Promiscuous receptor activation mediates glucagonostatic effects of GLP-1(9-36) and GLP-1(7-36)

Claudia Guida[1*], Caroline Miranda[2*], Ingrid Wernstedt Asterholm[2], Davide Basco[3], Anna Benrick[2], Belen Chanclon[2], Margarita V. Chibalina[1], Matthew Harris[4], Joely Kellard[1], Laura J. McCulloch[1], Joana Real[2], Nils J.G. Rorsman[1], Ho Yan Yeung[4], Frank Reimann[5], Makoto Shigeto[1], Anne Clark[1], Bernard Thorens[3], Patrik Rorsman[1,2,6], Graham Ladds[4] and Reshma Ramracheya[1†]

[1]Oxford Centre for Diabetes, Endocrinology and Metabolism, University of Oxford, Churchill Hospital, Headington, Oxford OX3 7LE, UK

[2]Metabolic Physiology Unit, Department of Physiology, Institute of Neuroscience and Physiology, University of Göteborg, Box 430, Göteborg, SE40530, Sweden.

[3]Center for Integrative Genomics, University of Lausanne, 1015, Lausanne, Switzerland

[4]Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1PD, UK

[5]Institute of Metabolic Science, Addenbrooke's Hospital, Cambridge CB2 0QQ, UK

[6]Oxford National Institute for Health Research, Biomedical Research Centre, Churchill Hospital, Oxford, OX3 7LE, UK

*Equal contribution (names in alphabetical order)

†Corresponding author (e-mail: reshma.ramracheya@ocdem.ox.ac.uk)

Running title: Glucagonostatic effects of GLP-1

Plasma glucose is controlled by the hormones glucagon and insulin. In type 2 diabetes, where insulin secretion is too low and glucagon secretion too high, elevated plasma glucose levels occur. Therefore, optimal therapeutic interventions aim to both enhance insulin secretion and inhibit glucagon release. The incretin hormone glucagon-like peptide 1 (GLP-1) possesses this capacity but the underlying mechanisms by which it suppresses glucagon release are unclear as glucagon-secreting α-cells express the receptor for GLP-1 at very low levels. Nevertheless, GLP-1 inhibit glucagon secretion by a direct (intrinsic) action in α-cells. Here, we examined the underlying mechanisms. We found that GLP-1 inhibits glucagon secretion at concentrations as low as 1-10 pM in isolated mouse and human islets. The degradation product GLP-1(9-36) inhibited glucagon secretion with similar potency. Whereas the effect of GLP-1 was sensitive to the PKA inhibitor 8-Br-Rp-cAMPS, GLP-1(9-36) exerted its effect by a PKA-independent mechanism that was sensitive to pretreatment with pertussis toxin (implicating an inhibitory GTP-binding protein). The glucagonostatic effect of GLP-1 was retained in islets from *Glp1r* knockout mice. Receptor signaling studies suggest that GLP-1(9-36) may activate glucagon receptors (GCGRs) and GCGR antagonism prevented the inhibitory effects of GLP-1(9-36). *In vivo*, GLP-1(9-36) reduced counter-regulatory glucagon secretion and enhanced the plasma glucose-lowering effect of exogenous insulin in mice fasted for 5-18h. We propose that GLP-1(7-36) and GLP-1(9-36) regulate glucagon secretion via interaction with both GLP-1R and GCGR.

Introduction

Glucagon is the body's principal hyperglycemic hormone (1). In both type 1 (T1D) and type 2 diabetes (T2D), hyperglycemia results from a combination of insufficient insulin secretion and oversecretion of glucagon (2). Whereas the insulin

secretion defect has attracted much attention, the dysregulation of glucagon secretion in diabetes remains, by comparison, an understudied area.

The incretin hormone GLP-1 inhibits glucagon secretion. This effect is thought to account for ~50% of its hypoglycemic action (3) with the remainder being due to the better-known stimulation of insulin secretion. The mechanisms by which GLP-1 inhibits glucagon secretion remain obscure. Most studies indicate that GLP-1 receptors (encoded by *Glp1r*) are expressed at very low levels (if at all) in glucagon-secreting α-cells (4-6) (but see (7) for a differing viewpoint). It was therefore postulated that GLP-1 exerts its inhibitory effect on glucagon secretion by a paracrine mechanism mediated by factor(s) secreted by the neighboring β- and δ-cells (e.g. insulin and somatostatin, respectively) within the pancreatic islets (8). Yet, GLP-1 (when tested at non-physiological nanomolar levels) robustly inhibited glucagon secretion in isolated islets by mechanisms that cannot be accounted for by paracrine signals (5, 6). We have proposed that this effect is mediated by activation of the few GLP-1 receptors present in α-cells and that this, via a small increase in cAMP, results in inhibition of the voltage-gated Ca^{2+} channels linked to exocytosis of glucagon-containing secretory granules. This concept is supported by the finding that the glucagonostatic effect (i.e. capacity to inhibit glucagon secretion) of GLP-1 (at 100 nM) is antagonized by the GLP-1 receptor antagonist exendin (9-39), prevented by the PKA inhibitor 8-Br-Rp-cAMPS and mimicked by low (nanomolar) concentrations of forskolin. We have now explored the role of the 'classical' GLP-1 receptor further by extending our studies to *Glp1r* knockout mice (9). We confirm that activation of GLP-1Rs only mediate part of the GLP-1's glucagonostatic effect. In addition, GLP-1 also exerts *Glp1r*- and PKA-independent effects. Here we have studied the effects of GLP-1(7-36) and its abundantly-occurring metabolite, GLP-1(9-36) on islet function, and explored possible alternative receptors and the intracellular signaling mechanisms involved.

Results

Dose-dependent inhibition of glucagon secretion by GLP-1(7-36) and GLP-1(9-36). We measured glucagon secretion in isolated mouse and human pancreatic islets exposed to 1 mM glucose (this represents a strong stimulus of glucagon secretion) and increasing concentrations of GLP-1(7-36) (Fig. 1A-B). In both mouse and human islets, GLP-1(7-36) produced a concentration-dependent inhibition of glucagon secretion that was maximal at 10 pM. High concentrations (10-100 nM) were paradoxically less efficacious than picomolar levels.

Following its release, GLP-1(7-36) is quickly degraded by dipeptidyl peptidase 4 (DPP-4) to form the metabolite GLP-1(9-36) (10). At any given point in time, over 80% of circulating GLP-1 occurs as GLP-1(9-36). In mouse islets, GLP-1(9-36) inhibited glucagon secretion as potently as GLP-1(7-36) (Fig. 1C). In human islets, GLP-1(9-36) was also strongly inhibitory and reduced glucagon secretion at 1 mM glucose by 50% (Fig. 1D). As was the case with GLP-1(7-36), high concentrations (≥1 nM) of the agonist were less efficacious than low concentrations (1-10 pM), a feature that may reflect receptor desensitization (35).

We excluded the possibility that GLP-1(9-36) inhibits glucagon secretion by a paracrine effect mediated by stimulation of insulin or somatostatin secretion (Supplementary Fig. 1A-D). Plasma GLP-1 levels range between 10-50 pM of which only <20% is GLP-1(7-36) (33). Collectively, these data therefore suggest that glucagon secretion is under strong tonic inhibition by circulating levels of GLP-1(7-36) and GLP-1(9-36) and that these effects are mediated by direct effects on the α-cells.

Glucagonostatic and insulinotropic effects of GLP-1 are mediated by pharmacologically distinct mechanisms. The insulinotropic effects of GLP-1(7-36) are shared with the GLP-1 receptor agonist exendin-4, which shows 53% homology to GLP-1(7-36) (11). Exendin-4 (10 pM) potentiated glucose-induced (6 mM) insulin secretion by 100% in mouse and human islets (Fig. 2A-B), comparable to that previously observed for GLP-1(7-36) (12). By contrast, the same concentration of

exendin-4 was without glucagonostatic effects in both mouse and human islets (Fig. 2C-D).

Collectively, these data suggest that GLP-1(7-36) stimulates insulin secretion and inhibits glucagon release by pharmacologically distinct mechanisms, raising the interesting possibility that the effects on glucagon secretion may involve receptors distinct from the previously characterized GLP-1 receptor.

GLP-1(7-36) and GLP-1(9-36) signal by PKA-dependent and -independent mechanisms, respectively. We compared the effects of GLP-1(7-36) and GLP-1(9-36) in the absence and presence of the PKA-inhibitor 8-Br-Rp-cAMPS. Under control conditions, GLP-1(7-36; 10 pM) inhibited glucagon secretion by 48%, which was reduced to 25% in the presence of 8-Br-Rp-cAMPS (10 µM; $p<0.01$). By contrast, the glucagonostatic effect of GLP-1(9-36; 10 pM) was not at all affected by the PKA inhibitor. In this experimental series, the inhibitory effect of GLP-1(9-36) averaged 42% and 40% ($p=0.7$) in the absence and presence of 8-Br-Rp-cAMPS, respectively (Fig. 3B). Thus, it appears that the inhibitory effects of GLP-1(7-36) are partially PKA-dependent, and those of GLP-1(9-36) are entirely PKA-independent. This difference provides additional evidence that the two GLP-1 amides may act by distinct mechanisms/receptors. Notably, the PKA inhibitor had no effect on glucagon secretion at 1 mM glucose, at variance with recent reports (13).

The glucagonostatic effect of GLP-1 is retained in Glp1r$^{-/-}$ *islets.* To substantiate whether the two GLP-1 amides act via different mechanisms, we compared the effects of GLP-1(7-36) and GLP-1(9-36) on insulin and glucagon secretion in islets isolated from control (*Glp1r*$^{+/+}$) and GLP-1 receptor knockout (*Glp1r*$^{-/-}$) mice (9).

In wild-type islets, increasing glucose from 1 to 6 mM increased insulin secretion by 100%. Treatment with GLP-1(7-36) (10 pM) resulted in an additional doubling of insulin secretion. In *Glp1r*$^{-/-}$ islets, increasing glucose from 1 to 6 mM also stimulated insulin secretion by 125% but GLP-1(7-36) (10 pM) did not lead to any further stimulation (Fig. 4A).

The effects of GLP-1(7-36) on glucagon secretion were tested at 1 mM glucose. In control islets, GLP-1(7-36) (10 pM) inhibited glucagon secretion by ~50%. Surprisingly, the inhibitory effects of GLP-1(7-36) persisted in *Glp1r*$^{-/-}$ islets where it amounted to a 55% reduction in glucagon release (Fig. 4B). Similarly, GLP-1(9-36) (10 pM) was equally inhibitory in *Glp1r*$^{-/-}$ and control islets, suppressing glucagon secretion by ~60% (Fig. 4C). The inhibitory effects of both, GLP-1(7-36) and GLP-1(9-36) on glucagon secretion were comparable to the suppression resulting from increasing the glucose concentration from 1 to 6 mM. The glucagonostatic effect of elevated glucose was not affected by ablation of *Glp1r* (Fig. 4B-C), at variance with was recently reported after cell-specific ablation of *Glp1r* in α-cells (7).

We point out that the mouse model used for these experiments is a general knockout and it can therefore be excluded that GLP-1 exerts its inhibitory action by a paracrine effect by activation of GLP-1Rs in ɗ- and β-cells. These data therefore suggest that whereas the insulinotropic effect of GLP-1 is mediated by the previously characterized (cloned) GLP-1 receptor, the glucagonostatic effects are mediated by activation of different receptor(s).

GLP-1(9-36) activates the glucagon receptor. In the search for alternative receptors that may mediate the *Glp1r*-independent effects of GLP-1(7-36) and those of its metabolite, we next tested whether GLP-1(9-36) could activate the glucagon receptor (*GCGR*), which is expressed in α-cells at low levels (but comparable to those found for *Glp1r)* in mouse and human β-cells (14-16). In HEK 293 cells expressing human *GCGR*, glucagon increased cAMP production with an EC_{50} of ~1 nM, an effect that was antagonized by the glucagon receptor antagonist L-168049 (17) (Fig. 5A). Intriguingly, GLP-1(9-36) also activated the glucagon receptor and increased cAMP content (Fig. 5B), an effect also prevented by L-168049.

Receptor activity-modifying proteins (RAMPs) modulate the pharmacology of G protein-coupled receptors (18). In human islets, RAMP2 is expressed at much higher (~20-fold) levels in the α- than in β-cells (14). Mouse α-cells also express RAMP2 (15).

Whereas the capacity of GLP-1(7-36) to activate the human GCGR is abolished when the receptor is co-expressed with RAMP2 (19), the activating potency of GLP-1(9-36) was enhanced 30-fold; the EC_{50} was reduced from 10 nM to 0.3 nM in this series of experiments in co-transfected HEK293 cells (Fig. 5C). Significantly, and in contrast to both GLP-1(7-36) and (9-36), exendin-4 was without effect on cAMP production in GCGR-expressing HEK 293 cells (Fig. 5D). The latter finding echoes the failure of exendin-4 to affect glucagon secretion.

We also considered the possibility that GLP-1 might suppress glucagon secretion by activation of GPR119, which are highly expressed in both mouse and human α-cells (14-16). However, both GLP-1(7-36) and (9-36) remained capable of inhibiting glucagon secretion in islets from *Gpr119*$^{-/-}$ mice (Supplementary Fig. 2) and neither GLP-(7-36) nor (9-36) activated GPR119 expressed in HEK293 cells (Supplementary Fig. 3). In addition, we have also excluded the possibility that the GLP-1R agonists mediate their actions through the activation of GIPRs (Supplementary Fig. 4).

Glucagon receptor antagonists abolish glucagonostatic effect of GLP-1(9-36). The receptor activation studies raise the interesting possibility that GLP-1(9-36) may act by activating the glucagon receptors. We further explored this hypothesis by comparing the glucagonostatic effects of GLP-1(9-36) in the absence and presence of the highly selective glucagon receptor antagonist L-168049 (17).

Under control conditions, GLP-1(9-36) (10 pM) inhibited glucagon secretion by ~50% (Fig. 6A). However, when tested in the presence of L-168049, GLP-1(9-36) failed to affect glucagon secretion. In the same islets, elevation of glucose to 6 mM inhibited glucagon secretion ~50% (confirming that the α-cells were functional).

These experiments were repeated in human islets. We observed that GLP-(9-36) (10 pM) inhibited glucagon secretion in human islets almost as strongly as 6 mM glucose (48% and 56%, respectively) and that the effect of the GLP-1 metabolite was

abolished by two other glucagon receptor antagonists, Peptide R (in 2 separate human islet preparations) and Peptide N (1 islet preparation) (20) (Fig. 6B).

GLP-1(9-36), but not GLP-1(7-36), signals by a G_i-dependent mechanism. The data shown in Fig. 3 suggests that GLP-1(9-36)-mediated glucagonostatic effects are resistant to PKA inhibition. We next compared the effects of GLP-1(7-36) and (9-36) in islets pretreated with pertussis toxin (PTX) to inhibit G_i (21). Under control conditions (without PTX pre-treatment), GLP-1(7-36) and (9-36) (both tested at 10 pM) inhibited glucagon secretion by >50%. Following pre-treatment with PTX (100 ng/ml for >4h), the inhibitory effect of GLP-1(9-36) was abolished whereas that of GLP-1(7-36) was unaffected (Fig. 7A). These divergent results provide additional support to the hypothesis that GLP-1(7-36) and (9-36) mediate their glucagonostatic effects by distinct mechanisms.

We next tested the capacity of GLP-1(7-36) to suppress glucagon secretion in islets from *Glp1r*$^{-/-}$ mice following inactivation of G_i. In keeping with the data in Fig. 4B, GLP-1(7-36) retained its capacity to inhibit glucagon secretion in *Glp1r*-deficient islets. However, unlike what was observed in control (wild-type) islets, GLP-1(7-36) did not inhibit glucagon secretion in PTX-pretreated *Glp1r*$^{-/-}$ islets (Fig. 7B).

The latter finding may suggest that the *Glp1r*-independent inhibitory effects of GLP-1(7-36) on glucagon secretion are actually mediated by the breakdown product GLP-1(9-36). To explore this hypothesis, we tested the effects of GLP-1(7-36) (10 pM) in the presence of the DPP-4 inhibitor sitagliptin (100 nM) (22) and compared the effects on glucagon secretion in the absence or presence of the GLP-1 receptor antagonist exendin(9-39) (100 nM) (Fig. 7C-D). Consistent with the data in *Glp1r*-deficient islets, GLP-1(7-36) when used at picomolar concentrations retained an inhibitory effect in the presence of exendin(9-39). However, co-application of sitagliptin and exendin(9-39) abolished the inhibitory effect of GLP-1(7-36) (Fig. 7D). Importantly, sitagliptin was without stimulatory effect on glucagon secretion when tested in the absence of exendin(9-39) (Fig. 7C).

GCGR is pleotropically coupled, activating not only stimulatory G_s but also the inhibitory G_i GTP-binding proteins (19, 23, 24). In HEK 293 cells expressing the GCGR, pretreatment with pertussis toxin increased cAMP accumulation in response to GLP-1(9-36) (Supplementary Fig. 5), indicating that GLP-1(9-36) can activate G_i.

GLP-1(7-36) and (9-36) remain inhibitory in Gcgr$^{-/-}$ islets. The studies with recombinant glucagon receptors and glucagon receptor antagonists suggest that GLP-1(9-36) may inhibit glucagon secretion by activation of glucagon receptors (GCGRs). We compared the effects of GLP-1(7-36) (10 pM) and (9-36) (10 pM) in islets from control and *Gcgr*$^{-/-}$ mice (Fig. 8A) (25). In wildtype islets, GLP-1(7-36) and GLP-1(9-36) applied at 1 mM glucose inhibited glucagon secretion as strongly as increasing glucose from 1 to 6 mM. Unexpectedly (given the data obtained with glucagon antagonists; Fig. 6), both GLP-1(7-36) and (9-36) remained as inhibitory in *Gcgr*$^{-/-}$ islets as in wildtype islets (Fig. 8A and B).

We reasoned that GLP-1(7-36) and (9-36) may still inhibit glucagon secretion in *Gcgr*$^{-/-}$ islets by activation of GLP-1Rs in α- or β-cells. If so, GLP-1(9-36) should not be inhibitory when both GCGR and GLP-1R signaling are disrupted. We tested this by application of GLP-1(9-36) to islets from *Glp1r*$^{-/-}$ in the presence of the glucagon receptor antagonist Peptide N. Under these conditions, GLP-1(9-36) was without any inhibitory effect (Fig. 8C).

Effects of GLP-1(9-36) in vivo. Plasma glucose reflects the balance between the hypoglycemic action of insulin and hyperglycemic action of glucagon. The observation that GLP-1(9-36) inhibits glucagon secretion *in vitro* therefore suggests that it may affect systemic glucose homeostasis. GLP-1(9-36) was administered intraperitoneally at a dose of 500 ng/g body weight as used previously by other investigators (26). Plasma GLP-1(9-36) peaked after 10 min, attaining a maximal concentration of 400 pM. The concentration then decayed exponentially but remained >60 pM (5-fold higher than basal) 60 min after injection of the peptide (Supplementary Fig. 6A), close to that producing the maximum glucagonostatic effect in the *in vitro*

measurements (cf. Fig. 1C) but it was without effect on plasma glucose and glucagon when administered to fed mice with a plasma glucose of 11±0.5 mM (n=7) (Supplementary Fig. 6B-C).

We reasoned that the systemic effects of GLP-1(9-36) might be more apparent when glucagon secretion is stimulated by insulin-induced hypoglycaemia. In mice fasted for 5h, insulin (0.5 U/kg body weight) lowered plasma glucose from a basal 7.5 mM to 4 mM (Fig. 9A) This was associated with an ~10-fold increase in plasma glucagon, an effect reduced by 40% when insulin was co-injected with GLP-1(9-36) (Fig. 9B). Despite the reduction of glucagon there was no effect of the GLP-1 metabolite on plasma glucose.

More dramatic effects were observed when the period of fasting was extended to 18h, the approximate transit time for ingested food in mice (27). In these mice, insulin-lowered plasma glucose fell from 5.5 mM to 3.5 mM and 2 mM in the absence and presence of co-injected GLP-1(9-36), respectively (Fig. 9C) and the experiment had to be terminated to comply with our animal license. Plasma glucagon levels measured at 30 min were the same in the absence and presence of GLP-1(9-36) (Fig. 9D).

It may seem surprising that GLP-1(9-36) seemingly failed to lower plasma glucagon compared to saline controls in these experiments but it should be noted the more pronounced hypoglycemia should have triggered a much stronger increase in plasma glucagon (28) and the absence of such an effect is therefore indicative of an inhibitory effect. Indeed, glucagon secretion measured in vitro was 2-fold higher at 2 mM glucose than 4 mM and inclusion of GLP-1(9-36) prevented this stimulation either in mouse or human isolated islets (Fig. 9E-F).

Discussion

Our data suggests that physiological (picomolar) concentrations of GLP-1 exert their glucagonostatic effects via mechanisms that are (at least in part) intrinsic to the

glucagon-secreting α-cells and that are independent of paracrine effects from the neighboring islet cells. A similar conclusion was previously made for pharmacological (nanomolar) concentrations of GLP-1(7-36) (5, 6).

The mechanisms by which GLP-1 inhibits glucagon secretion are clearly complex. Fig. 10 outlines a model that explains our findings and that integrates the function of GLP-1Rs and other GPCRs in α-cells. We propose that GLP-1(7-36) and GLP-1(9-36) inhibit glucagon secretion by a combination of (at least) two mechanisms that involve distinct receptors and intracellular signal transduction pathways. Whereas the two mechanisms normally cooperate, they may individually suffice to maximally suppress glucagon secretion under certain experimental conditions, which complicates the elucidation of the underlying mechanism. Accordingly, genetic ablation of one receptor subtype may not be enough to produce a clear phenotype and combination of genetic and pharmacological strategies are therefore required to unveil the role of the different receptors.

GLP-1(7-36) inhibits glucagon secretion by binding to GLP-1R. Activation of the GLP-1Rs leads to a small increase in intracellular cAMP, sufficient to activate PKA with resultant inhibition of glucagon secretion. This explains why the PKA-inhibitor Rp-cAMPS reduces the relative inhibitory effect of physiological concentrations of GLP-1(7-36) by 50%. In addition to this cAMP/PKA-dependent actions, GLP-1(9-36) also inhibits glucagon secretion by a pertussis toxin-sensitive (G_i) effect. The finding that GLP-1(7-36) is without effect when applied in the presence of exendin(9-39) (to block the GLP-1Rs) and sitagliptin (to inhibit the DPP4) further suggests that this effect is mediated following its degradation to GLP-1(9-36). The identity of the receptor that activates G_i remains to be conclusively established but receptor antagonist studies suggest it may be GCGR. Our data suggest that GLP-1(9-36) – but not GLP-1(7-36) – activates GCGR and G_i in α-cells. Exendin-4 had no effect on GCGR (unlike GLP-1(9-36)) and this difference may account for the failure of the GLP-1R agonist to suppress glucagon secretion. How GLP-1(9-36) and activation of G_i inhibits glucagon secretion

remains to be documented but we have previously shown that agents that activate G_i (including somatostatin) inhibit glucagon granule exocytosis in both mouse (29) and human (30) α-cells by depriming of release-competent secretory granules.

Coda: Beyond the α-cell. There have been multiple reports that GLP-1(7-36) and GLP-1(9-36) exert effects in the CNS, cardiovascular system, gastrointestinal tract, liver and muscle that persist in the absence of the classical GLP-1 receptor and which are likely to involve distinct receptor(s)/mechanisms (31-34). Our data suggest that an alternative GLP-1R does not exist and that *Glp1r*-independent effects of GLP-1(7-36) and GLP-1(9-36) instead reflect the simultaneous response to other (related) GPCRs (that normally mediate the effects of other ligands such as glucagon). The existence of an alternative GLP-1R has been inferred for a long time but attempts to identify it have failed and there is no obvious candidate in the genome (see http://www.glucagon.com/glp1secondreceptor.html). It is possible that the concept of receptor 'promiscuity' can be extended to other cases where a hormone exerts an effect without an obvious candidate receptor.

Materials and Methods

Human islets. Human pancreatic islets were isolated (with ethical approval and clinical consent) at the Diabetes Research and Wellness Foundation Human Islet Isolation Facility (OCDEM, Oxford, UK) from the pancreases of 24 nondiabetic donors. Donors (10 females, 14 males) were on average 48 years old (range 19–61) with a BMI of 28 (range 21–37). Islets were isolated as previously described (35). Human islets were usually released for experimental work within 24h of islet isolation. During the interval between islet isolation and the hormone secretion studies, islets were maintained in complete RPMI medium containing 5 mM glucose.

Animals and islet isolation. Most studies were conducted in 8-16 weeks old mice on a C57BL6/J or NMRI background. *Ethical approval.* All animal experiments were conducted in accordance with the ethical guidelines of the Universities of Oxford,

Lausanne, Copenhagen and Goteborg and were approved by the local Ethics Committees.

Mice were housed at up to six per cage and kept on 12h light-dark cycle with free access to chow diet and water.

In addition to the ordinary mice, three different mouse strains were used in this study: global *Glp1r*$^{-/-}$ mice (9); global *Gcgr*$^{-/-}$ mice (25); and α-cell-specific *Gpr119*$^{-/-}$ mice (GLU-Cre x GRP119(fx/fx) mice) (36).

For experiments with the mouse knockout models, sex- and age-matched wild type littermates were used as controls.

The mice were killed by cervical dislocation, the pancreas quickly resected and pancreatic islets isolated by liberase (Sigma) digestion. The islets were picked and grouped manually.

Reagents. GLP-1(7-36), GLP-1(9-36), exendin-4 and exendin(9-39) were purchased from Bachem (Weil am Rhein, Germany). The PKA inhibitor 8-Br-Rp-cAMPS (Rp-cAMPS) was purchased from BioLog Life Science Institute (Bremen, Germany), the SSTR2 antagonist CYN154806 from Tocris Bioscience (Bristol, UK), the Gpr119 agonist AS1269574 from Tocris and pertussis toxin from Sigma. All other reagents were of analytical grade. Human GLP-1(7-36)NH_2, GLP-1(9-36)NH_2, glucagon (GCG) and exendin-4 (Ex-4) were custom synthesised by Generon (Slough, U.K.). GPR119 small molecule agonist, AR231453, was purchased from Sigma (Dorset, U.K.) and glucagon receptor (GCGR) small molecule antagonist, L-168049 was purchased from Tocris (Bristol, U.K.). Rolipram and forskolin were purchased from Cayman Chemical Company (Michigan, U.S.). All peptide ligands were made up to 1mM stock solution in deionised water while small molecule compounds, except for rolipram (which was made up to 25mM), were made up to 10mM in dimethyl sulfoxide (DMSO). All compounds and peptide ligands were stored at -20°C before assays. cDNA constructs of the human GCGR was donated by Professor Patrick Sexton (Monash University, Australia) while cDNA constructs of human GPR119 were

purchased from cDNA.org. The LANCE® cAMP detection assay kits and 384-well white Optiplates were purchased from PerkinElmer Life Sciences (Waltham, U.S.A.). Minimal Essential Medium (MEM) and heat inactivated fetal bovine serum (FBS) were purchased from Gibco™ (Thermo-fisher Scientific, U.K.).

Measurements of islet hormone secretion. Mouse islets were used acutely, except for studies with PTX, where islets were treated with the toxin overnight, and were, pending the experiments, maintained in tissue culture for <24 h in RPMI medium containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 10 mM glucose prior to the measurements. Human islets were maintained in culture for up to 48h in RPMI medium containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 5 mM glucose.

Experiments were conducted using batches of 10–15 size-matched islets per tube (in triplicate) as previously described (35, 37). We note that glucagon secretion exhibits variability between preparations. To circumvent these confounds, each donor/group of mice were used as its own control when testing the effect of a compound.

Islets were washed twice in RPMI prior to preincubation in Krebs-Ringer buffer (KRB) containing 2 mg/ml BSA (S6003, Sigma-Aldrich) and 3 mM glucose for 1 h at 37ºC. Following this, islets were incubated in 0.3 ml KRB with 2 mg/ml BSA, supplemented with various glucose concentrations or compounds as indicated. After incubation, the supernatant was removed and quickly frozen and stored at −80°C. Insulin and glucagon were determined by radioimmunoassay (Millipore and Oxford Biosystem, respectively) according to the manufacturers' instructions.

Receptor binding studies. Cell Culture and transient transfection: HEK-293 cells lacking the calcitonin receptor (ΔCTR-HEK 293 cells) were given by Drs. David Hornigold, Jacqueline Naylor and Alessandra Rossi (MedImmune, Cambridge, UK), and used as described elsewhere(38). ΔCTR-HEK 293 cells were cultured in MEM supplemented with 10% heat-inactivated FBS plus 1% non-essential amino acids.

ΔCTR-HEK 293 cells were incubated in a humidified 95% air/5% CO_2 incubator at 37°C and were used between passages 1 to 5. ΔCTR-HEK 293 cells were transfected with polyethylenimine (PEI) as per manufacturer's protocol using 1:6 (w:v) DNA:PEI ratio on 24-well plate. The transfected cells were grown 48 h prior to assays.

cAMP accumulation assays: Assays were performed as previously described (19, 23, 39). In brief, ΔCTR-HEK 293 cells transiently transfected with GCGR or GPR119 were washed with phosphate buffered saline (PBS), resuspended in PBS containing 0.1% bovine serum albumin (BSA) plus 25μM rolipram, and seeded at 2000 cells/well in 384-well white Optiplates. Agonists were serially diluted at concentrations in the range of between the ranges of 100μM to 0.01 pM (dependent upon agonist used) in a 96 well-plate. GCGR antagonist L-168049 was added to the serially diluted peptide ligands in the antagonist assay and the DMSO content was kept at 2% across all wells. The cells were stimulated with ligands for 15 or 30 min as indicated and cAMP accumulation was measured using a LANCE® cAMP detection assay kit. Plates were read using a Mithras LB 940 multimode micro-plate reader (Berthold Technologies). Values were converted to concentration using a cAMP standard curve performed in parallel and standardised to cells stimulated with 100 μM forskolin, which provided the system maximum.

Plasma glucose measurements during insulin tolerance tests. Fed blood glucose levels (data point before fasting) were measured from a blood drop obtained by a tail vein nick using the Accu-Chek Aviva (Roche Diagnostic). The mice were then fasted for 5h prior to the experiments. Fast-acting human insulin (Actrapid, Novo Nordisk) was injected intraperitoneally at the indicated doses with a 25-gauge needle at time zero. GLP-1(9-39) was injected intraperitoneally with insulin as indicated. In the control experiments, insulin was co-injected with the solvent.

Tail vein blood glucose and glucagon levels were monitored using a glucometer before and an ELISA (Mercodia Glucagon ELISA, Uppsala, Sweden). Total GLP-1 was measured by ELISA (Crystal Chem, Zaandam, Netherlands).

Data analysis. All data are reported as mean ± SEM, unless otherwise stated. Statistical significance was defined as $P<0.05$. All statistical tests were conducted in Prism 5 (GraphPad Software, San Diego, CA). For two groupings, a t-test was conducted. If the data were nonparametric, a Mann–Whitney test was conducted. For more than two groupings, a one-way ANOVA was conducted. If there were two independent variables, a two-way ANOVA was conducted. For secretion data, a minimum of two human donors were used and each replicate was considered an individual experiment. For experiments on mouse islets, each replicate (using different groups of islets) was regarded as a separate experiment and the number of mice used for each experiment is stated and, in each case, at least 4 mice were used.

Study approval. All experiments on mouse islets were conducted in accordance with the United Kingdom Animals (Scientific Procedures) Act (1986) and the University of Oxford ethical guidelines. Breeding of *Gcgr* null mice were in compliance with an animal experiment license issued by the Danish Committee for Animal Research and approved by the local animal welfare committee at the University of Copenhagen. Breeding of *Glp1r*-deificient mice was performed at the University of Lausanne and were reviewed and approved by the Veterinary Office of Canton de Vaud.

Human pancreases were obtained with ethical approval of the NHS National Research Ethics Centre, Oxfordshire, United Kingdom (REC B) and clinical consent from heart-beating donors.

Author contributions

RR, CG, CM, IWA, DB, AB, BC, MVC, MH, JK, LJM, JR, NJGR, MS and HYY performed the experimental studies. Animal models were provided by FR and BT. GL directed the receptor binding studies. The study was conceived by PR and RR who

planned and designed the experiments. PR drafted the original version of the manuscript. CG and RR coordinated the editing of the final version of the manuscript. All co-authors contributed to the discussion and approved of the final version of the manuscript.

Acknowledgements

We thank Dr B. Svendsen and Professor J.J. Holst for supplying glucagon receptor knockout mice and valuable discussion. We thank Dr F. O'Harte (University of Ulster, Coleraine, Northern Ireland, UK) for the kind gift of the glucagon receptor antagonists Peptide N and R and Professor Niels Billestrup at the University of Copenhagen for access to his laboratory for the experiments on the GCGR mice.

The work was supported by a Diabetes UK RD Lawrence Fellowship to Dr Reshma Ramracheya, a Novo Nordisk University of Oxford postdoctoral fellowship to Dr Claudia Guida and an OXION Wellcome Trust studentship to Miss Joely Kellard.

Work in Oxford was also supported by a Wellcome Trust Senior Investigator Award.

Studies in Göteborg were covered by a Wallenberg Scholars Fellowship from the Knut and Alice Wallenbergs Stiftelse and an International Recruitment Award from the Swedish Research Council (VR) to Patrik Rorsman and a VR Research Grant to Ingrid Wernstedt Asterholm.

Studies in Cambridge were supported by the Rosetrees foundation (to H. Y. Y and G.L), a Wellcome Trust joint investigator award (FR) an MRC programme within the Metabolic Diseases Unit (FR). H.Y.Y was supported by an international scholarship from the Cambridge Trust.Work in Lausanne (BT) was supported by grants to B.T. from the Swiss National Science Foundation and the European Research Council.

References

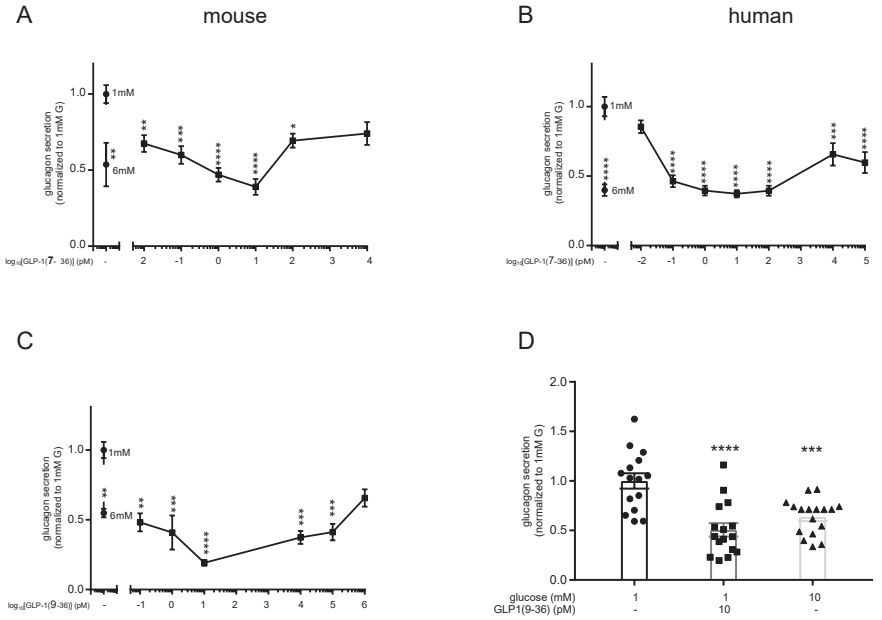
1. Cryer PE. Hypoglycemia-Associated Autonomic Failure in Diabetes: Maladaptive, Adaptive, or Both? *Diabetes.* 2015;64(7):2322-3.
2. Unger RH, and Orci L. The essential role of glucagon in the pathogenesis of diabetes mellitus. *Lancet.* 1975;1(7897):14-6.
3. Hare KJ, Vilsboll T, Asmar M, Deacon CF, Knop FK, and Holst JJ. The glucagonostatic and insulinotropic effects of glucagon-like peptide 1 contribute equally to its glucose-lowering action. *Diabetes.* 2010;59(7):1765-70.
4. Richards P, Parker HE, Adriaenssens AE, Hodgson JM, Cork SC, Trapp S, Gribble FM, and Reimann F. Identification and characterization of GLP-1 receptor-expressing cells using a new transgenic mouse model. *Diabetes.* 2014;63(4):1224-33.
5. De Marinis YZ, Salehi A, Ward CE, Zhang Q, Abdulkader F, Bengtsson M, Braha O, Braun M, Ramracheya R, Amisten S, et al. GLP-1 inhibits and adrenaline stimulates glucagon release by differential modulation of N- and L-type Ca2+ channel-dependent exocytosis. *Cell Metab.* 2010;11(6):543-53.
6. Ramracheya R, Chapman C, Chibalina M, Dou H, Miranda C, Gonzalez A, Moritoh Y, Shigeto M, Zhang Q, Braun M, et al. GLP-1 suppresses glucagon secretion in human pancreatic alpha-cells by inhibition of P/Q-type Ca(2+) channels. *Physiological reports.* 2018;6(17):e13852.
7. Zhang Y, Parajuli KR, Fava GE, Gupta R, Xu W, Nguyen LU, Zakaria AF, Fonseca VA, Wang H, Mauvais-Jarvis F, et al. GLP-1 Receptor in Pancreatic alpha-Cells Regulates Glucagon Secretion in a Glucose-Dependent Bidirectional Manner. *Diabetes.* 2019;68(1):34-44.
8. Orgaard A, and Holst JJ. The role of somatostatin in GLP-1-induced inhibition of glucagon secretion in mice. *Diabetologia.* 2017;60(9):1731-9.
9. Scrocchi LA, Brown TJ, MaClusky N, Brubaker PL, Auerbach AB, Joyner AL, and Drucker DJ. Glucose intolerance but normal satiety in mice with a null mutation in the glucagon-like peptide 1 receptor gene. *Nature medicine.* 1996;2(11):1254-8.
10. Baggio LL, and Drucker DJ. Biology of incretins: GLP-1 and GIP. *Gastroenterology.* 2007;132(6):2131-57.
11. Underwood CR, Parthier C, and Reedtz-Runge S. Structural basis for ligand recognition of incretin receptors. *Vitamins and hormones.* 2010;84(251-78.
12. Shigeto M, Ramracheya R, Tarasov AI, Cha CY, Chibalina MV, Hastoy B, Philippaert K, Reinbothe T, Rorsman N, Salehi A, et al. GLP-1 stimulates insulin secretion by PKC-dependent TRPM4 and TRPM5 activation. *The Journal of clinical investigation.* 2015;125(12):4714-28.
13. Yu Q, Shuai H, Ahooghalandari P, Gylfe E, and Tengholm A. Glucose controls glucagon secretion by directly modulating cAMP in alpha cells. *Diabetologia.* 2019;62(7):1212-24.
14. Blodgett DM, Nowosielska A, Afik S, Pechhold S, Cura AJ, Kennedy NJ, Kim S, Kucukural A, Davis RJ, Kent SC, et al. Novel Observations From Next-

Generation RNA Sequencing of Highly Purified Human Adult and Fetal Islet Cell Subsets. *Diabetes.* 2015;64(9):3172-81.

15. Adriaenssens AE, Svendsen B, Lam BY, Yeo GS, Holst JJ, Reimann F, and Gribble FM. Transcriptomic profiling of pancreatic alpha, beta and delta cell populations identifies delta cells as a principal target for ghrelin in mouse islets. *Diabetologia.* 2016;59(10):2156-65.
16. DiGruccio MR, Mawla AM, Donaldson CJ, Noguchi GM, Vaughan J, Cowing-Zitron C, van der Meulen T, and Huising MO. Comprehensive alpha, beta and delta cell transcriptomes reveal that ghrelin selectively activates delta cells and promotes somatostatin release from pancreatic islets. *Mol Metab.* 2016;5(7):449-58.
17. Cascieri MA, Koch GE, Ber E, Sadowski SJ, Louizides D, de Laszlo SE, Hacker C, Hagmann WK, MacCoss M, Chicchi GG, et al. Characterization of a novel, non-peptidyl antagonist of the human glucagon receptor. *J Biol Chem.* 1999;274(13):8694-7.
18. McLatchie LM, Fraser NJ, Main MJ, Wise A, Brown J, Thompson N, Solari R, Lee MG, and Foord SM. RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. *Nature.* 1998;393(6683):333-9.
19. Weston C, Lu J, Li N, Barkan K, Richards GO, Roberts DJ, Skerry TM, Poyner D, Pardamwar M, Reynolds CA, et al. Modulation of Glucagon Receptor Pharmacology by Receptor Activity-modifying Protein-2 (RAMP2). *J Biol Chem.* 2015;290(38):23009-22.
20. O'Harte FP, Franklin ZJ, and Irwin N. Two novel glucagon receptor antagonists prove effective therapeutic agents in high-fat-fed and obese diabetic mice. *Diabetes, obesity & metabolism.* 2014;16(12):1214-22.
21. Asano T, Katada T, Gilman AG, and Ross EM. Activation of the inhibitory GTP-binding protein of adenylate cyclase, Gi, by beta-adrenergic receptors in reconstituted phospholipid vesicles. *J Biol Chem.* 1984;259(15):9351-4.
22. Deacon CF. Peptide degradation and the role of DPP-4 inhibitors in the treatment of type 2 diabetes. *Peptides.* 2018;100(150-7.
23. Weston C, Poyner D, Patel V, Dowell S, and Ladds G. Investigating G protein signalling bias at the glucagon-like peptide-1 receptor in yeast. *British journal of pharmacology.* 2014;171(15):3651-65.
24. Wootten D, Reynolds CA, Smith KJ, Mobarec JC, Koole C, Savage EE, Pabreja K, Simms J, Sridhar R, Furness SGB, et al. The Extracellular Surface of the GLP-1 Receptor Is a Molecular Trigger for Biased Agonism. *Cell.* 2016;165(7):1632-43.
25. Gelling RW, Du XQ, Dichmann DS, Romer J, Huang H, Cui L, Obici S, Tang B, Holst JJ, Fledelius C, et al. Lower blood glucose, hyperglucagonemia, and pancreatic alpha cell hyperplasia in glucagon receptor knockout mice. *Proceedings of the National Academy of Sciences of the United States of America.* 2003;100(3):1438-43.
26. Day SM, Yang W, Ewin S, Zhou X, and Ma T. Glucagon-like peptide-1 cleavage product GLP-1 (9-36) amide enhances hippocampal long-term synaptic plasticity in correlation with suppression of Kv4.2 expression and eEF2 phosphorylation. *Hippocampus.* 2017;27(12):1264-74.

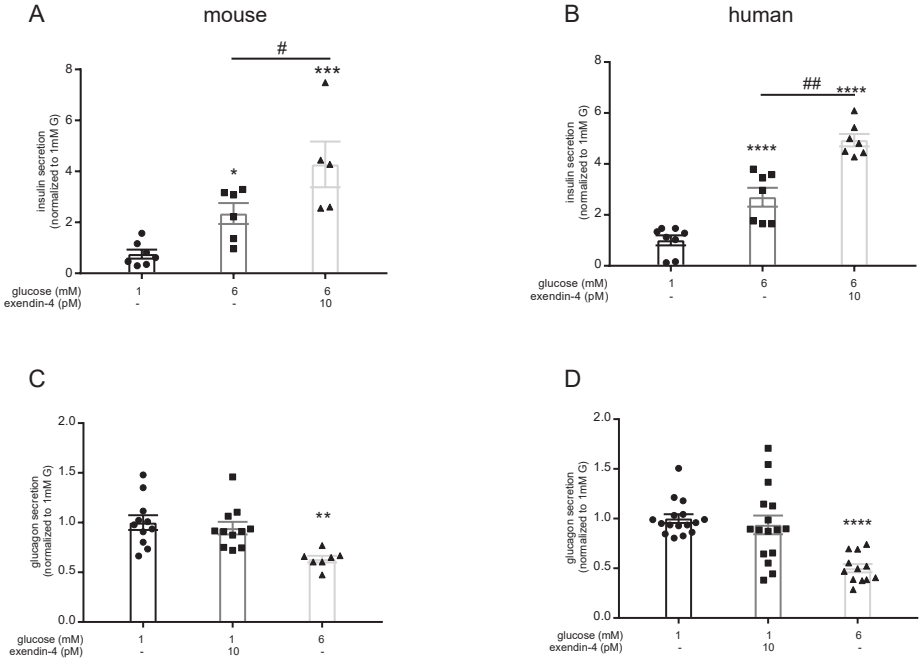
27. Padmanabhan P, Grosse J, Asad AB, Radda GK, and Golay X. Gastrointestinal transit measurements in mice with 99mTc-DTPA-labeled activated charcoal using NanoSPECT-CT. *EJNMMI research.* 2013;3(1):60.
28. Walker JN, Ramracheya R, Zhang Q, Johnson PR, Braun M, and Rorsman P. Regulation of glucagon secretion by glucose: paracrine, intrinsic or both? *Diabetes, obesity & metabolism.* 2011;13 Suppl 1(95-105.
29. Gromada J, Hoy M, Buschard K, Salehi A, and Rorsman P. Somatostatin inhibits exocytosis in rat pancreatic alpha-cells by G(i2)-dependent activation of calcineurin and depriming of secretory granules. *The Journal of physiology.* 2001;535(Pt 2):519-32.
30. Kailey B, van de Bunt M, Cheley S, Johnson PR, MacDonald PE, Gloyn AL, Rorsman P, and Braun M. SSTR2 is the functionally dominant somatostatin receptor in human pancreatic beta- and alpha-cells. *Am J Physiol Endocrinol Metab.* 2012;303(9):E1107-16.
31. Drucker DJ. The Cardiovascular Biology of Glucagon-like Peptide-1. *Cell Metab.* 2016;24(1):15-30.
32. Cantini G, Mannucci E, and Luconi M. Perspectives in GLP-1 Research: New Targets, New Receptors. *Trends in endocrinology and metabolism: TEM.* 2016;27(6):427-38.
33. Davidson MH. Cardiovascular effects of glucagonlike peptide-1 agonists. *Am J Cardiol.* 2011;108(3 Suppl):33B-41B.
34. Ussher JR, and Drucker DJ. Cardiovascular biology of the incretin system. *Endocrine reviews.* 2012;33(2):187-215.
35. Ramracheya R, Ward C, Shigeto M, Walker JN, Amisten S, Zhang Q, Johnson PR, Rorsman P, and Braun M. Membrane potential-dependent inactivation of voltage-gated ion channels in alpha-cells inhibits glucagon secretion from human islets. *Diabetes.* 2010;59(9):2198-208.
36. Moss CE, Glass LL, Diakogiannaki E, Pais R, Lenaghan C, Smith DM, Wedin M, Bohlooly YM, Gribble FM, and Reimann F. Lipid derivatives activate GPR119 and trigger GLP-1 secretion in primary murine L-cells. *Peptides.* 2016;77(16-20.
37. Vergari E, Knudsen JG, Ramracheya R, Salehi A, Zhang Q, Adam J, Asterholm IW, Benrick A, Briant LJB, Chibalina MV, et al. Insulin inhibits glucagon release by SGLT2-induced stimulation of somatostatin secretion. *Nature Communications.* 2019;10(1):139.
38. Bailey S, Harris M, Barkan K, Winfield I, Harper MT, Simms J, Ladds G, Wheatley M, and Poyner D. Interactions between RAMP2 and CRF receptors: The effect of receptor subtypes, splice variants and cell context. *Biochimica et biophysica acta Biomembranes.* 2019;1861(5):997-1003.
39. Knight A, Hemmings JL, Winfield I, Leuenberger M, Frattini E, Frenguelli BG, Dowell SJ, Lochner M, and Ladds G. Discovery of Novel Adenosine Receptor Agonists That Exhibit Subtype Selectivity. *Journal of medicinal chemistry.* 2016;59(3):947-64.
40. Schiele F, Ayaz P, and Fernandez-Montalvan A. A universal homogeneous assay for high-throughput determination of binding kinetics. *Analytical biochemistry.* 2015;468(42-9.

Figure 1: GLP-1(9-36) inhibits glucagon secretion



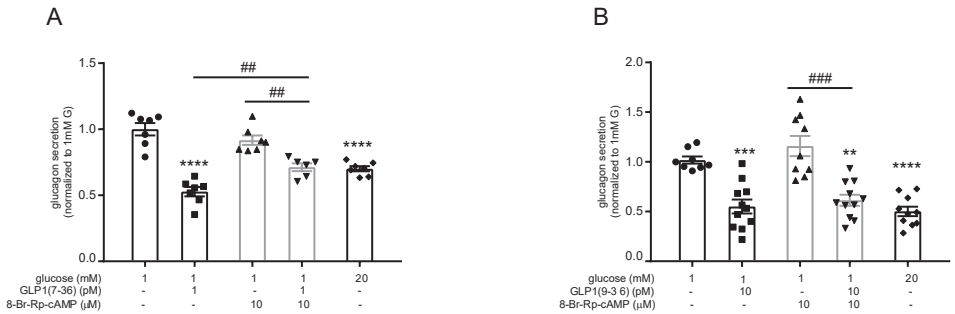
(**A-B**) Effects of increasing concentrations of GLP-1(7-36) on glucagon secretion in isolated mouse (A; n=8-11 using islets from 10 mice) and human (B; n=5-15 using islets from 4 donors) pancreatic islets. (**C-D**) As in (A and B) but using the degradation product GLP-1(9-36) (C; n=4 using islets from 4 mice, D; n=15-17 using islets from 4 donors). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 versus 1mM glucose; 1-way ANOVA with Dunnett's post-hoc test.

Figure 2: Differential pharmacology of insulinotropic and glucagonostatic effects of GLP-1



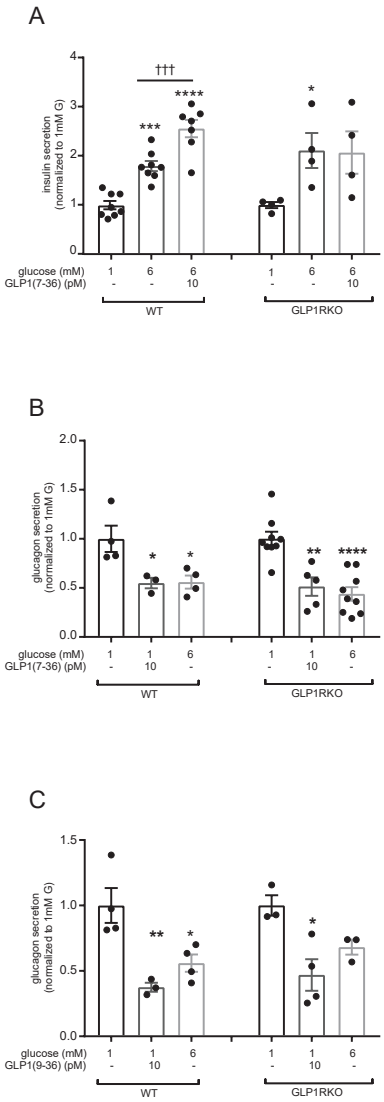
(**A-B**) Effects of 10 pM exendin-4 on insulin secretion in mouse (A; n=3 using islets from 3 mice) and human pancreatic islets (B; n=7 using islets from 2 donors) at 1 and 6mM glucose as shown. (**C-D**) Effects of GLP-1 receptor agonist exendin-4 on glucagon secretion at 1 and 6mM glucose in mouse (C; n=7-11 using islets from 12 mice) and human pancreatic islets (D; n=12-16 using islets from 4 donors).*P<0.05, **P<0.01, ****P<0.0001 versus 1mM glucose; ## P<0.01 for indicated comparison,1-way ANOVA with Dunnett's post-hoc test.

Figure 3: PKA-dependent and –independent glucagonostatic effects of GLP-1(7-36) and GLP-1(9-36)



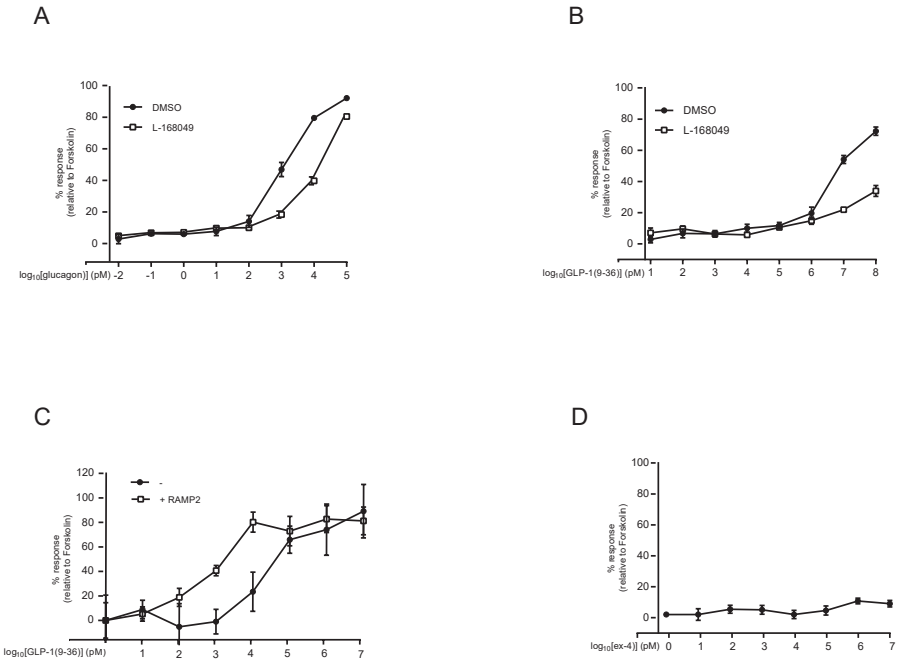
(**A**) Effects of GLP-1(7-36) on glucagon secretion in the absence and presence of the PKA-inhibitor Rp-cAMPS as indicated (n=7 using islets from 11 mice). Glucose was included at 1 and 20mM as indicated. (**B**) As in (A) but using GLP-1(9-36) (n=8-11 using islets from 10 mice). ***P<0.001, ****P<0.0001 versus 1mM glucose, ††P<0.01, †††P<0.001 for indicated comparisons; 1-way ANOVA with Dunnett's post-hoc test.

Figure 4: GLP-1(7-36) and GLP-1(9-36) retain glucagonostatic effects in Glp1r-/- mouse islets



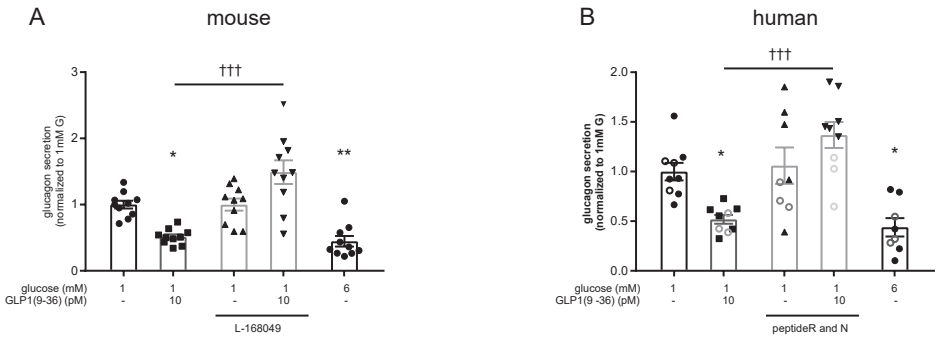
(**A)** Effects of 10 pM GLP-1 (7-36) on insulin secretion at 1 and 6mM glucose in islets isolated from wild type (WT) and Glp1r-/- (GLP1R-KO) mice (n=4-8 using islets from 4 mice of each genotype). (**B**) Effects of 10 pM GLP-1(7-36) on glucagon secretion in wild-type and Glp1r-/- islets (n=4-9 using islets from 4 mice of each genotype) at 1 and 6 mM glucose as shown. (**C**) As in (B) but testing effects of 10 pM GLP-1(9-36) (n=3-4 using islets from 3 mice of each genotype). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 versus 1mM glucose, †††P<0.001 for indicated comparison; 1-way ANOVA with Dunnett's post-hoc test.

Figure 6: GLP-1(9-36) activates the glucagon receptor



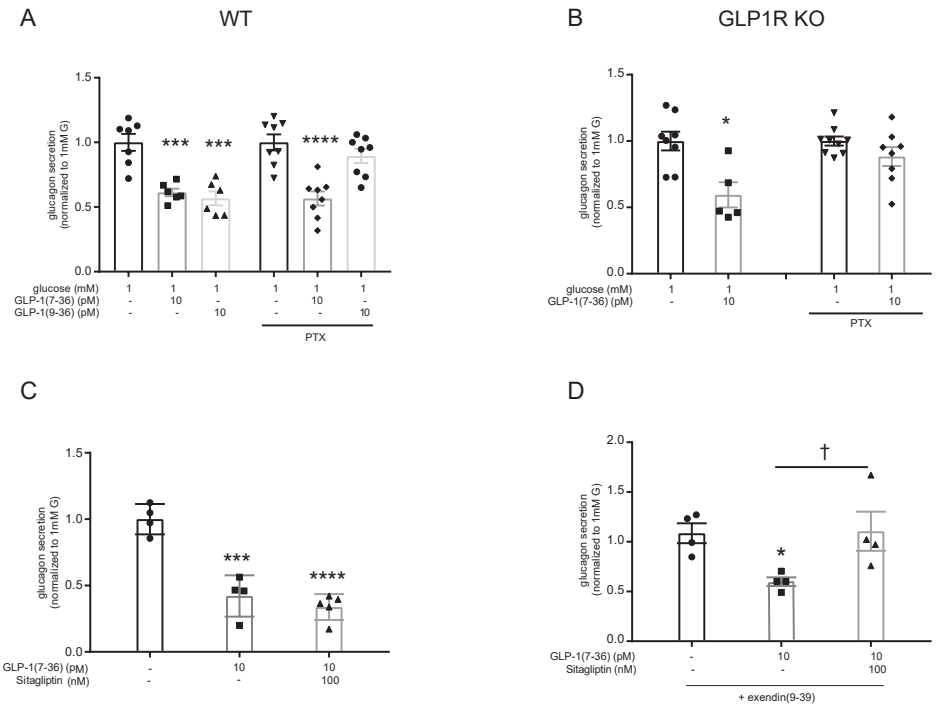
(**A-B**) cAMP accumulation was determined in [ΔCTR] HEK 293 cells transiently transfected with the GCGR and stimulated for 15 mins with glucagon (n=6) (A) and GLP-1(9-36) (n=10) (B) in the presence or absence of the selective competitive antagonist L-168049. (**C**) cAMP accumulation was determined in [ΔCTR]HEK 293 cells transiently transfected with the GCGR (-) or with GCGR +RAMP2 and stimulated for 15 mins with GLP-1(9-36) (n=4) Data expressed as percentage of cAMP production relative to GCGR alone. (**D**) cAMP accumulation was determined in [ΔCTR] HEK 293 cells transiently transfected with the GCGR and stimulated for 15 mins with exendin-4 (n=6).All data, except panel C, are expressed as percentage of cAMP production, determined using 100 μM forskolin stimulation.

Figure 6: GLP-1(9-36) glucagonostatic effects reversed by glucagon receptor antagonists



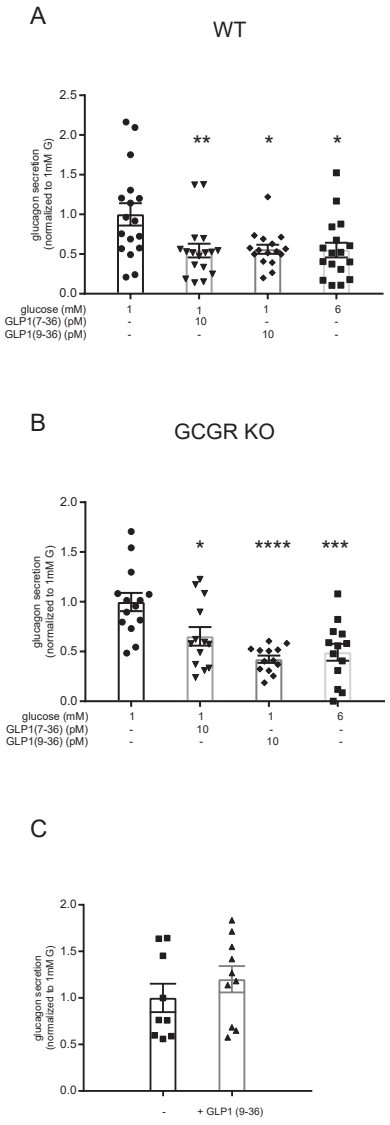
(**A**) Effects of 10 pM GLP-1(9-36) on glucagon secretion in the absence or presence of L-168049 (A; n=10 using islets from 7 mice). (**B**) Effect of 10 pM GLP-1(9-36) on glucagon secretion in human islets in absence and presence of Peptide R or Peptide N (n=8-9 from 3 donors); *P<0.05, **P<0.01 versus 1mM glucose; 1-way ANOVA with Dunnett's post-hoc test. Data from different donors have been color-coded (empty circles using Peptide N).

Figure 7: GLP-1(9-36) inhibits glucagon secretion by pertussis toxin-sensitive mechanism



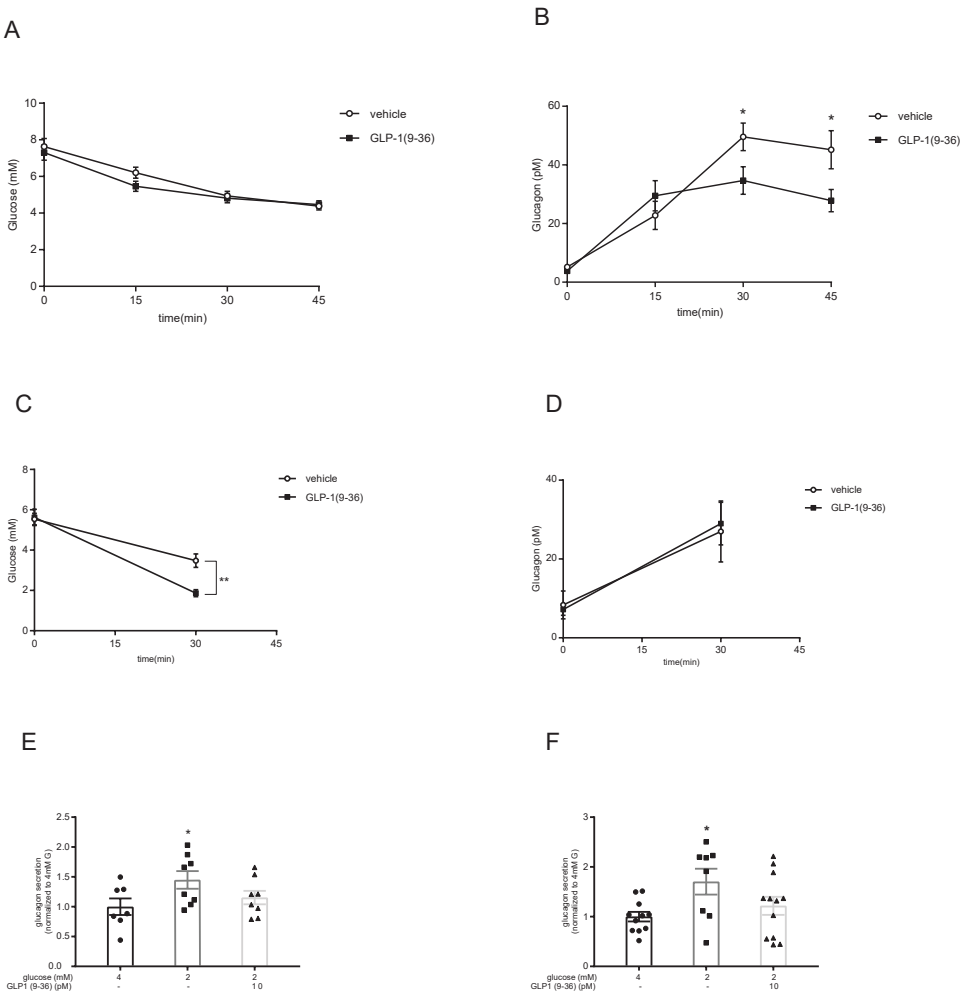
(**A**) Effects of 10 pM GLP-1(7-36) or GLP-1(9-36) on glucagon in wild type (WT) under control conditions and after pretreatment with pertussis toxin (PTX; n=6-8 using islets from 11 mice). (**B**) As in A but using islets from Glp1r-/- mice (GLP-1R KO; n=6-9 using islets from 10 mice). (**C-D**) Effects of 10 pM GLP-1(7-36) on glucagon secretion in the presence of the DPP4 inhibitor sitagliptin (100 nM) alone (C) and with the GLP-1 receptor antagonist exendin-9 (1μM) (D) (n=4 using islets from 4 mice). *P<0.05, ***P<0.001, ****P<0.0001 for indicated comparisons; 1-way ANOVA with Dunnett's post-hoc test.

Figure 8: GLP-1(9-36) inhibits glucagon secretion in islets from Gcgr-/- mice.



(**A-B**) Effects of 10 pM GLP-1(7-36) or 10 pM GLP-1(9-36) on glucagon secretion on glucagon secretion in islets from wild type (WT; A) and Gcgr-/- mice (B) exposed to 1 mM glucose (n=13-17 using islets from 12 mice of each genotype). The responsiveness to 6 mM glucose was also confirmed. (**C**) Effects of GLP-1(9-36) on glucagon secretion in islets from Glp1r-/- mice in presence of the glucagon receptor antagonist Peptide N (n=9 using islets from 12 mice). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 versus 1mM glucose; 1-way ANOVA with Dunnett's post-hoc test.

Figure 9:GLP-1(9-36) effects on glucose and glucagon secretion during insulin-induced hypoglycemia *in vivo.*



(**A-D**) Effect of GLP-1(9-36) injection on glucose (A and C) and on glucagon (B and D) after 15, 30 and 45 minutes from insulin administration, in mice fasted for 5h (A-B) (n=11-13) and in mice fasted for 18h (C-D) (n=5).*P<0.05, **P<0.01, for indicated comparisons. (**E-F**) Effect of 10 pM GLP-1(9-36) on glucagon secretion in mouse (E) (n=7-8 using islets from 4 mice) and human (F) islets (n=9-12 using islets from 3 donors) stimulated with 4mM and 2 mM glucose. *P<0.05 versus 4 mM glucose;1-way ANOVA with Dunnett's post-hoc test.

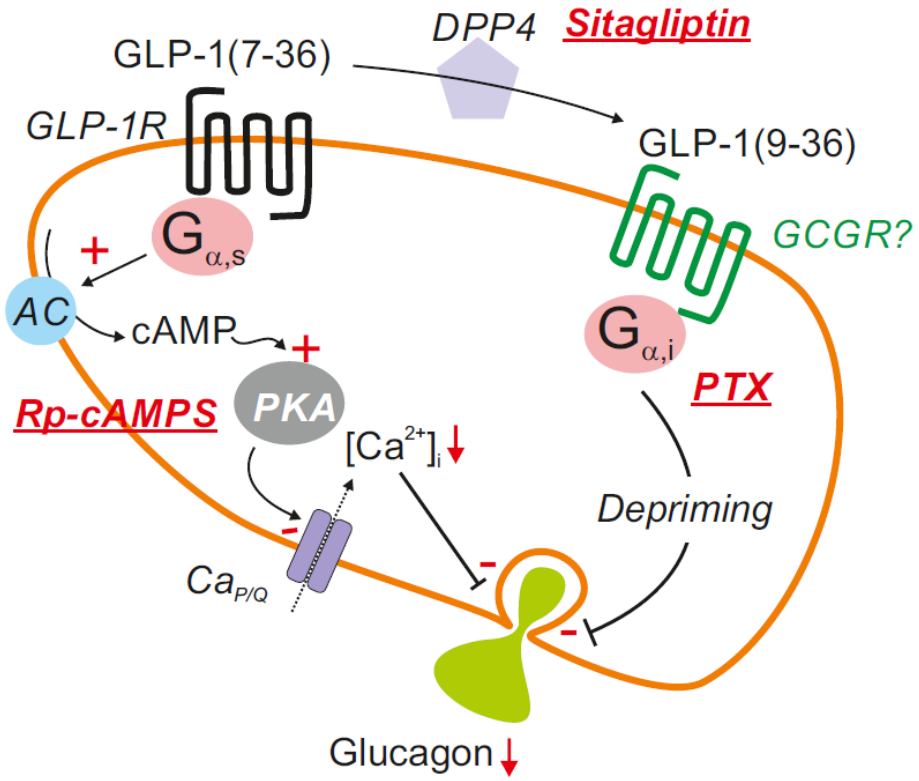


Figure 11. Schematic illustrating the regulation of glucagon secretion by GLP-1. Activation of GLP-1 receptor (GLP-1R) activates a stimulatory GTP-binding protein ($G_{\alpha,s}$) with resultant stimulation of adenylate cyclase (AC), elevation of intracellular cAMP and activation of protein kinase A (PKA). This leads to inhibition of voltage-gated P/Q-type Ca^{2+} channels and suppression of glucagon exocytosis via a reduction in cytoplasmic free Ca^{2+} ($[Ca^{2+}]_i$). This pathway is inhibited by the GLP-1R antagonist exendin(9-39) and the PKA-inhibitor Rp-cAMPS. In addition to this mechanism, GLP-1 may also (following its degradation to GLP-1(9-36), a reaction catalysed by dipeptidyl peptidase 4 [DPP4]) activate the glucagon receptor (GCGR). This pathway leads to activation of an inhibitor GTP-binding protein ($G_{\alpha,i}$) and culminates in depriming of secretory granules and reduced glucagon exocytosis. This pathway is inhibited by the DPP4 inhibitor sitagliptin, pertussis toxin (PTX) and GCGR antagonists such as L-168049. See main text for details.