Main Manuscript for

Increased RAMP3 expression enhances GLP-1 mediated Glucose Stimulated Insulin Secretion in a Manner Dependent on Ca2+ Mobilisation

Abigail Pearce[1], Claudia Sisk[1], Matthew Harris[1], Ho Yan Yeung[1], Sabrina Carvalho[1], Graham Ladds[1*].

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

*Graham Ladds

Email: grl30@cam.ac.uk

Author Contributions: A.P. and G.L. designed the study. A.P., C.M.S., H.Y.Y., S.C., M.H. performed the experiments. A.P. and G.L. wrote the manuscript. All authors proof-read and provided feedback on the manuscript.

Competing Interest Statement: The authors have no competing interest to declare

Classification: Biological Sciences; Pharmacology

Keywords: G Protein-coupled Receptor; Glucagon-Like Peptide-1; Receptor Activity-Modifying Protein; Signalling Bias; Diabetes

This PDF file includes:

Main Text
Figures 1 to 4

Abstract

The targeting of the Glucagon-like peptide-1 receptor (GLP-1R) for diabetes and obesity is not a novel strategy, with recent therapeutics showing efficacy in weight loss and glycaemic control. However, they are also associated with side effects, including gastrointestinal disruptions and pancreatitis. Developing agonists with different signalling profiles, or that exert some tissue selectivity can circumvent these on-target, unwanted effects. Receptor activity-modifying proteins (RAMPs) offer the potential to do both, through modulation of agonist binding and signalling, as well as surface expression. The GLP-1R was found to interact with RAMP3, with the heterodimer able to bind agonist at the cell surface. RAMP3 expression biased the receptor towards Ca2+ mobilisation, away from the canonical cAMP-driven signalling. When examining G protein coupling, the interaction with RAMP3 reduced activation of the cognate Gαs, but increased secondary couplings to Gαq and Gαi. These increased couplings led to an elevation in glucose-stimulated insulin secretion when cells overexpressing RAMP3 were stimulated with GLP-1. The effects of this interaction can then inform selection of models and peptide design when targeting this receptor for therapeutic intervention.

Significance Statement

G Protein-Coupled Receptors, such as the Glucagon-Like Peptide-1 (GLP-1) Receptor are common drug targets, although not without side effects due to on-target, unwanted signalling outputs. This study identifies an interacting protein, which alters its signalling to promote insulin secretion. This may improve the therapeutic potential of GLP-1 mimetics, by enhancing effectual signalling over that associated with unwanted effects, by directly targeting this receptor complex.

Main Text

Introduction

Obesity and type 2 diabetes mellitus (T2DM) are increasingly prevalent diseases, with the incidence expected to rise to 51% of the global population by 2035 (https://data.worldobesity.org/publications/?cat=19). Finding treatments for these diseases has been an ongoing mission, with efforts more recently focused on simultaneous targeting of both conditions, with the aim to improve T2DM by resolving the underlying obesity.

A recent therapeutic advance has been the approval of peptides targeting the Glucagon-Like Peptide-1 (GLP-1) receptor for obesity and weight loss. The incretin effect, whereby oral glucose elevates plasma insulin levels to a greater degree than IV administration, was first observed in 1964 (1). This is mediated by incretin hormones, the best known of which are GLP-1 and the glucose-dependent insulinotropic polypeptide (GIP). As such, mimetics of the endogenous GLP-1 hormone, the first of which was exenatide (exendin-4), were approved for T2DM in 2005 (2). However, newer mimetics semaglutide (trade names of Ozempic and Wegovy) or tirzepatide (sold under Mounjaro or Zepbound), the latter of which targets both the GLP-1R and the GIP Receptor, have garnered much attention through their dual effects on lowering plasma glucose and aiding weight loss. Semaglutide has since been reported to protect against neurodegeneration and neuroinflammation (3), as well as cardiovascular disease (4). This culminated in semaglutide being labelled as the 2023 breakthrough of the year (5). However, with their increased use, attention is being drawn to the unwanted side effects of GLP-1 mimetics. Common side effects are nausea and vomiting, as well as other gastrointestinal discomforts. However, more serious side effects are also known, such as pancreatitis, kidney failure, and gallbladder problems (6). There is therefore the unmet need to develop mimetics with improved therapeutic potential that exhibit fewer side effects.

One method of reducing on-target unwanted effects is to selectively activate signalling pathways associated with the positive outcome, relying on the concept of signalling bias. The GLP-1R is a class B1 G protein-coupled receptor (GPCR), which predominantly couples to Gαs, leading to an increase in cyclic-adenosine monophosphate (cAMP) production. However, like other members of class B1, the GLP-1R exhibits

pleiotropy, coupling to other Gα families and recruiting β-arrestins (7, 8), leading to a multifaceted signalling output. For other pleiotropic GPCRs, the activation of these different pathways can be influenced by the agonist, giving rise to signalling bias. This has been studied most heavily for the μ-opioid receptor, where it is thought agonist tolerance is mediated by β-arrestins, leading G protein-biased agonists to be preferred (9).

Signalling bias can also be influenced by the tissue, due to differential expression of signalling proteins, as well as allosteric modulators. A common protein allosteric modulator of Class B1 GPCRs are the receptor activity-modifying proteins (RAMPs). First discovered for the calcitonin-like receptor, with which they form obligate heterodimers, RAMPs influence the expression, affinity, and signalling bias of the receptor. For the calcitonin family, this leads to receptors considered functionally distinct (10, 11), although smaller effects are observed on other receptors e.g. the vasoactive intestinal peptide receptor 1 ($VPAC_1$) or glucagon receptor (GCGR) when co-expressed with RAMP2 (12). Importantly, the effects of RAMPs are agonist- and pathway-dependent, with RAMP2 increasing $G\alpha_s$ coupling at GCGR in response to glucagon and oxyntomodulin (an endogenous dual agonist of both GCGR and GLP-1R) but decreasing the response to GLP-1 and liraglutide (a synthetic, lipidated GLP-1 mimetic, approved in the treatment of T2DM) (13).

RAMPs have been implicated in diabetes and obesity, although their role appears multifaceted. Of the GPCRs which interact with RAMPs, many are involved in the regulation of glucose levels and body weight. In addition to glucagon, another pancreatic hormone, amylin, binds a class B1 GPCR, with the three amylin receptors heterodimers between the calcitonin receptor (CTR) and RAMP1-3. RAMPs are expressed in many of the tissues of the endocrine system, such as the thyroid and hypothalamus, and global RAMP knock out in rodents causes dysregulation of body weight and glycaemic control (14, 15) (the role of RAMPs in diabetes and obesity is further reviewed in Malcharek et al., 2024 (16)). Importantly, RAMPs are co-expressed with the incretin hormone receptors across regions of the brain, and the pancreas (Human Protein Atlas as of January 2025). The interaction between GIPR and RAMPs, and the consequences for glucose tolerance have been studied previously (17), but the involvement of GLP-1R is less known. GLP-1R has been previously shown to interact with all three RAMPs (18, 19), with depressive effects on cAMP accumulation observed. However, specific interactions at the plasma membrane have not been studied, nor have any effects of RAMP expression on physiological roles of the receptor, such as glucose-stimulated insulin secretion (GSIS).

Herein, we measured a specific membrane interaction between GLP-1R and RAMP3, but not RAMP1 or 2. The two proteins were observed to form a complex able to bind agonist, but no effect was observed on affinity of the receptor. RAMP3 expression decreased cAMP accumulation and increased mobilisation of Ca^{2+} from intracellular stores (Ca^{2+}_i). This was determined to be primarily due to an increase in $G\alpha_{i/o}$ coupling. In contrast, no effect was observed on internalisation, with only a decrease in maximal β-arrestin recruitment. This elevation in $(Ca^{2+})_i$ was shown to lead to enhanced GSIS. There is therefore the potential for improved GLP-1 mimetics, which show bias towards the GLP-1R-RAMP3 complex, with subsequent effects on tissue selectivity and therapeutic profile.

Results

GLP-1R specifically interacts with RAMP3

Although GLP-1R expression has been unable to promote RAMP membrane trafficking, interactions have been observed at the molecular level, e.g. using BRET (19). Therefore, to determine if these observed interactions are present at the plasma membrane, a cell surface BRET interaction assay was used as previously described (17). Nluc-GPCR and SNAP-RAMP were transiently transfected into Cos7 cells, which do not endogenously express RAMPs, CTR or CLR (20), to reduce any interference from unlabelled proteins. SNAP-RAMPs were irreversibly conjugated to SNAP-Surface Alexa Fluor 488 to specifically measure cell surface interactions; the conjugate was cell impermeable so only labelled SNAP-RAMPs expressed at the cell surface. All three RAMPs displayed an interaction with CLR, as observed by the saturating increase in BRET ratio. GLP-1R only displayed a saturating increase in BRET with SNAP-RAMP3 (Figure 1A). Overexpression of HA-CLR was not shown to promote an interaction between Nluc-

GLP-1R and SNAP-RAMP1 or 2, indicating that expression of RAMP at the membrane is not sufficient to induce an interaction (Supplementary Figure S1).

To validate the formation of a GLP-1R-RAMP3 complex, we measured binding of a fluorescent ligand to the protein heterodimer. We utilised NanoBiT, with LgBiT preceding the N-terminus of the receptor, and SmBiT upstream of the N-terminus of the RAMP (illustrated in Figure 1B). We first demonstrated that only RAMP3, and not RAMPs 1 or 2, interacted with GLP-1R; an interaction was measured by increased luminescence, as only when the proteins interact can a full luciferase form (Supplementary Figure S1). Binding of the Tag-Lite GLP-1 receptor red ligand, based on exendin-4 (Ex-4-Red) was then observed through an increase in the BRET ratio, with a calculated K_d of 6.7nM (Figure 1C). This was not observed for SmBiT-RAMP1 or -RAMP2. The BRET ratio could be reduced by injection of 1μM unlabelled GLP-1, competing with the fluorescent agonist and further demonstrating binding had occurred to the heterodimer (Figure 1D).

FLAG-RAMP3 has no effect on GLP-1R expression or agonist binding

When measuring their effects on GPCR signalling, RAMPs are commonly co-expressed at a 1:1 ratio, due to the stoichiometry of the interaction with CLR (21). However, the maximal interaction between GLP-1R and RAMP3 in the BRET assay was observed when RAMP3 was expressed at twice that of GLP-1R (by weight of expression plasmid, Figure 1A). We therefore measured the effect of increasing RAMP3 expression on GLP-1R, either co-expressing (1:1 ratio) or overexpressing (2:1 ratio) RAMP3. It was confirmed that the expression of FLAG-RAMP3 was increased at the 2:1 ratio compared to 1:1, but no difference was observed on GLP-1R expression (Figure 1E, Table S1).

In addition to receptor expression, differences in signalling could be driven by changes in affinity. As RAMP modulation of GPCR signalling can be agonist dependent, effects were examined across a range of agonists. GLP-1 and oxyntomodulin are endogenous agonists, the latter with a reduced potency. GLP-1 9-36, a product of the degradation of GLP-1 which can show weak partial agonism, was also tested. Examples of incretin mimetics, exendin-4 and liraglutide were also included. FLAG-RAMP3 had little influence on binding affinity for any peptide, as measured through a NanoBRET competition binding assay (Figure 1F, Table S2, Supplementary Figure S1).

Second messenger signalling is Influenced by RAMP3 expression

As with most Class B1 GPCRs, the primary signalling mediator for the GLP-1R is $G\alpha_s$, leading to a increase in cAMP (7, 22). When stimulated with GLP-1 for 8-minutes, a dose-dependent increase in cAMP was observed (pEC_{50} value of 11.4±0.3). This was reduced by RAMP3 expression, with a reduction in the potency at 8-minutes when RAMP3 was over expressed (to 10.4±0.2) (Figure 2A, Table S3). The pattern was preserved across exendin-4, liraglutide, and oxyntomodulin, with reductions in both potency and efficacy observed when RAMP3 was overexpressed (Figure 2B, Supplementary Figure S2A). GLP-1 9-36 did not elicit sufficient cAMP production over the concentration range tested to calculate potency and efficacy values.

When increasing the stimulation period to 30-minutes, there was again a reduction in the potency of the response to GLP-1, with over-expression significantly reducing the pEC_{50} value (Figure 2C, Table S3). Effects on other agonists were reduced, with significant differences no longer observed (Figure 2D, Supplementary Figure S2B).

GLP-1R is a pleiotropic receptor, able to couple to G proteins other than $G\alpha_s$ (7, 8, 23). In many studies, the receptor has been found to couple to the $G\alpha_{q/11}$ family of G proteins, with GLP-1R activity also associated with increased intracellular Ca^{2+} concentrations $(Ca^{2+})_i$ due to mobilisation from intracellular stores. The effect of RAMP3 were therefore tested against $(Ca^{2+})_i$ mobilisation (Figure 2E-F, Table S3). FLAG-RAMP3 expression enhanced the $(Ca^{2+})_i$ mobilisation in response to GLP-1, increasing the potency of the response (Figure 2E). The dataset was again extended to other agonists to determine any agonist-dependent effects. Again, GLP-1 9-36 was unable to elicit any $(Ca^{2+})_i$ mobilisation in the absence of

RAMP3, meaning signalling parameters could not be calculated. However, when RAMP3 was co-expressed, a response to GLP-1 9-36 could begin to be observed (Supplementary Figure S2C).

There was a significant trend for RAMP3 to increase maximal signalling in response to all agonists (*$p<0.0001$*) (Figure 2E-F, Table S3). Expression in a 1:1 ratio significantly increased the maximal responses to exendin-4 (from 43.6±5.30 to 71.61±4.64 percent of 10 μM ionomycin, *$p<0.05$*) and oxyntomodulin (from 21.7±3.5 to 63.6±10.6 percent of 10 μM ionomycin, *$p<0.01$*). Over-expression also significantly increased the maximal response of these two agonists to 97.6±6.8 and 52.3±8.5 respectively (*$p<0.0001$, $p<0.05$*), as well as increasing the potency of the response to GLP-1 (from a pEC_{50} of 7.2±0.2 to 8.1±0.2, *$p<0.05$*). There were no significant effects on the response to liraglutide, highlighting agonist dependency.

As changes in signalling can result from altered receptor desensitisation, we investigated the effects of RAMP3 expression on β-arrestin recruitment and internalisation. This is especially pertinent when concerning RAMP3, as it is known to have effects on receptor internalisation and trafficking for other class B1 GPCRs (24–26). Recruitment was reduced for all agonists, although only when RAMP3 was expressed at a 2:1 ratio of receptor. For GLP-1, exendin-4, and oxyntomodulin, there was no subsequent effect on internalisation, consistent with the observation that GLP-1R internalisation is β-arrestin-independent (27) (Supplementary Figure SF3, Table S4). However, RAMP3 overexpression led to a significant decrease in the potency of internalisation in response to liraglutide (Supplementary Figure SF3).

In order to make comparisons across pathways and agonists, logarithmic values of relative activity (LogRA) were calculated as metrics of bias for second messenger signalling assays (cAMP and $(Ca^{2+})_i$ mobilisation) and desensitisation (Figure 2G-H, Tables S3, S4). The effects of RAMPs overall varied when looking at different peptides. GLP-1 showed a decrease in cAMP accumulation, and an increase in $(Ca^{2+})_i$ mobilisation, with little effect on desensitisation. However, liraglutide displayed lesser effects on signalling, with only small reductions in cAMP accumulation, but reduced internalisation.

RAMP3 expression directly influences G protein coupling

To determine the underlying changes in G protein coupling associated with this biased second messenger production, the TRUPATH G protein biosensor platform was used (28) (Figure 3A). Three major families were examined, as they are closely linked with changes in cAMP accumulation and $(Ca^{2+})_i$ mobilisation; $G\alpha_s$, $G\alpha_{i/o}$, and $G\alpha_{q/11}$. There are reports that the TRUPATH system struggles to detect $G\alpha_q$ coupling at classically $G\alpha_s$ coupled receptors (28, 29). We therefore tested the wild type $G\alpha_q$, and the R183Q mutant ($G\alpha_q(Q)$), in response to all four full agonists. No quantifiable response was observed using wild type $G\alpha_q$ (Supplementary Figure S4), as has been observed previously (29).

Whilst no effect was observed for GLP-1, exendin-4, or liraglutide, there was a significant reduction in the maximal $G\alpha_s$ dissociation elicited by oxyntomodulin when RAMP3 was expressed (reduced to 58.9% ±5.8 of receptor alone) (Figure 3B, Table S5). There was no effect on the $G\alpha_q(Q)$ mutant response to exendin-4, liraglutide, or oxyntomodulin, but RAMP3 did significantly increase the maximal dissociation elicited by GLP-1 (to 119 ±5.5% of receptor alone) (Figure 3C, Table S5).

Whilst the effects on $G\alpha_s$ and $G\alpha_q$ provide some insights as to how RAMP3 exerts its effects on second messenger signalling, it is not conclusive. Linked with both a reduction in cAMP, and an increase in $(Ca^{2+})_i$ is $G\alpha_{i/o}$ (illustrated in Figure 3C). GLP-1R coupling to this family of G proteins has been observed previously (7, 8), so the effects of RAMP3 were studied across the family. GLP-1R showed little coupling to $G\alpha_z$, which was unchanged by RAMP3 expression (Supplementary Figure S4). However, for other members of the family, there was a significant increase in the potency of coupling as increasing concentrations of RAMP3 were expressed ($G\alpha_{i1}$ showed in Figure 3D, Supplementary Figure S4, Table S6).

To better enable comparisons between the different Gα subunits, LogRA values were calculated, relative to the response to receptor alone (Figure 3E, Table S5, S6). The changes at $G\alpha_s$ and $G\alpha_q(Q)$ were slight, mirroring the effects when only looking at the potency (pEC_{50}) and efficacy (E_{max}) parameters. Larger

changes were observed in $G\alpha_{i/o}$ coupling, with significant decreases in the LogRA for $G\alpha_{i1}$ when RAMP3 was overexpressed in cells stimulated with exendin-4 and oxyntomodulin.

Signalling in an Insulin Secreting Cell Line

GLP-1R signalling is inherently linked with insulin secretion, as receptor activation significantly enhances GSIS. This is linked to both cAMP accumulation and $(Ca^{2+})_i$ mobilisation, enhancing sensitivity of the insulin secretion machinery (Figure 6A), with β-arrestin recruitment and internalisation both positively and negatively implicated in GSIS (30, 31). To confirm the changes observed in HEK293T cells are applicable to a more physiological setting, the above signalling assays were repeated in INS-1 832/3 cells, a rat insulinoma cell line. These cells endogenously express the GLP-1R, alongside low levels of RAMP2 and 3 (Supplementary Figure SF5). RAMP3 was transfected into these cells, maintaining the endogenous expression of GLP-1R to look at the second messenger signalling. Assaying at 8- and 30-minute stimulation, no effect was observed on cAMP accumulation in response to GLP-1 when RAMP3 was expressed (Figure 4B, Table S7). However, as observed in HEK293T cells, RAMP3 did increase the potency of $(Ca^{2+})_i$ mobilisation (from a pEC_{50} of 6.94±0.11 to 7.41±0.14) (Figure 4C-D, Table S7).

When looking at β-arrestin recruitment or internalisation, INS-1 832/3 cells without endogenous GLP-1R expression (32) were used to avoid any potential interference. Cells were transfected with GLP-1R-Nluc and either vector or RAMP3, and β-arrestin1/2-YFP for β-arrestin recruitment, or RIT-Venus for internalisation. RAMP3 had no effect on β-arrestin1 recruitment but increased maximal β-arrestin2 recruitment in this cell line (Supplementary Figure SF5, Table S8). This shows a system dependency to the effects of RAMP3, with the opposite effect observed in HEK293T cells. As in HEK293T cells, there was no effect on internalisation (Supplementary Figure SF5, Table S8). To summarise the overall effect of RAMP3 on GLP-1R signalling in INS-1 832/3 cells, LogRA values were again calculated. Relative to vector-transfected cells, there was no significant effect of RAMP3 on relative activity, however the greatest observed effect was the bias towards $(Ca^{2+})_i$ mobilisation (Figure 6E, Tables 8-9).

Insulin Secretion is Enhanced by RAMP3

INS-1 cells overexpressing RAMP3 displayed significantly greater insulin secretion in response to 10nM GLP-1 than those expressing vector; GLP-1 increased insulin secretion 2.36±0.30 fold over 2.8mM glucose compared to 3.94±0.33 when RAMP3 is overexpressed (Figure 4F, Table S9). This was not observed when cells were transfected with RAMP1, although both RAMP1 and 3 increased insulin secretion in response to 10nM GIP, whose receptor interacts with both proteins (17) (Figure 4F, Table S9). The effect of RAMP3 was confirmed for the other GLP-1R agonists, whose signalling has also been affected by RAMP3 expression, although the increase was not significant for exendin-4.

To determine how this increase in insulin secretion occurs, inhibitors of different G protein-mediated signalling pathways were employed (schematic shown in Figure 4G). Data have been normalised to the response to GLP-1 in the absence of inhibitor.

As no specific inhibitor of $G\alpha_s$ is available, inhibitors of proteins whose signalling is downstream of cAMP accumulation were used. Inhibition of Protein Kinase A (PKA) with Rp-8-Br-cAMPs had no effect on insulin secretion, irrespective of the expression of RAMP3 (Figure 4H, Table S10). However, inhibition of exchange factor directly activated by cAMP1/2 (EPAC1/2) with the dual inhibitor ESI-09 significantly decreased GLP-1 mediated potentiation of GSIS in both vector and RAMP3 transfected cells (Figure 4H, Table S10).

Inhibition of $G\alpha_{q/11}$ activation with 100nM YM-254890, or $G\alpha_{i/o}$ signalling with pertussis toxin (PTX) decreased insulin secretion significantly only when RAMP3 was overexpressed. It is therefore suggested that RAMP3 enhances $G\alpha_{q/11}$ and $G\alpha_{i/o}$ activation, enhancing $(Ca^{2+})_i$ mobilisation and GSIS.

Discussion

Insulin secretion following ingestion of food high in carbohydrates is greatly potentiated by signalling of incretin hormones. Manipulation of the signalling of the incretins is therefore a valuable strategy for treating diabetes. However, development of therapeutics targeting the GLP-1R has been made difficult by the variability in the signalling of the receptor depending on the study. Part of this variability is attributed to the cell background in which the assay is performed, and the different interacting proteins expressed therein. We therefore sought to identify any interactions with the RAMP family of proteins, which modulate signalling at many Class B1 GPCRs, and determine any subsequent effects on signalling of exogenously and endogenously expressed receptors. We then further delved into the effect of these changes in signalling on GSIS.

GLP-1R was found to interact with RAMP3, but not the other members of the RAMP family of proteins. Previous studies have shown interactions with RAMP1 and 2, although at a reduced level. However, these studies have used isolated lysates (18), or whole cell resonance energy transfer (17, 19). Neither technique discerns between interactions in different subcellular compartments, and therefore does not confirm a functional receptor complex. Use of a cell impermeable dye to irreversibly label SNAP-RAMP enables measurement of only interactions at the plasma membrane, suggesting interactions previously observed with RAMP1 and 2 occur in intracellular compartments. Furthermore, adaptation of the NanoBiT system for ligand binding determined it is only the GLP-1R-RAMP3 complex capable of agonist binding, and not the other two RAMPs. This interaction had no effect on membrane expression of either GLP-1R of RAMP3, nor was there any effect on binding of GLP-1 to the receptor. Unlike other RAMPs, RAMP3 is able to translocate to the plasma membrane in the absence of an interacting GPCR (12). It is therefore possible that it displays interactions with an increased number of GPCRs through proximity, as opposed to a specific interaction (26). However, increasing the expression of RAMP1 at the membrane through overexpression of CLR had no effect on the interaction observed.

RAMP3 expression did, however, modulate G protein signalling at the receptor. Consistent with previous observations (19), cAMP accumulation was decreased in the presence of RAMP3, especially at early time points. However, unlike previous reports, an increase in $(Ca^{2+})_i$ mobilisation was observed with increasing expression of RAMP3. These findings were validated by examining the effects on G protein coupling, using the TRUPATH biosensor platform. At individual agonists, small perturbations could be observed. $G\alpha_s$ coupling in response to oxyntomodulin was increased by RAMP3, and $G\alpha_q$ coupling was increased when cells were stimulated with GLP-1. However, for the remaining agonists, no effects were observed. However, $G\alpha_{i/o}$ coupling, whilst small, was increased by RAMP3 in response to all agonists tested.

In correlation with the effects on cAMP production, β-arrestin recruitment was decreased by RAMP3 overexpression, although not for the weaker agonist oxyntomodulin. There were no subsequent effects on internalisation for most agonists, but the response to liraglutide was reduced. The GLP-1R has been shown to internalise through multiple avenues, with no β-arrestin dependency (27), so a lack of correlation is not surprising. Regarding liraglutide, it is possible the bias towards β-arrestin observed for the agonist correlates with β-arrestin-dependent internalisation. Analysis of GRK recruitment, which is thought paramount for GLP-1R internalisation, might help uncover the agonist dependent effects observed.

Insulin secretion is enhanced by overexpression of RAMP3. This was observed for all GLP-1R agonists tested, but not high glucose alone. It is therefore unlikely the increase in RAMP3 expression itself directly increases insulin secretion. Furthermore, increasing RAMP1 expression (which does not interact with GLP-1R) had no effect on the potentiation of GSIS by GLP-1R, but did enhance GIPR mediated GSIS, consistent with findings that reducing RAMP1 expression reduced glucose tolerance in mice (17).

GLP-1-mediated GSIS was unaffected by inhibition of PKA by Rp-8-Br-cAMPs in the presence or absence of RAMP3. Previous studies have shown potentiation of GSIS to be both PKA dependent (33) and independent (34), with this study agreeing with the latter, although this might be due to reduced affinity of the compound for the rat kinases. Inhibition of EPAC1/2 with ESI-09 significantly reduced insulin secretion, although a non-significant reduction was also observed in cells stimulated with high glucose alone (Supplementary Figure S5), suggesting importance in GSIS independent of the incretin hormones. EPAC1/2 has been previously shown to be important for cAMP-dependent potentiation of GSIS (35), which

this data supports. As cAMP accumulation was unaffected in the INS-1 832/3 cell line by the overexpression of RAMP3, it is not surprising that there was no interactive effect of RAMP3 when PKA or EPAC1/2 were inhibited. YM-254890 and PTX, inhibitors of $G\alpha_{q/11}$ and $G\alpha_{i/o}$ respectively, however, only reduced insulin secretion in cells overexpressing RAMP3. This is consistent with the elevation in $(Ca^{2+})_i$ mobilisation when RAMP3 was overexpressed, dependent on enhanced $G\alpha_{i/o}$ coupling. $G\alpha_{i/o}$-mediated $(Ca^{2+})_i$ mobilisation has been shown to depend on active $G\alpha_{q/11}$ (36), making the resolution of the respective contributions of the two G protein families difficult. However, the effect of YM-254890 was greater than PTX, suggesting a contribution from both families.

RAMP3 has therefore been shown to influence agonist bias at the GLP-1R, shifting the receptor towards $(Ca^{2+})_i$ mobilisation, which enhances insulin secretion. As RAMP3 expression varies between tissues, it is possible that by directly targeting this complex, tissue specific receptor activation could be facilitated. However, of more relevance is the consideration of RAMP expression when developing new GLP-1 mimetics, or small molecule agonists and allosteric modulators for the treatment of T2DM and obesity. Agonists which have reduced affinity for the RAMP3 complex over receptor alone might show reduced therapeutic potential, which might not be detected when only looking at the GLP-1R alone. Furthermore, it is important to ensure allosteric modulators do not disrupt the interaction, as is the case for the CLR-RAMP1 antagonist BIBN 4096 BS (37), as this could lead to a reduction in GLP-1R activity in cells expressing RAMP3.

Materials and Methods

Cell culture, BRET assays, second messenger signalling assays, flow cytometry, RNA expression quantification, and insulin secretion assays performed in the study are available in *SI Appendix, Materials and Methods*.

Acknowledgments

A.P. is funded by the Biotechnology and Biological Sciences Research Council (BBSRC; BB/W014831/1). C.S. and HYY were supported by the Cambridge Trust International Scholarship with HYY also being funded by the Rosetrees Trust. SC was supported by an AstraZeneca studentship. G.L. is a Royal Society Industry Fellow (INF/R2/212001).

References

1. H. ELRICK, L. STIMMLER, C. J. HLAD JR., Y. ARAI, Plasma Insulin Response to Oral and Intravenous Glucose Administration1. *J Clin Endocrinol Metab* **24**, 1076–1082 (1964).

2. J. Guyton, M. Jeon, A. Brooks, Glucagon-like peptide 1 receptor agonists in type 1 diabetes mellitus. *American Journal of Health-System Pharmacy* **76**, 1739–1748 (2019).

3. W. Wang, *et al.*, Associations of semaglutide with first-time diagnosis of Alzheimer's disease in patients with type 2 diabetes: Target trial emulation using nationwide real-world data in the US. *Alzheimer's & Dementia* **20**, 8661–8672 (2024).

4. D. H. Ryan, *et al.*, Semaglutide Effects on Cardiovascular Outcomes in People With Overweight or Obesity (SELECT) rationale and design. *Am Heart J* **229**, 61–69 (2020).

5. J. Couzin-Frankel, Obesity meets its match. *Science (1979)* **382**, 1226–1227 (2023).

6. M. M. Smits, D. H. Van Raalte, Safety of Semaglutide. *Front Endocrinol (Lausanne)* **12** (2021).

7. C. Weston, D. Poyner, V. Patel, S. Dowell, G. Ladds, Investigating <scp>G</scp> protein signalling bias at the glucagon-like peptide-1 receptor in yeast. *Br J Pharmacol* **171**, 3651–3665 (2014).

8. C. Avet, *et al.*, Effector membrane translocation biosensors reveal G protein and βarrestin coupling profiles of 100 therapeutically relevant GPCRs. *Elife* **11** (2022).

9. A. E. Conibear, E. Kelly, A Biased View of μ-Opioid Receptors? *Mol Pharmacol* **96**, 542–549 (2019).

10. D. L. Hay, M. L. Garelja, D. R. Poyner, C. S. Walker, Update on the pharmacology of calcitonin/CGRP family of peptides: IUPHAR Review 25. *Br J Pharmacol* **175**, 3–17 (2018).

11. G. Christopoulos, *et al.*, Multiple Amylin Receptors Arise from Receptor Activity-Modifying Protein Interaction with the Calcitonin Receptor Gene Product. *Mol Pharmacol* **56**, 235–242 (1999).

12. A. Christopoulos, *et al.*, Novel Receptor Partners and Function of Receptor Activity-modifying Proteins*. *Journal of Biological Chemistry* **278**, 3293–3297 (2003).

13. C. Weston, *et al.*, Modulation of Glucagon Receptor Pharmacology by Receptor Activity-modifying Protein-2 (RAMP2). *Journal of Biological Chemistry* **290**, 23009–23022 (2015).

14. S. Arrigoni, *et al.*, A selective role for receptor activity-modifying proteins in subchronic action of the amylin selective receptor agonist NN1213 compared with salmon calcitonin on body weight and food intake in male mice. *European Journal of Neuroscience* **54**, 4863–4876 (2021).

15. R. Dackor, K. Fritz-Six, O. Smithies, K. Caron, Receptor Activity-modifying Proteins 2 and 3 Have Distinct Physiological Functions from Embryogenesis to Old Age*. *Journal of Biological Chemistry* **282**, 18094–18099 (2007).

16. M. A. Malcharek, A. Pearce, C. A. Brighton, D. C. Hornigold, G. Ladds, The role of receptor activity-modifying proteins in obesity and diabetes mellitus. *J Mol Endocrinol* **73**, e240056 (2024).

17. M. Harris, *et al.*, RAMPs regulate signalling bias and internalisation of the GIPR. *bioRxiv* 2021.04.08.436756 (2021). https://doi.org/10.1101/2021.04.08.436756.

18. E. Lorenzen, *et al.*, “Multiplexed analysis of the secretin-like GPCR-RAMP interactome” (2019).

19. L. Shao, *et al.*, Modulating effects of RAMPs on signaling profiles of the glucagon receptor family. *Acta Pharm Sin B* **12**, 637–650 (2022).

20. S. Bailey, *et al.*, Interactions between RAMP2 and CRF receptors: The effect of receptor subtypes, splice variants and cell context. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1861**, 997–1003 (2019).

21. Y.-L. Liang, *et al.*, Cryo-EM structure of the active, Gs-protein complexed, human CGRP receptor. *Nature* **561**, 492–497 (2018).

22. M. Marti-Solano, *et al.*, Combinatorial expression of GPCR isoforms affects signalling and drug responses. *Nature* **587**, 650–656 (2020).

23. C. Koole, *et al.*, Allosteric Ligands of the Glucagon-Like Peptide 1 Receptor (GLP-1R) Differentially Modulate Endogenous and Exogenous Peptide Responses in a Pathway-Selective Manner: Implications for Drug Screening. *Mol Pharmacol* **78**, 456–465 (2010).

24. J. M. Bomberger, N. Parameswaran, C. S. Hall, N. Aiyar, W. S. Spielman, Novel Function for Receptor Activity-modifying Proteins (RAMPs) in Post-endocytic Receptor Trafficking. *Journal of Biological Chemistry* **280**, 9297–9307 (2005).

25. J. M. Bomberger, W. S. Spielman, C. S. Hall, E. J. Weinman, N. Parameswaran, Receptor Activity-modifying Protein (RAMP) Isoform-specific Regulation of Adrenomedullin Receptor Trafficking by NHERF-1. *Journal of Biological Chemistry* **280**, 23926–23935 (2005).

26. D. I. Mackie, *et al.*, RAMP3 determines rapid recycling of atypical chemokine receptor-3 for guided angiogenesis. *Proceedings of the National Academy of Sciences* **116**, 24093–24099 (2019).

27. S. M. McNeill, *et al.*, The role of G protein-coupled receptor kinases in GLP-1R β-arrestin recruitment and internalisation. *Biochem Pharmacol* **222**, 116119 (2024).

28. R. H. J. Olsen, *et al.*, TRUPATH, an open-source biosensor platform for interrogating the GPCR transducerome. *Nat Chem Biol* **16**, 841–849 (2020).

29. D. Safitri, *et al.*, Cancer-Associated Mutations Enhance The Sensitivity Of The Trupath GαQ/11 System. *bioRxiv* 2022.09.01.506210 (2022). https://doi.org/10.1101/2022.09.01.506210.

30. N. Sonoda, *et al.*, "Arrestin-1 mediates glucagon-like peptide-1 signaling to insulin secretion in cultured pancreatic cells" (2008).

31. B. Jones, *et al.*, Targeting GLP-1 receptor trafficking to improve agonist efficacy. *Nat Commun* **9** (2018).

32. J. Naylor, *et al.*, Use of CRISPR/Cas9-engineered INS-1 pancreatic β cells to define the pharmacology of dual GIPR/GLP-1R agonists. *Biochemical Journal* **473**, 2881–2891 (2016).

33. P. E. Light, J. E. Manning Fox, M. J. Riedel, M. B. Wheeler, Glucagon-Like Peptide-1 Inhibits Pancreatic ATP-Sensitive Potassium Channels via a Protein Kinase A- and ADP-Dependent Mechanism. *Molecular Endocrinology* **16**, 2135–2144 (2002).

34. M. Shigeto, *et al.*, GLP-1 stimulates insulin secretion by PKC-dependent TRPM4 and TRPM5 activation. *Journal of Clinical Investigation* **125**, 4714–4728 (2015).

35. Y. Kashima, *et al.*, Critical Role of cAMP-GEFII·Rim2 Complex in Incretin-potentiated Insulin Secretion*. *Journal of Biological Chemistry* **276**, 46046–46053 (2001).

36. E. M. Pfeil, *et al.*, Heterotrimeric G Protein Subunit Gαq Is a Master Switch for Gβγ-Mediated Calcium Mobilization by Gi-Coupled GPCRs. *Mol Cell* **80**, 940-954.e6 (2020).

37. P. S. Miller, *et al.*, Non-peptidic antagonists of the CGRP receptor, BIBN4096BS and MK-0974, interact with the calcitonin receptor-like receptor via methionine-42 and RAMP1 via tryptophan-74. *Biochem Biophys Res Commun* **391**, 437–442 (2010).

Figures

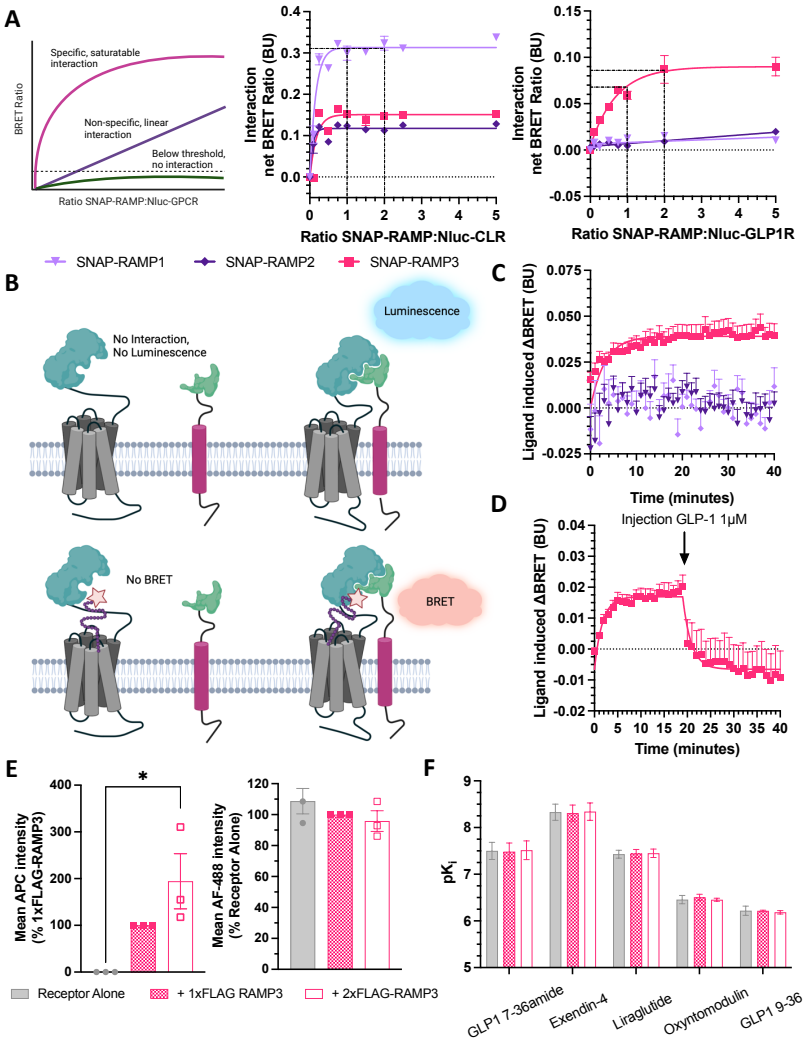

Figure 6 GLP-1R and RAMP3 interact and can bind agonists as a complex. (A) Cell-surface NanoBRET assay measuring interactions between Nluc-CLR (left) and Nluc-GLP-1R (right) with SNAP-RAMPs labelled with SNAP-Surface Alexa Fluor 488 in Cos7 cells. (B) Schematic of a NanoBiT assay for measuring GLP-1R-RAMP interactions and fluorescent ligand binding to the complex. (C) Binding of Ex-4-Red to the LgBiT-GLP-1R SmBiT-RAMP3 complex in HEK293T cells, which can then be displaced by addition of 1μM unlabelled GLP-1 (D). Expression of FLAG-RAMP3 (E) or Nluc-GLP-1R (F) in HEK293T cells transfected with Nluc-GLP-1R and increasing amounts of FLAG-RAMP3. (F) Binding affinities (pK_i) values for GLP-1, exendin-4, liraglutide, oxyntomodulin, and GLP-1 9-36 at Nluc-GLP-1, co-expressed with FLAG-RAMP3. Mean values are shown ± SEM.

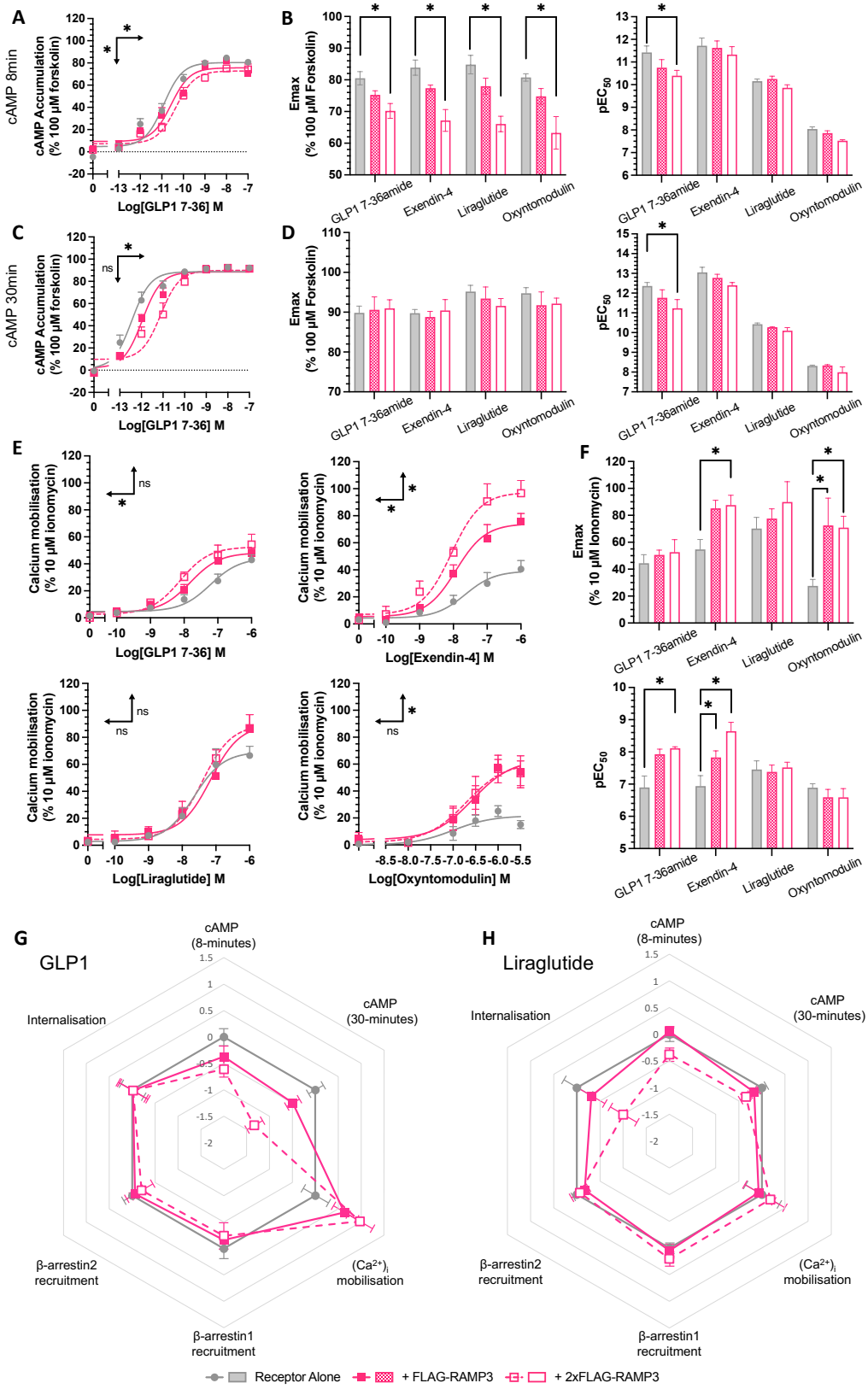
A
cAMP 8min
cAMP Accumulation (% 100 µM forskolin)
Log[GLP1 7-36] M
B
Emax (% 100 µM Forskolin)
pEC50
GLP1 7-36amide
Exendin-4
Liraglutide
Oxyntomodulin
C
cAMP 30min
D
E
Calcium mobilisation (% 10 µM ionomycin)
Log[Exendin-4] M
Log[Liraglutide] M
Log[Oxyntomodulin] M
F
Emax (% 10 µM Ionomycin)
G
GLP1
H
Liraglutide
cAMP (8-minutes)
cAMP (30-minutes)
(Ca2+)i mobilisation
β-arrestin1 recruitment
β-arrestin2 recruitment
Internalisation
Receptor Alone
+ FLAG-RAMP3
+ 2xFLAG-RAMP3

Figure 7 RAMP3 decreases cAMP accumulation but increases $(Ca^{2+})_i$ mobilisation. cAMP accumulation mediated by Nluc-GLP-1R, expressed with increasing concentrations of FLAG-RAMP3, in response to 8- minute stimulation with GLP-1 (A), with E_{max} and pEC_{50} values plotted for GLP-1, exendin-4, liraglutide, or oxyntomodulin (B). (C-D) As before but for 30-minute stimulation. (E) Mobilisation of $(Ca^{2+})_i$ by Nluc-GLP-1R with FLAG-RAMP3, in response to stimulation with GLP-1, exendin-4, liraglutide, or oxyntomodulin, with E_{max} and pEC50 values plotted (F). Log RA values for second messenger signalling and receptor desensitisation for GLP-1R, measured in HEK293T cells, in response to GLP-1 (G) or liraglutide (H).

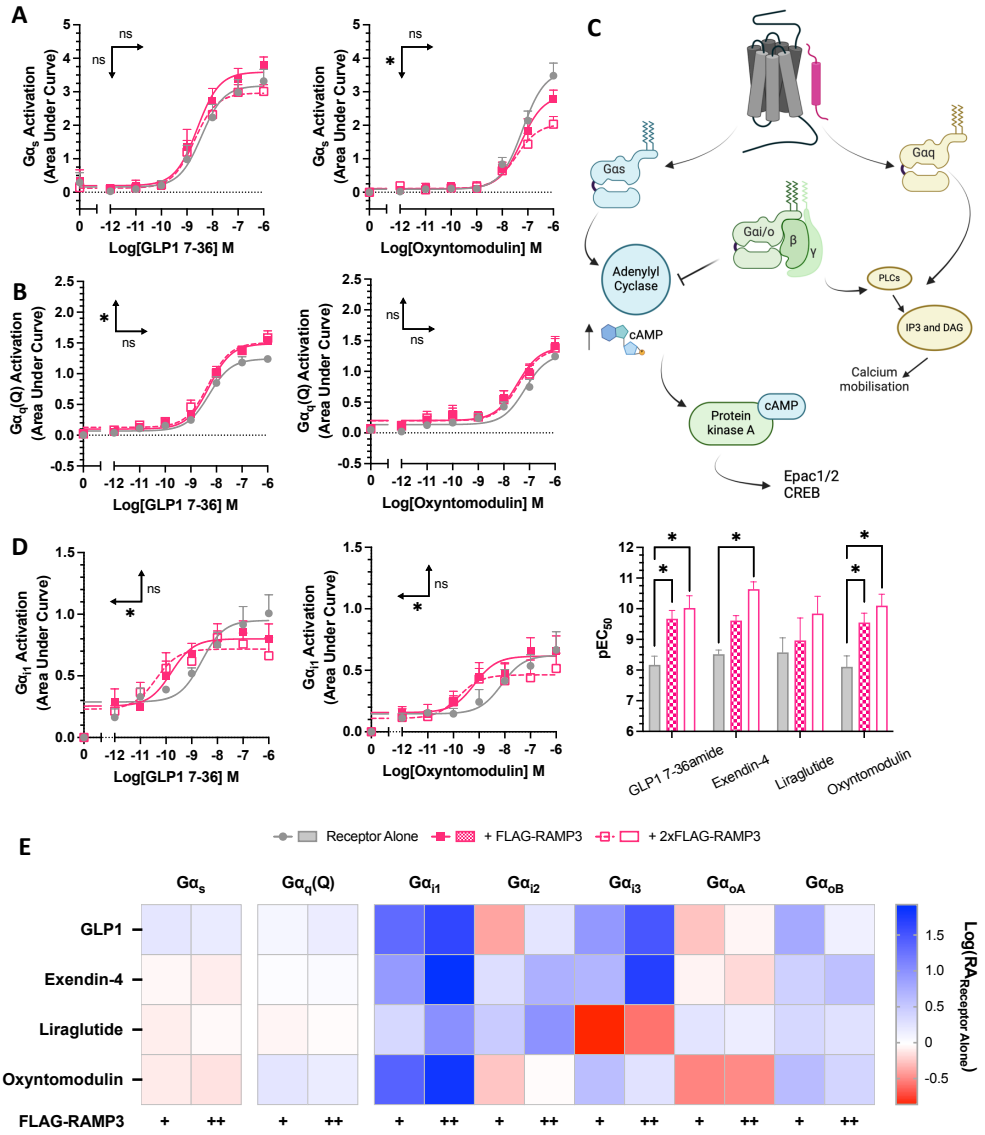

Figure 8 RAMP3 alters G protein coupling of GLP-1R differentially depending on the peptide agonist. (A) Schematic of the TRUPATH biosensor platform for G protein coupling, with the G protein families of interest depicted. (B-D) Measurement of G protein coupling using the TRUPATH biosensor platform, expressed in HEK293T cells alongside GLP-1R and increasing concentrations of FLAG-RAMP3. (E) LogRA values for G protein coupling, expressed relative to the response in the absence of FLAG-RAMP3.

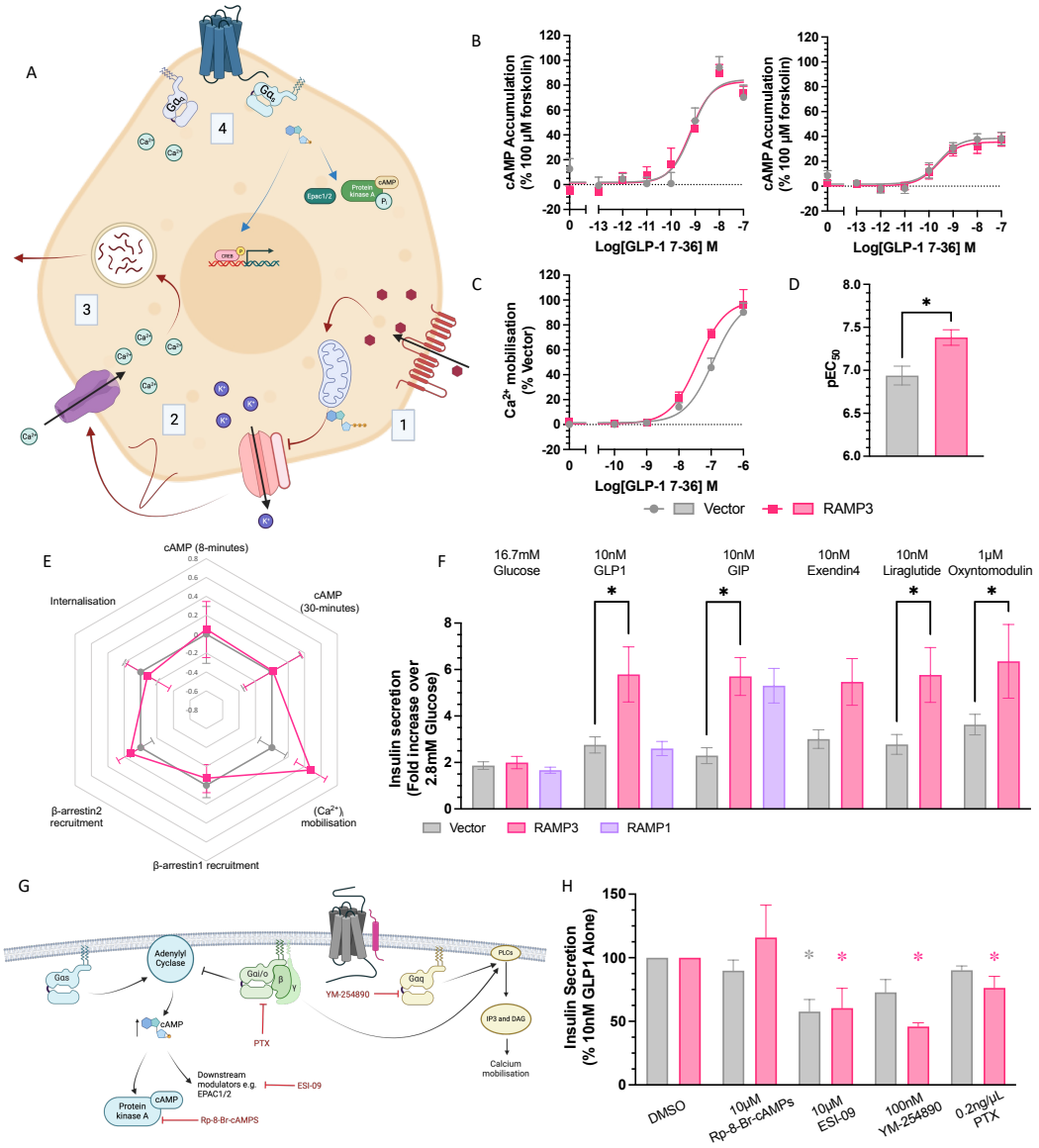

Figure 4 Increased $(Ca^{2+})_i$ mobilisation when RAMP3 is overexpressed increases GSIS in a pancreatic β-cell model. (A) Schematic for GPCR-mediated regulation of GSIS. 1. Glucose entry and metabolism leads to an increase in ATP synthesis. 2. ATP acts on K^+ channels, depolarising the membrane and opening voltage gated Ca^{2+} channels. 3. Increased $(Ca^{2+})_i$ leads to vesicle exocytosis. 4. GPCR activation increases $(Ca^{2+})_i$ and leads to signalling cascades that enhances the β-cell's sensitivity to changes in glucose, increasing insulin secretion. (B) cAMP accumulation in INS-1 832/3 cells transfected with vector or RAMP3, stimulated with GLP-1 for 8 (left) or 30 (right) minutes. (C-D) $(Ca^{2+})_i$ mobilisation in INS-1 cells transfected with vector or RAMP3, stimulated with GLP-1. (E) LogRA values for signalling and desensitisation of GLP-1R expressed in INS-1 832/3 cells, stimulated with GLP-1, in cells transfected with vector or RAMP3. (F) GSIS in INS-832/3 cells, transfected with vector, RAMP3, or RAMP1 and stimulated with a range of peptide agonists. (G) Schematic illustrating the action of different small molecule inhibitors of G protein signalling, and their effects on insulin secretion in INS-1 832/3 cells transfected with vector or RAMP3. (H) Insulin secretion in INS-1 832/3 cells transfected with vector or RAMP3 and stimulated with 10nM GLP-1. Cells were pretreated and co-stimulated with inhibitors of G protein signalling. * $p < 0.05$.