ± 1.9            |
| [oA2]GIP/Glucagon | > 8                           | 177.8 ± 32.8*                    | 134.5 ± 5.9                                                        | 100.1 ± 11.6           |
| [oA2]GcG/GP       | > 8                           | 308.7 ± 32.6*                    | 194.2 ± 19.3                                                       | 91.11 ± 6.2            |
| [sA2]GIP/Glucagon | > 8                           | 384.8 ± 15.4*                    | 250.2 ± 27.1*                                                       | 73.28 ± 4.9            |

* p < 0.01 compared to respective control.
** p < 0.05 compared to respective control.
*** p < 0.001 compared to respective control.

Effects of Twice Daily Administration of [DA2]GLP-1/GcG or Exenatide on Body Weight, Energy Intake, Nonfasting Plasma Glucose, and Insulin Concentrations in High Fat-fed Mice—Twice daily administration of exenatide or [DA2]GLP-1/GcG had no significant effect on accumulated energy intake over the course of the 21 days (Fig. 3a). There was an obvious trend for reduced body weight gain with [DA2]GLP-1/GcG treatment, and this was significant on days 10 and 16 when compared with the saline control group (Fig. 3b). Similarly, nonfasting plasma glucose levels were not significantly different between groups at the individual observation points, but the overall glucose exposure during the 21-day period was significantly (p < 0.05) reduced by [DA2]GLP-1/GcG treatment (Fig. 3c). Circulating insulin concentrations were significantly (p < 0.01) increased on day 21 in exenatide and [DA2]GLP-1/GcG mice, with accompanying elevations (p < 0.05 and p < 0.01; respectively) of overall insulin levels during the entire 21-day treatment period (Fig. 3d).

Effects of Twice Daily Administration of [DA2]GLP-1/GcG or Exenatide on Glucose Tolerance, Plasma Insulin Response to Glucose, and Insulin Sensitivity in High Fat-fed Mice—Following intraperitoneal (Fig. 4, a and b) or oral (Fig. 4, c and d) glucose challenge on day 21, plasma glucose levels had a strong tendency to be reduced in exenatide and [DA2]GLP-1/GcG-treated mice, but this failed to reach significance (Fig. 4, a and c). Furthermore, there was a significant (p < 0.05 to p < 0.01) elevation of both post-injection and 0–60 min overall AUC glucose-stimulated plasma insulin concentrations in exenatide and [DA2]GLP-1/GcG mice following either intraperitoneal or oral glucose administration on day 21 (Fig. 4, b and d). The effects of [DA2]GLP-1/GcG and exenatide were broadly similar (Fig. 4, a–d). In addition, the hypoglycemic action of exogenous insulin was substantially and similarly (p < 0.001) augmented in exenatide and [DA2]GLP-1/GcG mice 30 and 60 min post-insulin injection on day 21 (Fig. 4e). This was corroborated from overall 0–60 min glucose values, where both treatment groups significantly (p < 0.05) improved insulin action compared with saline controls (Fig. 4f).

Effects of Twice Daily Administration of Exenatide or [DA2]GLP-1/GcG on Locomotor Activity and Metabolic Rate in High Fat-fed Mice—There were no differences in O2 consumption, CO2 production, respiratory exchange ratio, and energy expenditure in any of the groups of mice on day 21 (data not shown). However, although there were also no significant differences in ambulatory activity between groups (Fig. 5, a and b), treatment with exenatide and [DA2]GLP-1/GcG significantly (p < 0.05 and p < 0.01; respectively) increased rearing and jumping episodes during the light phase, as assessed by Z beam breaks (Fig. 5c). In addition, [DA2]GLP-1/GcG administration significantly (p < 0.01) increased Z beam breaks during the dark phase (Fig. 5d).

[DA2]GLP-1/GcG-induced cAMP Production in GLP-1, GIP, and Glucagon R-transfected Cells—A shown in Fig. 5, [DA2]GLP-1/GcG stimulated cAMP production in an almost identical fashion to native GLP-1 in GLP-1 R-transfected cells (Fig. 5e). In harmony, cAMP production capabilities of [DA2]GLP-1/GcG were essentially similar to native GIP and glucagon in GIP and glucagon R-transfected cells, respectively (Fig. 5, f and g).

Glucose-lowering and Insulinotropic Actions of [DA2]GLP-1/GcG in GLP-1, GIP, and Double Incretin R Knock-out Mice—As would be expected, native GIP and GLP-1 were without biological effects in GIP and GLP-1 R knock-out mice, respectively (Fig. 6, a–d). In addition, administration of GIP or GLP-1 had no consequence in double incretin R knock-out mice (Fig. 6, e and f). In contrast, [DA2]GLP-1/GcG significantly (p < 0.05 to p < 0.001) increased glucose-stimulated insulin secretion in GLP-1, GIP, and double incretin R knock-out mice (Fig. 6, b, d, and f). The glucose-lowering actions of [DA2]GLP-1/GcG were particularly evident in GIP R knock-out mice, with significant...
reductions in 30 and 60 min post-injection values \((p < 0.001)\), as well as overall 0–60 min AUC \((p < 0.05)\) values, when compared with glucose controls (Fig. 6a). In addition, there was also a strong trend for decreased glucose levels with \([\text{DA}^2]\text{GLP-1/GcG}\) treatment in GLP-1R knock-out mice (Fig. 6c), and increased sample size may have improved the statistical power of this experiment. Interestingly, glucagon induced significant \((p < 0.05\) to \(p < 0.01)\) elevations of the overall glycemic excursion in GIP and double incretin R knock-out mice, but not in GLP-1 R knock-out mice (Fig. 6).

**DISCUSSION**

In the present study, we have evaluated the biological actions and therapeutic applicability of a series of novel GLP-1/GIP/glucagon hybrid peptides. These peptides were engineered to combine the energy liberating action of glucagon (8), with the robust insulin-releasing actions of GIP and GLP-1 (6), in a single compound. Unlike native GIP, GLP-1, or glucagon, all novel peptides were completely stable to enzymatic breakdown by DPP-IV and exhibited significantly enhanced insulinotropic actions in clonal beta-cells. These observations are in harmony with previous studies that clearly reveal that the N-terminal modifications employed in the current study mask the DPP-IV-binding site and increase intrinsic biological activity of GIP, GLP-1, and glucagon (11–13). Thus, importantly our data show that the modified hybrid peptides still retain the ability to activate important corresponding pathways that lead to insulin secretion.

To screen acute in vivo properties of positive control and novel hybrid peptides, they were co-administered with glucose to normal mice. Only \([\text{DS}^2]\text{GcG/GIP}\), \([\text{DA}^2]\text{GLP-1/GcG}\), and \([\text{DA}^2]\text{GIP/GLP-1}\) exhibited significantly improved glucose-lowering and insulin-releasing actions when compared with glucose control. This suggests that these modifications did not have deleterious effects on three-dimensional peptide structures and their ability to bind to G protein-coupled receptors on the surface of beta-cells. Indeed, acute biological actions of \([\text{DS}^2]\text{GcG/GIP}\), \([\text{DA}^2]\text{GLP-1/GcG}\), and \([\text{DA}^2]\text{GIP/GLP-1}\) were essentially similar to positive control peptides. It also reinforces the concept that GLP-1, GIP, and glucagon signaling are all comparatively important in terms of maintaining normal glycemic control (20). To evaluate the potential anti-diabetic pro-
pensity of [DS²]GcG/GIP, [DA²]GLP-1/GcG, and [DA²]GIP/GLP-1, similar studies were carried out in ob/ob mice. The Aston ob/ob mouse model employed in the current study presents with hyperglycaemia and severe obesity because by the defective production of leptin and is a particularly robust model of obesity and diabetes (18). Under these conditions, only [DA²]GLP-1/GcG retained convincing beneficial actions. Moreover, [DA²]GLP-1/GcG had marked glucose homeostatic and insulin-releasing effects when administered 8 h previously in high fat-fed mice, which were significantly superior to native GLP-1. These effects are reminiscent of other related modified gluco-regulatory hormones (6) and provide a strong basis for the subsequent 21-day study conducted in high fat-fed mice.

Chronic treatment with [DA²]GLP-1/GcG or exenatide in high fat-fed mice resulted in similar significant improvements in metabolic status. This included prominent elevations in non-fasting insulin levels with concomitant reductions in glycemic status. Notably, [DA²]GLP-1/GcG was appreciably more effective than exenatide in terms of glucose lowering action. Furthermore, [DA²]GLP-1/GcG, but not exenatide, induced sig-
significant reductions in body weight gain. Thus, it follows that combining the activity of two or more regulatory hormones, to concomitantly activate related biological pathways, offers a more favorable approach for the treatment of obesity and diabetes than activation of lone pathways (2). The slightly greater weight loss with [DA²]GLP-1/GcG, as opposed to exenatide treatment, could also be an important factor. The observed effects of [DA²]GLP-1/GcG were independent of changes in energy intake, despite related satiating effects of GLP-1 and glucagon (21, 22). The lack of effect of exenatide on energy intake and body weight likely reflects up-regulation of inherent adaptive mechanisms to normalize energy balance and body weight regulation when only one signaling pathway is activated, with similar observations reported previously in our laboratory and others (23, 24). However, this does contrast with similar studies in genetically obese or diabetic animals showing that longer term administration of exenatide significantly reduced food intake, causing weight loss (24–26). The most plausible explanation for the lack of such chronic effects of exenatide in the current study therefore lies with the dose, possible GLP-1 receptor desensitization, or background genetics. It seems unlikely to us that the dose is an issue, because other studies with exenatide in animal models have employed doses of 1 nmol/kg (27), 24 nmol/kg (25), or 50 nmol/kg (24). The alter-
native that GLP-1 receptor desensitization occurs is perhaps plausible from in vitro studies (28), although this phenomenon has not been observed to any appreciable extent in vivo (29, 30). Thus, the observation of lack of effect of exenatide on energy intake and body weight regulation would appear to be species- and animal model-specific.

Intraperitoneal and oral glucose tolerance were marginally improved to a similar extent by 21-day twice daily treatment with [DA2]GLP-1/GcG and exenatide. This was associated with significantly increased insulin levels following nutrient challenge. Thus, as would be expected, the metabolic benefits of [DA2]GLP-1/GcG and exenatide are likely mediated predominately by direct insulin secretory actions (21). Interestingly, substantial similar insulin-induced reductions of blood glucose levels were observed in [DA2]GLP-1/GcG and exenatide mice, highlighting beneficial effects independent of pancreatic beta-cell function. This facet of biological action does not appear to be a direct consequence of reduced adipose tissue mass and thus most likely reflects actions of GLP-1 to improve insulin resistance (31). Thus, [DA2]GLP-1/GcG appears to have both beneficial effects of pancreatic beta-cell function and also a direct or indirect augmentation of peripheral insulin action. We were unable to perform pharmacokinetic analysis of [DA2]GLP-1/GcG because of the requirement for generation of a specific antibody. Thus, the possibility that [DA2]GLP-1/GcG has altered binding kinetics or an extended half-life as compared with exenatide cannot be discounted.

To further clarify the mechanism behind the observed effects of [DA2]GLP-1/GcG or exenatide, we assessed aspects of locomotor activity and metabolic rate following 21-day treatment. Locomotor activity was unchanged, but explorative episodes (Z-beam breaks) were elevated during the light phase in both treatment groups, but only by [DA2]GLP-1/GcG, and not exenatide, during the dark phase. This is interesting because the activity of mice is normally much less during the light phase and merits further investigation. Given the prominent effects of glucagon and GLP-1 on energy balance (32), the elevations of energy expenditure may have been predicted in the current study. However, this was not the case, because neither treatment regimen altered energy expenditure or the respiratory exchange ratio. In contrast, the beneficial metabolic actions of other co-agonists reported to date generally appear to center around effects on thermogenesis in brown adipose tissue and increased energy expenditure (2). However, it is unclear
whether this effect would be fully translated to the obese insulin-resistant human form of type 2 diabetes. Thus, [DA²]GLP-1/GcG may possess a distinct advantage over other similar co-
agonists and represent a particularly attractive candidate for further development.

Finally, in an attempt to delineate the receptors involved in the biological actions of [DA²]GLP-1/GcG, we conducted studies in genetically transfected cells and knock-out mice. Interestingly, [DA²]GLP-1/GcG stimulated cAMP production in GLP-1, GIP, and glucagon R-transfected cells with comparable, or even increased, efficacy when compared with the native peptide. Thus, our data clearly illustrate that [DA²]GLP-1/GcG is a potent triple agonist, with cross-talk between GLP-1, GIP, and glucagon Rs. The rationale as to why a GLP-1/glucagon hybrid would efficiently activate GIP Rs is unclear, but it does demonstrate the marked similarities and sequence overlap between peptides and receptors of the same glucagon superfamily (33).

Moreover, recent data relating to a modified glucagon/GIP peptide hybrid clearly show that this molecule was capable of activating GLP-1 Rs (17). Further studies in GIP, GLP-1, and double incretin R knock-out mice confirmed our initial in vitro findings, with [DA²]GLP-1/GcG displaying prominent insulin secretory actions in all three models, corroborating triple agonist properties. However, the glucose-lowering action of [DA²]GLP-1/GcG was different in GIP and GLP-1 R knock-out mice, despite comparable insulin-releasing actions, indicating possible differences in insulin action between knock-out models.

In conclusion, the present study has demonstrated the hybrid peptide analog, [DA²]GLP-1/GcG, is a DPP-IV resistant, potent, triple acting GIP, GLP-1, and glucagon R agonist. [DA²]GLP-1/GcG has robust insulin secretory actions and improves both glucose tolerance and insulin resistance in high fat-fed mice. Whether the beneficial actions of [DA²]GLP-1/
GcG are due to concurrent activation of receptors on the same or distinct cell types, with subsequent stimulation of complementary signaling pathways, still needs to be determined. However, it is clear that multitargeting peptides are an attractive new class of therapeutics for the treatment of type 2 diabetes.

Acknowledgments—We thank B. Thorens (University of Lausanne) for GLP-1 R- and GIP R-transfected Chinese hamster lung cells and C. Unson (Rockefeller University) for glucagon-R-transfected HEK293 cells. We also thank B. Thorens (University of Lausanne) for supplying GIP R and double incretin R KO mice and D. Drucker (University of Toronto) for GLP-1 R KO mice.

REFERENCES
1. Murphy, K. G., and Bloom, S. R. (2006) Gut hormones and the regulation of energy homeostasis. Nature 444, 854–859
2. Sadry, S. A., and Drucker, D. J. (2013) Emerging combinatorial hormone therapies for the treatment of obesity and T2DM. Nat. Rev. Endocrinol. 9, 425–433
3. Ionut, V., Burch, M., Youdim, A., and Bergman, R. N. (2013) Gastrointestinal hormones and bariatric surgery induced weight loss. Obesity 21, 1093–1103
4. Gribble, F. M. (2012) The gut endocrine system as a coordinator of postprandial nutrient homeostasis. Proc. Nutr. Soc. 71, 456–462
5. Gault, V. A., Kerr, B. D., Harriott, P., and Flatt, P. R. (2011) Administration of an acylated GLP-1 and GIP preparation provides added beneficial glucose-lowering and insulino tropic actions over single incretins in mice with Type 2 diabetes and obesity. Clin. Sci. 121, 107–117
6. Flatt, P. R., Bailey, C. J., and Green, B. D. (2009) Recent advances in antidiabetic drug therapies targeting the enteroinferal axis. Curr. Drug Metab. 10, 125–137
7. Dunning, B. E., and Gerich, J. E. (2007) The role of α-cell dysregulation in fasting and postprandial hyperglycemia in type 2 diabetes and therapeutic implications. Endocr. Rev. 28, 253–283
8. Parker, J. A., McCullough, K. A., Field, B. C., Minnion, J. S., Martin, N. M., Ghatei, M. A., and Bloom, S. R. (2013) Glucagon and GLP-1 inhibit food intake and increase c-fos expression in similar appetite regulating centres in the brainstem and amygdala. Int. J. Obes. (Lond.) 37, 1391–1398
9. Gelling, R. W., Vuguin, P. M., Du, X. Q., Cui, L., Romer, J., Pederson, R. A., Leiser, M., Sorensen, H., Holst, J. J., Fledelius, C., Johansen, P. B., Fleischer, N., McIntosh, C. H., Nishimura, E., and Charron, M. J. (2009) Pancreatic beta-cell overexpression of the glucagon receptor gene results in enhanced beta-cell function and mass. Am. J. Physiol. Endocrinol. Metab. 297, E695–707
10. Deacon, C. F. (2005) What do we know about the secretion and degradation of incretin hormones? Regul. Pept. 128, 117–124
11. Pospisilik, J. A., Hinke, S. A., Pederson, R. A., Hoffmann, T., Rosche, F., Schlenzig, D., Glund, K., Heiser, U., McIntosh, C. H., and Demuth, H. (2001) Metabolism of glucagon by dipeptidyl peptidase IV (CD26). *Regul. Pept.* 96, 133–141
12. Hinke, S. A., Gelling, R. W., Pederson, R. A., Manhart, S., Nian, C., Demuth, H. U., and McIntosh, C. H. (2002) Dipeptidyl peptidase IV-resistant [D-Ala(2)]glucose-dependent insulinotropic polypeptide (GIP) improves glucose tolerance in normal and obese diabetic rats. *Diabetes* 51, 652–661
13. Xiao, Q., Giguere, J., Parisien, M., Jeng, W., St-Pierre, S. A., Brubaker, P. L., and Wheeler, M. B. (2001) Biological activities of glucagon-like peptide-1 analogues in *vitro* and *in vivo*. *Biochemistry* 40, 2860–2869
14. Gault, V. A., Porter, D. W., Irwin, N., and Flatt, P. R. (2011) Comparison of sub-chronic metabolic effects of stable forms of naturally occurring GIP(1–30) and GIP(1–42) in high-fat fed mice. *J. Endocrinol.* 208, 265–271
15. McClanahan, N. H., Barnett, C. R., Ah-Sing, E., Abdel-Wahab, Y. H., O’Harte, F. P., Yoon, T. W., Swanston-Flatt, S. K., and Flatt, P. R. (1996) Characterization of a novel glucose-responsive insulin-secreting cell line, BRIN-BD11, produced by electrotusion. *Diabetes* 45, 1132–1140
16. Flatt, P. R., and Bailey, C. J. (1981) Abnormal plasma glucose and insulin responses in heterozygous lean (ob/+ ) mice. *Diabetologia* 20, 573–577
17. Bhat, V. K., Kerr, B. D., Vasu, S., Flatt, P. R., and Gault, V. A. (2013) A DPP-IV-resistant triple-acting agonist of GIP, GLP-1 and glucagon receptors with potent glucose-lowering and insulinotropic actions in high-fat-fed mice. *Diabetologia* 56, 1417–1424
18. Bailey, C. J., Flatt, P. R., and Atkins, T. W. (1982) Influence of genetic background and age on the expression of the obesity hyperglycaemic syndrome in Aston ob/ob mice. *Int. J. Obes.* 6, 11–21
19. Hamilton, A., and Hölscher, C. (2009) Receptors for the incretin glucagon-like peptide-1 are expressed on neurons in the central nervous system. *Neuroreport* 20, 1161–1166
20. Cho, Y. M., Merchant, C. E., and Kieffer, T. J. (2012) Targeting the glucagon receptor family for diabetes and obesity therapy. *Pharmacol. Ther.* 135, 247–278
21. Baggio, L. L., and Drucker, D. J. (2007) Biology of incretins. GLP-1 and GIP. *Gastroenterology* 133, 2131–2157
22. Habegger, K. M., Heppner, K. M., Geary, N., Bartness, T. J., DiMarchi, R., and Tschöp, M. H. (2010) The metabolic actions of glucagon revisited. *Nat. Rev. Endocrinol.* 6, 689–697
23. Irwin, N., Hunter, K., Montgomery, J. A., and Flatt, P. R. (2013) Comparison of independent and combined metabolic effects of chronic treatment with (pGlu-Gln)-CCK-8 and long-acting GLP-1 and GIP mimetics in high-fat-fed mice. *Diabetes Obes. Metab.* 15, 650–659
24. Szayna, M., Doyle, M. E., Betkey, J. A., Holloway, H. W., Spencer, R. G., Greig, N. H., and Egan, J. M. (2000) Exendin-4 decelerates food intake, weight gain, and fat deposition in Zucker rats. *Endocrinology* 141, 1936–1941
25. Young, A. A., Gedulin, B. R., Bhavsar, S., Bodkin, N., Jodka, C., Hansen, B., and Denaro, M. (1999) Glucose-lowering and insulin-sensitizing actions of exendin-4. Studies in obese diabetic (ob/ob, db/db) mice, diabetic fatty Zucker rats, and diabetic rhesus monkeys (*Macaca mulatta*). *Diabetes* 48, 1026–1034
26. Greig, N. H., Holloway, H. W., De Ore, K. A., Jani, D., Wang, Y., Zhou, J., Garant, M. J., and Egan, J. M. (1999) Once daily injection of exendin-4 to diabetic mice achieves long-term beneficial effects on blood glucose concentrations. *Diabetologia* 42, 45–50
27. Al-Barazanji, K. A., Arch, J. R., Buckingham, R. E., and Tadaryon, M. (2000) Central exendin-4 infusion reduces body weight without altering plasma leptin in (fa/fa) Zucker rats. *Obes. Res.* 8, 317–323
28. Wang, Q., and Brubaker, P. L. (2002) Glucagon-like peptide-1 treatment delays the onset of diabetes in 8 week-old db/db mice. *Diabetologia* 45, 1263–1273
29. Delmeire, D., Flamez, D., Moens, K., Hinke, S. A., Van Schravendijk, C., Pipeleers, D., and Schuit, F. (2004) Prior *in vitro* exposure to GLP-1 with or without GIP can influence the subsequent beta cell responsiveness. *Biochem. Pharmacol.* 68, 33–39
30. Baggio, L. L., Kim, J. G., and Drucker, D. J. (2004) Chronic exposure to GLP-1R agonists promotes homologous GLP-1 receptor desensitization *in vitro* but does not attenuate GLP-1R-dependent glucose homeostasis *in vivo*. *Diabetes* 53, S205–S214
31. Parlevliet, E. T., de Leeuw van Weenen, J. E., Romijn, J. A., and Pijl, H. (2010) GLP-1 treatment reduces endogenous insulin resistance via activation of central GLP-1 receptors in mice fed a high-fat diet. *Am. J. Physiol. Endocrinol. Metab.* 299, E318–E324
32. Baggio, L. L., Huang, Q., Brown, T. J., and Drucker, D. J. (2004) A recombinant human glucagon-like peptide (GLP-1)-albumin protein (albugon) mimics peptideergic activation of GLP-1 receptor-dependent pathways coupled with satiety, gastrointestinal motility, and glucose homeostasis. *Diabetes* 53, 2492–2500
33. Brubaker, P. L., and Drucker, D. J. (2002) Structure-function of the glucagon receptor family of G protein-coupled receptors. The glucagon, GIP, GLP-1, and GLP-2 receptors. *Receptors Channels* 8, 179–188