Modulation of Glucagon Receptor Pharmacology by RAMP2

Cathryn Weston[1*], Jing Lu[2], Naichang Li[2], Kerry Barkan[1], Gareth O. Richards[3], David J. Roberts[3], Timothy M. Skerry[3], David Poyner[4], Meenakshi Pardamwar[5], Christopher A. Reynolds[5*]Simon J. Dowell[6] Gary B. Willars[2] and Graham Ladds[1,7*]

[1] Division of Biomedical Cell Biology, Warwick Medical School, University of Warwick, Coventry, CV4 7AL, UK.
[2] Department of Cell Physiology and Pharmacology, University of Leicester, Leicester, LE1 9HN, UK.
[3] The Mellanby Centre for Bone Research, Department of Human Metabolism, University of Sheffield, Sheffield, S10 2RX, UK.
[4] School of Life Sciences, Aston University, Aston Triangle, Birmingham, UK, B4 7ET.
[5] School of Biological Sciences, University of Essex, Wivenhoe Park, Colchester CO4 3SQ, UK.
[6] Department of Biological Sciences, Molecular Discovery Research, GlaxoSmithKline, Hertfordshire, SG1 2NY, UK.
[7] Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1PD, UK.

[*]Running Title: RAMP2 engenders signal bias at the glucagon receptor

To whom correspondence should be addressed: Cathryn Weston, Division of Biomedical Cell Biology, Warwick Medical School, University of Warwick, Coventry CV4 7AL, UK. Tel: +44 (0)2476 524203, E-mail: caw38@leicester.ac.uk or grl30@cam.ac.uk.

Keywords: G protein-coupled receptors (GPCRs); glucagon receptor (GCGR); glucagon, glucagon-like petide-1; pharmacology; type 2 diabetes; signal bias; receptor activity modifying proteins (RAMPs); signal transduction

Background: The glucagon and glucagon-like peptide-1 (GLP-1) receptors are important targets for treating type 2 diabetes.
Results: We describe novel glucagon receptor pharmacology, through interaction with the receptor activity modifying protein-2 (RAMP2).
Conclusion: RAMP2 regulates both ligand binding and G protein selectivity of the glucagon receptor.
Significance: The effect of RAMP2 should be considered when designing anti-diabetic treatments.

ABSTRACT

The glucagon and glucagon-like peptide-1 (GLP-1) receptors play important, opposing roles in regulating blood glucose levels. Consequently, these receptors have been identified as targets for novel diabetes treatments. However, drugs acting at the GLP-1 receptor, whilst having clinical efficacy, have been associated with severe adverse side-effects and targeting of the glucagon receptor has yet to be successful. Here we use a combination of yeast reporter assays and mammalian systems, to provide a more complete understanding of glucagon receptor signaling considering the effect of multiple ligands, association with the receptor-interacting protein, receptor activity modifying protein-2 (RAMP2) and individual G protein α-subunits. We demonstrate that RAMP2 alters both ligand selectivity and G protein preference of the glucagon receptor. Importantly, we also uncover novel cross-reactivity of therapeutically used GLP-1 receptor ligands at the glucagon receptor that is abolished by RAMP2 interaction. This study reveals the glucagon receptor as a previously unidentified target for GLP-1 receptor agonists and highlights a role for RAMP2 in regulating its pharmacology. Such previously unrecognized functions of RAMPs highlight the need to consider all receptor-interacting proteins in future drug development.

INTRODUCTION

Glucagon, released from pancreatic α-cells is generally considered as a counter-regulatory hormone to insulin (1). It acts via the glucagon receptor (GCGR), a G protein-coupled receptor (GPCR), to stimulate the release of glucose from the liver into the blood (2). High blood glucose levels, caused by an imbalance in plasma levels of

insulin and glucagon, are characteristic of type 2 diabetes. The stimulation of insulin release, via activation of the glucagon-like-peptide-1 (GLP-1) receptor, (a closely related GPCR to the GCGR) has been the focus of intense research resulting in the approval of several drugs (3). Due to the role excess GCGR activity may play in the development of hyperglycaemia, compounds acting at this receptor (to antagonize signaling) are also highly sort after (4). However, despite several years of research with many patents for small molecule compounds filed (5), there are currently no successful drugs approved for clinical use.

The GCGR mediates its effects predominantly through the generation of intracellular cAMP via coupling to heterotrimeric G proteins containing Gαs (6). However, the GCGR can adopt multiple active conformations thereby regulating other downstream pathways, including members of the inhibitory Gαi family (7), which antagonize cAMP production. Distinct cellular outcomes can therefore be driven, through the stabilisation of different receptor conformations (8) by interacting molecules (including ligands, G proteins and accessory proteins). Understanding how each of the interactions influences GCGR signaling could lead to the development of ligands that selectively engage therapeutically beneficial pathways, thereby producing more efficacious drugs with greater specificity and fewer side-effects. To this end, we sort to investigate the effect of association with a class of interacting proteins, the receptor-activity modifying proteins (RAMPs), on GCGR pharmacology. RAMPs have previously been shown to modulate the ligand preference for other family B GPCRs (9,10). However, no comprehensive study of their role on ligand and G protein selection has thus far been undertaken.

The three RAMPs (RAMP1, RAMP2 and RAMP3) were first identified as being essential components of the receptors for calcitonin gene-related peptide (CGRP) and adrenomedullin (AM) (11). In addition to this well-characterized interaction with the calcitonin-like-related receptor (CLR)[1] where they are required to facilitate trafficking to the cell surface, RAMPs also associate with other family B GPCRs including the calcitonin receptor to modulate ligand and G protein selection (12, 13) the vasoactive intestinal polypeptide / pituitary AC-activating peptide 1 (VPAC1) receptor and the GCGR (9, 10).

Although the GCGR interacts with RAMP2 (9) the impact of this association on signaling and physiology has not been determined. Here we report that RAMP2 association significantly alters the pharmacology of all GCGR ligands. This modulation is dependent upon the activating ligand and the downstream G protein pathway. Furthermore we show that ligands of the GLP-1 receptor are able to act as agonists at the GCGR but that this is abolished by RAMP2 interaction. These results demonstrate the complex interplay between the ligand, the GCGR and the RAMP that alters the signaling bias of the receptor. Importantly, the study highlights the GCGR as a novel and potentially significant target for drugs designed to provide agonism at the GLP-1 receptor, which may now be tractable if the role of RAMP2 is considered. The outcome of our work could therefore impact upon new or improved drugs to treat type 2 diabetes.

EXPERIMENTAL PROCEDURES

Peptides - Glucagon, oxytomodulin and GLP-1 (7-36) amide were synthesized by Alta Biosciences (University of Birmingham, Birmingham, UK) and prepared as 1 mM stocks in water. Liraglutide, Exenatide and Lixisenatide were supplied by George Eliot Hospital NHS Trust (Nuneaton, UK). CGRP and AM were purchased from BACHEM (Bubendorf, Switzerland) and made to 1 mM stocks in water. The radioligand, [^{125}I]glucagon was purchased from PerkinElmer (Life and Analytical Sciences, Waltham, MA). The GCGR antagonist, des-His1-[Glu9]-glucagon (1-29) amide was purchased from Tocris (Bristol, UK) and prepared as a 0.1 mM stock in water. Yeast nitrogen base and yeast extract were purchased from Difco (Franklin Lakes, NJ). Flurorescein-Di-β-D-glucopyranoside (FDGlu) was purchased from Invitrogen (Paisley, UK). All other reagents were purchased from Sigma-Aldrich (St Louis, MO).

Constructs and DNA manipulation - The plasmid for the generation of direct, in-frame mCherry fusion proteins was kindly donated by Steve Royale (University of Warwick). cDNA constructs of the human GLP-1R (containing an N-terminal myc-tag) and the human glucagon receptor were donated by Professor Patrick Sexton (Monash University, Australia) and Dr Run Yu (Geffen School of Medicine at UCLA, Los Angeles, CA) respectively. Constructs for the expression of N-terminally FLAG-tagged human RAMPs were a described previously (10). The cDNA construct

containing a myc-tagged CLR was provided by Dr Michel Bouvier (University of Montreal, Canada). RAMP-GFP constructs were purchased from Cambridge Bioscience (Cambridge Bioscience Ltd, Cambridge, UK). DNA manipulations were performed using standard methods. Oligonucleotides were supplied by Invitrogen. PCR amplification used FastStart Taq polymerase (Roche Diagnostics, Burgess Hill, UK). All constructs were sequenced by GATC (GATC Biotech, London, UK) prior to use.

Cell culture and transfections - Dulbecco's modified Eagles's medium (DMEM) (Invitrogen) supplemented with 10% fetal calf serum and 2 mM L-glutamine was used to culture human embryonic kidney (293T) (HEK-293) cells provided by Dr Jügen Müller (University of Warwick) in a humidified 5% CO_2-95% air incubator at 37^{o}C. Cells were transfected with Fugene 6 (Roche) in accordance with the manufacture's instructions using a 1:3 (w:v) DNA:Fugene ratio. Transfected cell lines were grown for 48 h prior to assaying. Where appropriate, PTX (200 ng/ml) was added to ADP-ribosylate Gαi for 16 h prior to assaying, thereby uncoupling receptor-mediated $G\alpha_i$-dependent inhibition of cAMP production.

Analysis of cell-surface expression by ELISA - Cells were seeded into 24-well plates coated with poly-D-lysine and transiently transfected with receptors, RAMPs and vector controls as appropriate. Following 48 h growth in supplemented DMEM, media was replaced with 3.7% formaldehyde for 15 min. Cells were washed 3 times with 500 µl phosphate-buffered saline (PBS) and incubated with 1 % BSA in PBS for 45 min to prevent nonspecific antibody binding. To determine receptor expression, 250 µl of primary antibody (mouse anti-myc, (Fisher Scientific, Loughborough, UK)), diluted 1:2500 in 1 % BSA PBS) was added for 1 h. RAMP expression was similarly assessed using an anti-FLAG M2 primary antibody (mouse, (Sigma-Aldrich) diluted 1:3500 in PBS with 1% BSA). Cells were washed 3 times with 500 µl PBS and re-blocked (500 µl PBS + 1 % BSA) for 15 min before the addition of the secondary antibody (HRP-conjugated anti-mouse IgG (GE-Healthcare, Pittsburg, PA)) diluted 1:2500 in 1 % BSA PBS for 1 h. Following 3 further washes with PBS HRP, activity was determined using SigmaFast O-phenylenediamine tablets (Sigma-Aldrich) according to the manufacturer's instructions. Values were normalised to myc-GLP-1R (for receptor expression) or FLAG-RAMP2/CRLR (for RAMP expression) as 100% and cells transfected with empty vector as 0%.

cAMP accumulation assays - Transfected cells were washed in PBS, resuspended in stimulation buffer (PBS containing 0.1% BSA and 0.5 mM IBMX) and seeded at 2000 cells per well in 96-well white Optiplates. Ligands were added in the range of 1 pM to 1mM and cAMP accumulation was measured after 30 min stimulation using LANCE® cAMP Detection Kit (PerkinElmer). Values were converted to concentration using a cAMP standard curve performed in parallel.

Competition-binding assays - Homologous and heterologous competition-binding assays were performed on whole cells transfected with GCGR in the absence and presence of RAMP2 using [^{125}I]glucagon as the radioligand. Cells were seeded into 6-well plates and grown to 80% confluency before transfection with 1.5 µg plasmid DNA and incubated for a further 24 hours. Media was then replaced with fresh DMEM, cells transferred onto a 96-well plate coated with poly-D-lysine and incubated for 24 h to achieve confluence prior to assay. Binding was performed using an adaptation of the method outlined in (14) in a final volume of 180 µl with all components diluted in assay buffer [(Krebs'-HEPES buffer plus BSA, KHB); 10 mM HEPES, 4.2 mM $NaHCO_3$, 11.7 mM D-glucose, 1.18 mM $MgSO4{\cdot}7H_2O$, 1.18 mM KH_2PO_4, 4.69 mM KCl, 118 mM NaCl, and 1.3 mM $CaCl_2{\cdot}2H_2O$ 0.1% BSA (w:v)]. For the assay, cells were washed in assay buffer, 0.1 nM of radioligand and various concentrations of the peptides were added and the plate incubated for 16 h at 4^{o}C. Following washing with ice-cold assay buffer, 100 µl NaOH (0.1M) was added to each well and the plate incubated for 5 min on ice. Cells were removed and the plate washed with 100 µl HCl (0.1M). Radioactivity was determined in 2 ml Safefluor scintillant (PerkinElmer) using a liquid scintillation counter with a count time of 3 min per sample. Values were corrected for non-specific binding (NSB) as determined by the amount of radioligand detected bound to cells not expressing GCGR.

Live cell imaging in HEK-293 cells - Transfected cells were seeded into 8-well microscope slides (Thistle Scientific, Galsgow, UK) and incubated (37^{o}C, 5% CO_2-95% air) for 25 h. Prior to imaging, growth media was replaced with a Hepes buffer as detailed in (15) pre-warmed to 37^{o}C. Cells were viewed on a Personal

DeltaVision system (Applied Precision, Issaquah, WA) equipped with a Photometric CoolSNAP HQ camera (Roper Scientific) and deconvolution was applied to images for visual clarity as described previously (16).

Yeast strain construction and assay - General yeast procedures were performed as described previously (17, 18). *S. cerevisiae* dual reporter strains expressing chimeras of the yeast GPA1, 1-467, (GPA1/Gα) with the five C-terminal amino acids of human Gα protein corresponding to Gαs or Gαi3 (MMY84 and MMY89 respectively, (19)) were used in this study since we have previously shown that both the GLP-1 receptor and GCGR couple to these subunits (17)). Mammalian GPCRs and RAMPs were introduced to the yeast strains under the control of the GAPDH promoter using plasmids containing either *ura3* (p426-GPD) or *leu2* (p425-GPD). Plasmids were transformed in a 1:1 ratio to enable equal expression levels of both the RAMP and receptors. Transformation was achieved using the lithium acetate/single-stranded DNA/polyethylene glycol method as previously described (20). Positive transformants were selected and maintained on media lacking uracil and leucine. Receptor signalling was measured using the yeast growth assay (21) adapted as described in (17). Initially cell growth was performed in SD-URA-LEU media at 30^{o}C to select for only those expressing both plasmids. Cells were then cultured to remove basal activity in SD-URA-LEU-HIS media overnight at 30^{o}C and assayed using FDGlu supplemented media in the presence of different concentrations of ligand (0.01 nM-100 mM). Fluoresence was measured on a TECAN Infinite M200 microplate reader (TECAN Ultra Evolution, Reading, UK).

Scintillation proximity assay (SPA) for G protein activation **-** Receptor/G-protein activation profiles were determined using a scintillation proximity assay as described previously (22). Cells were grown to confluence in DMEM with GlutaMAX, supplemented with 10% FCS and 1x penicillin/streptomycin, in 5% CO_2-95% air at 37 ^{o}C. The cells were harvested using trypsin/EDTA (Sigma-Aldrich), washed with PBS, and resuspended in electroporation buffer (20 mM HEPES, 135 mM KCl, 2 mM $MgCl_2$, 2 mM ATP, 5 mM glutathione, 0.5% (v:v) Ficoll 400 adjusted to pH 7.6 using KOH) at a concentration of ~4 million cells in 4mm gap electroporation cuvettes (York Biosciences, UK) before addition of the required DNA (5 μg receptor, 15 μg RAMP constructs). The cells were then electroporated at 0.25 kV and 960 μF using a Gene Pulser (Biorad, Hemel Hempstead, UK) and then cultured for 48 h. The cells were then homogenized in ice cold PBS using a Dounce homogenizer and centrifuged at 300 g for 10 min at 4^{o}C in a final volume of 40 ml. The supernatant was collected in a fresh tube and centrifuged at 50,000 g for 25 min at 4^{o}C. The resulting pellet was resuspended in ice-cold SPA buffer (50 mM HEPES, 100 mM NaCl, 5 mM $MgCl_2$, 0.5% (w:v) BSA, pH 7.4).

SPA protocol - Concentration-response curves were constructed by incubating increasing agonist concentrations with membranes (10μg) prepared from cells transfected with receptor and 0.1 μM GDP in HEPES buffer (100 mM NaCl, 50 mM HEPES, 5 mM $MgCl_2$, 0.5% (w:v) BSA Fraction V, adjusted to pH 7.5 with KOH) in a total volume of 200 μl in white Optiplates (PerkinElmer). The assay was initiated by addition of 0.5 nM [^{35}S]GTPγS and incubated for 1 h at 35^{o}C. The assay was terminated by addition of Nonidet P40 at a final concentration of 0.3% (v:v) (Roche) and incubated at room temperature for 30 min on a plate shaker. A 10 μl aliquot of anti-G-protein antibody (Gs sc-383, Gi sc-262) at a concentration of 60 μg/μl, was then added, followed by a further 30 min at room temperature before addition of 75 μl anti-rabbit PVT SPA beads (PerkinElmer). The plate was then sealed, incubated at 4^{o}C for 20 h, spun at 1300 g for 10 min before reading in a TopCount scintillation counter (PerkinElmer). Data are shown as mean ± S.E.M for 3 independent experiments, each performed in triplicate.

Data analysis – Data were analysed using Prism 6.0e (Graphpad Software, San Diego, CA). EC_{50} and E_{max} values were obtained through fitting of a three-parameter logistic equation. Relative efficacy (log τ) and equilibrium dissociation constants (log K_A) were generated through use of an operational model for partial agonism (23). Fluorescent image analysis was performed using ImageJ 1.46b using the QUIMP plugin as previously described (17, 24). Statistical differences were analysed before data were normalised, using a Student's test or one-way ANOVA with Bonferroni's or Dunnett's multiple comparisons as appropriate and a probability (p) < 0.05 was considered significant.

RESULTS

RAMP2 increases glucagon potency and efficacy at the GCGR - RAMP proteins require association with a GPCR, such as the CLR, in the endoplasmic reticulum to enable efficient translocation to the cell surface. Using an enzyme-linked immunosorbent assay (ELISA), little or no cell surface expression of the RAMPs was seen when transfected alone into HEK-293 cells. However, all three FLAG-tagged RAMPs were detected at the cell surface, upon co-transfection with the CLR (Fig. 1A). In contrast, the GLP-1 receptor was unable to translocate any of the RAMPs to the cell surface. Upon co-transfection of each of the RAMPs with the GCGR, only RAMP2 resulted in detectable cell surface expression (Fig. 1). Similar data were obtained using RAMP-GFP fusion constructs such that co-expression of the CLR was required to enable observation of plasma membrane-associated RAMPs and the GCGR located only RAMP2 at the plasma membrane (Fig. 1B) demonstrating a positive interaction between the GCGR and RAMP2 in mammalian cells.

Like other family B GPCRs, upon ligand binding, the GCGR preferentially activates Gαs to stimulate adenylate cyclase and generate cAMP. To investigate the effect of RAMP2 association on GCGR pharmacology we constructed cAMP concentration-response curves to glucagon in HEK-293 cells transiently co-transfected with the GCGR and each of the individual RAMPs. Co-expression of RAMP2 induced a 2-fold increase in the maximal cAMP level (E_{max}) and a 10-fold increase in the potency (EC_{50}) of glucagon compared to the vector control (Fig. 1C, Table 1). In contrast, co-expression of either RAMP1 or RAMP3 did not influence the E_{max} or EC_{50} of glucagon-mediated cAMP generation (Fig. 1B). In order to assess if agonist responses of the GCGR were influenced by endogenously expressed RAMPs in HEK-293 cells, we utilized the absolute requirement of the CLR receptor to interact with a RAMP to produce a functional receptor at the plasma membrane (11). In the absence of co-transfected RAMPs, we were unable to detect the CLR at the cell surface by ELISA using a myc-tag (Fig. 1D). Furthermore, cAMP accumulation was not observed following stimulation with either CGRP or AM (Fig. 1E). These data indicate that there is insufficient endogenous RAMP expression in HEK-293 cells to interact with the CLR, so effectively providing a null background in which to study the effect of RAMP2 association with the GCGR.

Changes in GCGR pharmacology are not due to an increase in cell surface expression or ligand binding affinity - The cell surface expression and ligand binding properties of several family B receptors (10, 11) are enhanced through association with RAMPs. We used both quantitative image analysis of C-terminal receptor-fluorescent protein fusion constructs, (Fig. 2A) and cell surface ELISAs of myc-tagged receptors (Fig. 2B) to determine the effect of RAMP2 on receptor trafficking. In contrast to the CLR, which was only observed (Fig. 2A, arrows) or detected (Fig. 2B) at the plasma membrane when co-transfected with a RAMP, no change in the level of GCGR at the cell surface could be determined. Increases in the amount of RAMP2 relative to GCGR transfected into HEK-293 cells resulted in an elevated level of FLAG-tagged RAMP2 at the plasma membrane but did not enhance the level of myc-GCGR detected (Fig. 2C), although a RAMP2-dependent increase in both potency and E_{max} was observed as measured through glucagon-stimulated cAMP (Fig. 2D). Further, a homologous competition-binding assay on cells expressing the GCGR in the presence and absence of RAMP2 revealed no change in receptor affinity for glucagon (Fig. 2E). These data suggest that the elevated cAMP production and increased glucagon potency observed upon association of the GCGR with RAMP2 (Fig. 1B) do not arise due to changes in ligand-binding affinities or enhanced cell surface expression.

Modulation of GCGR pharmacology by RAMP2 is G protein-dependent - Intracellular levels of cAMP can be regulated by GPCRs both positively (most notably via activation of Gαs) and negatively (via stimulation of Gαi). The GCGR couples to both of these competing G protein subunits in mammalian cells (25). Further, expression of the GCGR in a yeast system containing chimeric (yeast-human) G proteins that allows the isolation of individual GPCR-G protein interactions, has revealed functional coupling of the receptor to both Gαs and Gαi subunits (17). The chimeric G proteins in this *S. cerevisiae* system allow the coupling of human GPCRs to the endogenous yeast-mating pathway. This pathway has been modified to include a growth reporter and thereby provides the opportunity to analyze the proportion of a receptor's response attributable to individual pathways (18, 26, 27) by assessing the

extent of reporter activity following stimulation with a range of ligands. Co-expression of RAMP2 (but not RAMP1) with the GCGR increased the potency and maximal response to glucagon when the receptor coupled to the chimeric protein of the yeast α-subunit and mammalian Gαs (GPA1/Gαs) (Fig. 3A). However, a reduction in response was observed in strains expressing GPA1/Gαi (Fig. 3B; Table 2). Using a scintillation proximity assay (SPA), we determined the activation of specific G proteins in transfected mammalian cells (Fig. 3C and D).. RAMP2 co-expression resulted in no change in glucagon-stimulated Gαs activation; however, the maximal level of Gαi activation achieved was reduced 2-fold (Table 2). These results demonstrate that RAMP2 expression in mammalian cells results in a similar change to the GCGR-mediated activation of individual G proteins to that seen in the yeast assay Taken together, these data demonstrate that the ability of RAMP2 to alter glucagon-mediated responses at the GCGR is G protein-dependent: a RAMP2 interaction with GCGR appears to specifically reduce Gαi activation.

Since Gαs and Gαi have opposing influence on adenylate cyclase activity in mammalian cells, these observations could explain the RAMP2-dependent elevation in glucagon-induced cAMP generation in HEK-293 cells (Fig. 2B). RAMP2 may selectively reduce coupling of the GCGR to Gαi thereby leaving activation of Gαs un-opposed. To assess this, we pre-treated HEK-293 cells with pertussis toxin (PTX) to prevent receptor-mediated Gαi activation (28). In cells treated with PTX, the potency and E_{max} of glucagon-stimulated cAMP generation by the GCGR were increased similar to those in cells co-expressing RAMP2 (but not PTX treated) (Fig. 3E). PTX treatment had no effect on the potency or maximal response in cells co-expressing the GCGR and RAMP2 (Fig. 3E, Table 1). These data suggest that RAMP2 uncouples the GCGR from Gαi; this is entirely consistent with the data from the yeast cell and SPA assays.

RAMP2 modulates GCGR pharmacology in a ligand-dependent manner - To further investigate the effect of RAMP2 on GCGR pharmacology we next analysed changes in signaling in response to the related glucagon ligand, oxyntomodulin. In HEK-293 cells, challenge of the GCGR with oxyntomodulin resulted in a concentration-dependent increase in cAMP but with weaker potency than glucagon (Fig. 4A, Table 3). However, similar to the effects observed with glucagon, RAMP2 co-transfection increased both the potency and maximal cAMP production without affecting the binding affinity of oxyntomodulin (Fig. 4B). In *S. cerevisiae,* although the co-expression of RAMP2 potentiated the response to oxyntomodulin in the GPA1/Gαs yeast strain, it did not affect responses in the GPA1/Gαi strain (Fig. 4C-D). This is in contrast to the inhibitory impact of RAMP2 association on glucagon-mediated GCGR responses in the GPA1/Gαi expressing yeast. Furthermore, PTX treatment of HEK-293 cells expressing the GCGR had no effect on the potency of oxyntomodulin-mediated cAMP responses although there was a small increase in E_{max}. Intriguingly, this increase occurred independently of RAMP2 expression (Fig. 4A). In RAMP2 expressing cells, there was however, a small decrease in the potency of oxyntomodulin-induced cAMP production following incubation with PTX. These data indicate that the effect of RAMP2 on the GCGR Gαi response is sensitive to the activating ligand.

GLP-1 is a partial agonist at the GCGR in the absence of RAMP2 - Tissue-specific processing of pro-glucagon produces several peptides including oxyntomodulin, glucagon and GLP-1 (29) that, in its major post-prandial active form (GLP-1 7-36 amide, hereafter GLP-1), is considered to act in opposition to glucagon by stimulating insulin secretion. Whilst oxyntomodulin and glucagon can also stimulate the GLP-1 receptor (17, 29, 30), a limited number of studies have suggested that GLP-1 cannot bind to the GCGR (31, 32). Given that similarities between both ligands (Fig. 5A) and receptors (33) of this family are comparable or greater than those in other systems (e.g. the calcitonin or PTH families) that show common binding properties, we wished to determine if a reciprocal activation of the GCGR by GLP-1 was possible. Initially, we used yeast strains expressing the GCGR and either the GPA1/Gαs or GPA1/Gαi chimera, and we determined that GLP-1 was able to weakly activate the GCGR thereby acting as a partial agonist compared to glucagon (Fig. 5B-C, Table 4 and 5). The observed difference in potencies between GLP-1 and glucagon are unlikely to reflect the ability of each peptide to penetrate the yeast cell wall since alternative rank orders of activity have

been reported for these ligands at the GLP-1 receptor (17).

Due to its role in insulin secretion, the GLP-1 receptor has received significant attention as a possible therapeutic target for treating type 2 diabetes, resulting in the approval of three peptide analogues of GLP-1 (liraglutide, exenatide and lixisenatide) for clinical use. These mimetics have been designed to activate the GLP-1 receptor but also to resist proteolytic degradation, particularly by dipeptidyl peptidase IV, thereby considerably enhancing their plasma half-life beyond the 1-2 min of natural GLP-1. Significantly, amongst the clinically approved drugs tested here, only liraglutide (with 97% homology to GLP-1) stimulated a response in GCGR-expressing yeast strains (Fig. 5B-C, Table 4 and 5). Similar to GLP-1, liraglutide displayed partial agonism at the GCGR with a reduced potency compared to glucagon. Intriguingly, co-expression of RAMP2 prevented both peptides from activating the GCGR in the yeast strains (Fig. 5B-C). This effect was specific to the RAMP2-GCGR receptor interaction since co-expression of RAMP2 with the GLP-1 receptor had no effect on receptor pharmacology in either yeast or cAMP assays (Fig. 6).

RAMP2 expression prevents GLP-1 binding to the GCGR - Having observed a RAMP2-specific modulation of GLP-1-mediated activation of the GCGR in yeast cells, we next examined these interactions in mammalian cells. Both GLP-1 and liraglutide were partial agonists of cAMP generation compared to glucagon at the GCGR (Figs. 7A and B, Table 3). This agonism by the GLP-1 receptor ligands was abolished upon co-transfection of RAMP2. These effects were specific to a RAMP2-GCGR interaction: RAMP2 did not affect GLP-1-mediated cAMP generation by the GLP-1 receptor in HEK-293 cells (Fig. 6C). Further, the addition of a GCGR antagonist (des-His1-[Glu9]-glucagon (1-29) amide) inhibited GLP-1 and liraglutide signalling through the GCGR (Figs. 7A and B). Finally, ligand binding assays revealed that, contrary to previous reports, GLP-1 and the related mimetic, liraglutide bound to the GCGR in competition with glucagon. Significantly, both GLP-1 and liraglutide failed to bind to the GCGR upon RAMP2 co-transfection (Fig. 7C). Titration experiments, to increase the concentration of RAMP2 revealed that the GLP-1-mediated GCGR cAMP response is extremely sensitive to the presence of RAMP2 (Fig. 7D). These data demonstrate that RAMP2 specifically regulates binding and thereby signal transduction of GLP-1 ligands at the GCGR. Previous work on the ability of GLP-1 receptor ligands to activate the GCGR has not considered the influence of RAMPs, which may have obscured such an interaction.

DISCUSSION

RAMPs have long been known to modify the pharmacology of family B GPCRs: altering the efficacy and potency of ligands and changing their ligand preference entirely, thereby having the ability to create distinct receptors (11-13). Despite having been identified over 10 years ago (9), this study is the first to investigate the pharmacological consequences of the interaction between RAMP2 and the GCGR. Here we have shown, using a combination of both mammalian and yeast cell assays, that co-expression with RAMP2 causes a marked change in GCGR pharmacology through alterations in G protein-coupling that are also dependent upon the activating ligand.

One particular strength of our approach, using both the mammalian and yeast systems, is that we have identified subtle differences in signaling that may not have been apparent through the use of a single assay. For example, in HEK-293 cells, the impact of RAMP2 on cAMP responses to glucagon and oxyntomodulin were essentially the same, with a potentiation in cAMP accumulation in both cases (Figs. 3 and 4). However, in the yeast assay, in which the activation of individual (competing) G protein pathways can be assessed, there were clear ligand-dependent differences in the impact of RAMP2 on signaling by the GCGR. In the case of glucagon, RAMP2 potentiated the response mediated through the GPA1/Gαs chimera (Fig. 3A), but reduced activation through the GPA1/Gαi chimera (Fig. 3B). In contrast, RAMP2 modulation of the oxyntomodulin response only occurred through the GPA1/Gαs chimera (Fig. 4A-B).

In HEK-293 cells, co-expression of RAMP2 with the GCGR resulted in potentiation of both glucagon- and oxyntomodulin-stimulated cAMP. However, the GCGR couples to both stimulatory (Gαs) and inhibitory (Gαi) G proteins that combined regulate cAMP production following receptor stimulation (25). Through a variety of different techniques we demonstrated that the mechanisms by which the RAMP2 achieved this affect is both ligand- and G protein-specific. The

data suggest that when glucagon is used as the ligand, the presence of RAMP2 reduces the Gαi component. In contrast, the affect on the oxyntomodulin response occurs through the potentiation of the Gαs activity when measured in the yeast assay. This is broadly supported in the cAMP response; with an increase in E_{max} being observed following PTX treatment of HEK-293 cells. However, PTX appeared to reduce the affect of RAMP2 on the potency of oxyntomodulin. This possibly indicates the presence of another competing G protein-mediated pathway for example Gαq (7) which, through the promotion of protein kinase C activation has been reported to affect PTX-sensitivity of other Gαs/Gαi coupled family B GPCRs (34). The elevation of intracellular calcium by GCGR activation has been relatively well described and has been demonstrated to occur in transfected HEK-293 cells via both Gαi and Gαq-mediated pathways (7). It is somewhat surprising that little attention has been given to the ability of the GCGR to signal via Gαi and our data suggest that further work is required to fully elucidate the physiological role of the Gαi coupling.

The GCGR is one of a number of receptors that regulate glucose homeostasis within the body (6). Its primary role is to promote the release of glucose from the liver to maintain suitable levels of blood glucose. The opposing role is played by the related GLP-1 receptor that, through a number of actions but primarily through the induction of insulin release, promotes the lowering of blood glucose levels following food ingestion. We (17) and others (30-31) have previously reported the ability of the GCGR ligands to activate the GLP-1 receptor. Significant findings of the present study are the potential for GLP-1 receptor ligands to bind and stimulate the GCGR and the ability of RAMP2 to inhibit this. Both GLP-1 (7-36) amide, the major active post-prandial circulating form of GLP-1, and the synthetic, clinically used peptide liraglutide displayed partial agonism at the GCGR. Intriguingly, neither lixisenatide nor exenatide, both long-acting, clinically used GLP-1 mimetics, were able to stimulate the GCGR. Significantly, activation of the GCGR by the GLP-1 receptor ligands was abolished upon co-expression of RAMP2 and this was independent of the G protein present. These results suggest that unlike glucagon and oxyntomodulin, RAMP2 exerts its effects on GLP-1-mediated GCGR activation via preventing ligand binding. Given the differential expression of RAMP2 across mammalian cell lines these results offer a possible explanation for previous studies that have been unable to observe GLP-1 (and liraglutide) activity at the GCGR (31-32). Furthermore, given that RAMPs are expressed across many different tissues (35) including many where GCGR activity has also been reported (36-37) our results may also provide insight into physiological observations. For example RAMP2 expression in the heart has been shown to be significantly up-regulated in mouse models of heart failure (38). Given the observations by Ban *et al* that GLP-1 has a cardioprotective role acting via a receptor other than GLP-1R (39) and our results demonstrating that RAMP2 prevents GLP-1-GCGR activity it would be interesting to determine if RAMP2 interacts with the GCGR to alter signal transduction in cardiomyocytes and if this interaction is perhaps preventing the actions of GLP-1 leading to heart failure. However, no studies to date have simultaneously assessed the cellular content of both RAMP2 and GCGR.

Interestingly, the binding experiments presented in this study suggest that GLP-1 and liraglutide display a higher affinity for the GCGR than the native peptide. However, this did not translate into an increase in potency relative to glucagon when measured as cAMP production. It is possible that the high affinity binding of these ligands promotes the activation of alternative GCGR-mediated pathways with greater potency than that seen for cAMP but further investigation is required to determine if GLP-1 and liraglutide are truly biased ligands at the GCGR.

Indeed, other receptors for GLP-1R ligands have been suggested (39-40). In such reports, some effects of GLP-1 ligands have been observed in the absence of the GLP-1 receptor either through the use of specific antagonists or knockout animals. Given the results presented here, and the tissue distribution of GCGR expression (36-37) we suggest that some of these effects may be mediated via the GCGR. However, in some assay systems the ability of GLP-1 receptor ligands to cross-react with the GCGR may have been missed due the presence of RAMP2. These data highlight the need to consider GCGR and RAMP2 expression when designing novel therapies to prevent unwanted activity. Hybrid peptides that modulate both the GCGR and GLP-1 receptor in different ways have also been suggested as potentially beneficial (41).

Previously, it has been thought that such a compound would have to display agonist activity at the GLP-1 receptor whilst antagonising the GCGR. However, our data reveal the possibility of using an agonist to both receptors that is biased to different downstream pathways. For example, a ligand that stimulates the GLP-1 receptor-Gαs and GCGR-Gαi pathways would have a similar effect to an agonist-antagonist combination. Due to the observed ligand and RAMP-engendered signaling bias observed, our data offer new mechanistic information that could be useful in the development of such co-agonists.

Structure-activity relationship studies for the GLP-1 receptor have suggested various important residues required in both the receptor and ligand for binding and activation (42). Many of these reported residues, are also found within the TM regions of the GCGR and are required for interaction of the N-terminus of the ligand (which is relatively well-conserved across the ligands used in this study (Fig. 5A)). Given that RAMPs have been shown to modify the ligand-GPCR interaction for other receptors (12, 43) it is possible that the formation of a RAMP2-GCGR complex subtly changes the arrangement of key residues involved in GLP-1 binding, thereby preventing its interaction. It would therefore be interesting to observe how the presence of RAMP2 alters that of the recently published crystal structure of the GCGR (44). Initial homology models, built upon the assumption that GLP-1 binds in a similar mode to the GCGR extracellular domain as it does to the GLP-1 receptor suggest that the interaction of RAMP2 with GCGR may block GLP-1 binding. Loop 5 of the GCGR extracellular domain is shorter than its GLP-1R counterpart having an unusual type I' turn (45) that creates a more constrained environment for the GLP-1 C-terminus. If RAMP2 bound to the GCGR in a similar way to that of the CLR (46) then the additional steric constraints in the vicinity of the GLP-1 C-terminus may discourage binding. It should be noted that these conclusions are speculative and required further in depth analysis before definitive conclusion can be drawn. The two hydrophobic regions found within glucagon and oxyntomodulin required for binding to the extracellular domain of the receptor and forming interactions with the extracellular loop 1 (33) are partially conserved in the C-termini of both GLP-1 and liraglutide but not in exenatide and lixisenatide (boxed regions in Fig. 5A). These observations provide a possible explanation for the lack of activity at the GCGR displayed by the latter two ligands.

The idea that ligands can influence pathway selectivity of GPCRs, termed biased agonism, has been suggested for many years (8) and is supported by the observed structural links between the ligand-binding pocket, via TM domains, to the intracellular G protein binding site (47-48). Furthermore, it has been shown that different G protein subunits can influence the structure adopted by a GPCR thereby influencing ligand binding (10). Additionally, Udawela *et al* have demonstrated that the different domains of RAMP proteins enable them to influence both ligand and G protein selectivity of GPCRs (49). Studies using the calcitonin receptor have revealed that RAMP modulation of ligand potencies can be pathway specific. Here they show that RAMP expression has a greater affect on amylin potency when cAMP is accumulation is used as a measure of receptor activity compared to when calcium mobilisation is monitored demonstrating that RAMPs may have a direct effect on G-protein coupling efficiency (13). Combining these reports with our own observations suggests that there is a complex interplay between ligand, RAMP, receptor and G protein that combine to bring about signal transduction, with each component being able to affect the binding or activity of another. Although the GCGR is expressed in the liver, where its main biological function is to counterbalance insulin actions, it is also expressed in a vast array of other tissues (50) including the lung, pancreas and kidney. Interestingly, in mouse studies, varying levels of RAMP2 mRNA have been reported in these tissues (51). RAMP2 expression was higher in the pancreas, where GCGR stimulation enhances glucagon secretion (50), than in the liver where receptor activity stimulates glucose release (6). Understanding how the GCGR signals within each of these tissues will enable the design of biased drugs that specifically engage the most therapeutically beneficial pathways and reducing unwanted side effects. Our data, which reveal a role for RAMP2 in determining both ligand selectivity and the downstream signaling pathways of the GCGR highlight the critical need to consider all components present in target cells when designing new therapeutics.

ACKNOWLEDGMENTS
This work was supported by a Warwick Impact Fund (C.W., G.L.), the BBSRC (G.L. - BB/G01227X/1), (T.S., G.R., D.R. - BB/F008392/1), (D.P. - BB/M007529/1 and BB/M000176/1), Warwick Research Development Fund (C.W., G.L.) grant number (RD13301) and the Birmingham Science City Research Alliance (G.L). We would like to thank Vinod Patel, Paul O'Hare for provision of some reagents and Debbie Hay and Harriet Watkins useful discussions.

CONFLICT OF INTEREST STATEMENT
The authors declare they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS
CW, JL, NL, KB, GOR, DJR, MP, CAR performed experiments. CW, JL, NL, TMS, DP, CAR, SJD, GBW and GL analysed data. CW, TMS, CAR, GBW and GL designed experiments. CW and GL wrote the manuscript. All authors reviewed and approved the final manuscript.

REFERENCES
1. Habegger, K.M., Heppner, K.M., Geary, N., Bartness, T.J., DiMarchi, R. and Tschöp, M.H. (2010) The metabolic actions of glucagon revisited. *Nat Rev Endocrinol* **6**, 689-697.
2. Unger, R.H. (1985) Glucagon physiology and pathophysiology in the light of new advances. *Diabetologia* **28**, 574-578.
3. Scott, D.A., Boye, K.S., Timlin, L., Clark, J.F. and Best, J.H. (2013) A network meta-analysis to compare glycaemic control in patients with type 2 diabetes treated with exenatide once weekly or liraglutide once daily in comparison with insulin glargine, exenatide twice daily or placebo. *Diabetes Obes Metab* **15**, 213-223.
4. Bagger, J.I., Knop, F.K., Holst, J.J. and Vilsbøll, T. (2011) Glucagon antagonism as a potential therapeutic target in type 2 diabetes. *Diabetes Obes Metab* **13**, 965-971.
5. Shen, D.M., Lin, S. and Parmee, E.R. (2011) A survey of small molecule glucagon receptor antagonists from recent patents (2006-2010). *Expert Opin Ther Pat* 21(8): 1211-1240.
6. Ahrén, B. (2009) Islet G protein-coupled receptors as potential targets for treatment of type 2 diabetes. *Nat Rev Drug Discov* **8**, 369- 85.
7. Xu, Y. and Xie, X. (2009) Glucagon receptor mediates calcium signaling by coupling to Gαq/11 and Gαi/o in HEK293 cells. *J Recept Sig Transduct Res* **29**, 318-325.
8. Kenakin, T. and Miller, L.J. (2010) Seven transmembrane receptors as shapeshifting proteins: The impact of allosteric modulation and functional selectivity on new drug discovery. *Pharmacol Rev* **62**, 265–304.
9. Christopoulos, A., Christopoulos, G., Morfis, M., Udawela, M., Laburthe, M., Couvineau, A., Kuwasako, K., Tilakaratne, N. and Sexton, P.M. (2003) Novel receptor partners and function of receptor activity-modifying proteins. *J Biol Chem* **278**, 3293-3297.
10. Wootten, D., Lindmark, H., Kadmiel, M., Willcockson, H., Caron, K.M., Barwell, J., Drmota, T. and Poyner, D.R. (2013) Receptor activity modifying proteins (RAMPs) interact with the VPAC2 receptor and CRF1 receptors and modulate their function. *Br J Pharmacol* **168**, 822-834.
11. McLatchie, L.M., Fraser, N.J., Main, M.J., Wise, A., Brown, J., Thompson, N., Solari, R., Lee, M.G. and Foord, S.M. (1998) RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. *Nature* **393**, 333-339.
12. Hay, D.L., Christopoulos, G., Christopoulos, A. and Sexton, P.M. (2006) Determinants of 1-piperidinecarboxamide, N-[2-[[5-amino-l-[[4-(4-pyridinyl)-l-piperazinyl] carbonyl] pentyl] amino]-1-[(3, 5-dibromo-4-hydroxyphenyl) methyl]-2-oxoethyl]-4-(1, 4-dihydro-2-oxo-3 (2H)-quinazolinyl)(BIBN4096BS) affinity for calcitonin gene-related peptide and amylin receptors—the role of receptor activity modifying protein 1. *Mol Pharmacol* **70**, 1984-1991.

13. Morfis, M., Tilakaratne, N., Furness, S.G., Christopoulos, G., Werry, T.D., Christopoulos, A. and Sexton, P.M. (2008) Receptor activity-modifying proteins differentially modulate the G protein-coupling efficiency of amylin receptors. *Endocrinology* **149**, 5423-5431.
14. Coopman, K., Wallis, R., Robb, G., Brown, A.J.H., Wilkinson, G.F., Timms, D. and Willars, G.B. (2011) Residues within the transmembrane domain of the glucagon-like peptide-1 receptor involved in ligand binding and receptor activation: Modelling the ligand-bound receptor. *Mol Endocrinol* **25**, 1804-1818.
15. Coopman, K., Huang, Y., Johnston, N., Bradley, S.J., Wilkinson, G.F. and Willars, G.B. (2010) Comparative effects of the endogenous agonist glucagon-like peptide-1 (GLP-1)-(7-36) amide and the small-molecule ago-allosteric agent 'compound 2' at the GLP-1 receptor. *J Pharmacol Exp Ther* **334**, 795–808.
16. Croft, W., Hill, C., McCann, E., Bond, M., Esparza-Franco, M.A., Bennett, J., Rand, D., Davey, J. and Ladds, G. (2013) A physiologically required GPCR-RGS interaction that compartmentalizes RGS activity. *J Biol Chem* **288**, 27327-27342.
17. Weston, C., Poyner, D., Patel, V., Dowell, S. and Ladds, G. (2014) Investigating G protein signaling bias at the glucagon-like peptide-1 receptor in yeast. *Br J Pharmacol* **171**, 3651-3665.
18. Dowell, S.J. and Brown, A.J. (2002) Yeast assays for G-protein-coupled receptors. *Receptors Channels* **8**, 343-352.
19. Brown, A.J., Goldsworthy, S.M., Barnes, A.A., Eilert, M.M., Tcheang, L., Daniels, D., Muir, A.I., Wigglesworth, M.J., Kinghorn, I., Fraser, N.J., Pike, N.B., Strum, J.C., Steplewski, K.M., Murdock, P.R., Holder, J.C., Marshal, F.H., Szkeres, P.G., Wilson, S., Ignar, D.M., Foord, S.M., Wise, A. and Dowell, S.J. (2003) The orphan G protein-coupled receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids. *J Biol Chem* **278**, 11312–11319.
20. Gietz, R.D. and Schiestl, R.H. (2007) High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nat. Protoc.* **2,** 31-34.
21. Bertheleme, N., Singh, S., Dowell, S.J., Hubbard, J. and Byrne, B. (2013) Loss of constitutive activity is correlated with increased thermostability of the human adenosine A2A receptor 1. *Brit. J. Pharmacol.* **169,** 988–998.
22. Usdin, T.B., Hoare, S.R., Wang, T., Mezey, E. and Kowalak, J.A. (1999) TIP39: a new neuropeptide and PTH2-receptor agonist from hypothalamus. *Nat Neurosci* **2**, 941-943.
23. Black, J.W. and Leff, P. (1983) Operational models of pharmacological agonism. *Proc R Soc Lond B Biol Sci* **220**, 141–162.
24. Bond, M., Croft, W., Tyson, R., Bretschneider, T., Davey, J. and Ladds, G. (2013) Quantitative analysis of human ras localization and function in the fission yeast *Schizosaccharomyces pombe*. *Yeast* **30**, 145–156.
25. Grady, T., Fickova, M., Tager, H.S., Trivedi, D. and Hruby, V.J. (1987) Stimulation and inhibition of cAMP accumulation by glucagon in canine hepatocytes. *J Biol Chem* **262**, 15514-15520.
26. Stewart, G.D., Valant, C., Dowell, S.J., Mijaljica, D., Devenish, R.J., Scammells, P.J., Sexton P.M. and Christopoulos, A. (2009) Determination of adenosine A1 receptor agonist and antagonist pharmacology using Saccharomyces cerevisiae: implications for ligand screening and functional selectivity. *J Pharmacol Exp Ther* **331**, 277-286.
27. Brown, A.J., Daniels, D.A., Kassim, M., Brown, S., Haslam, C.P., Terrell, V.R., Brown, J., Nichols, P.L., Staton, P.C., Wise, A. and Dowell, S.J. (2011) Pharmacology of GPR55 in yeast and identification of GSK494581A as a mixed-activity glycine transporter subtype 1 inhibitor and GPR55 agonist. *J Pharmacol Exp Ther* **337**, 236-246.
28. Rudling ,J.E., Kennedy, K., and Evans, P.D. (1999) The effect of site-directed mutagenesis of two transmembrane serine residues on agonist-specific coupling of a cloned human a2A-adrenoceptor to adenylyl cyclase. *Br J Pharmacol* **127**, 877-886.
29. Schepp, W., Dehne, K., Riedel, T., Schmidtler, J., Schaffer, K. and Classen, M. (1996) Oxyntomodulin: a cAMP-dependent stimulus of rat parietal cell function via the receptor for glucagon-like peptide-1 (7-36) NH2. *Digestion* **57**, 398–405.

30. Wynne, K., Park, A.J., Small, C.J., Patterson, M., Ellis, S.M., Murphy, K.G., Wren, A.M., Frost, G.S., Meeran, K., Ghatei, M.A. and Bloom, S.R. (2005) Subcutaneous oxyntomodulin reduces body weight in overweight and obese subjects a double-blind, randomized, controlled trial. *Diabetes* **54**, 2390-2395.
31. Runge, S., Wulff, B.S., Madsen, K., Bräuner-Osborne, H. and Knudsen, L.B. (2003) Different domains of the glucagon and glucagon-like peptide-1 receptors provide the critical determinants of ligand selectivity. *Br J Pharmacol* **138**, 787-794.
32. Jørgensen, R., Kubale, V., Vrecl, M., Schwartz, T.W. and Elling, C.E. (2007) Oxyntomodulin differentially affects glucagon-like peptide-1 receptor β-arrestin recruitment and signaling through Gα. *J Pharmacol Exp Ther* **322,** 148-154.
33. Hollenstein, K., de Graaf, C., Bortolato, A., Wang, M.W., Marshall, F.H. and Stevens, R.C. (2014) Insights into the structure of class B GPCRs. *Trends Pharmacol Sci* **35**, 12-22.
34. Shyu, J.F., Zhang, Z., Hernandez-Lagunas, L., Camerino, C., Chen, Y., Inoue, D., Baron, R. and Horne, W. C. (1999). Protein kinase C antagonizes pertussis-toxin-sensitive coupling of the calcitonin receptor to adenylyl cyclase. *Eur J Biochem* **262**, 95-101.
35. McLatchie, L.M., Fraser, N.J., Main, M.J., Wise, A., Brown, J., Thompson, N., Solari, R., Lee, M.G. and Foord, S.M. (1998) RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. *Nature* **393,** 333-339.
36. Marks, J., Debnam, E.S., Dashwood, M.R., Srai, S.K. and Unwin, R.J. (2003) Detection of glucagon receptor mRNA in the rat proximal tubule: potential role for glucagon in the control of renal glucose transport. *Clin Sci* (Lond). **104**, 253-258.
37. Ali, S., Ussher, J.R., Baggio, L.L., Kabir, M.G., Charron, M.J., Ilkayeva, O., Newgard, C.B. and Drucker, D.J. (2015) Cardiomyocyte glucagon receptor signaling modulates outcomes in mice with experimental myocardial infarction. *Mol metab* **4,** 132-143.
38. Totsune, K., Takahashi, K., Mackenzie, H. S., Murakami, O., Arihara, Z., Sone, M., Mouri, T., Brenner, B.M. and Ito, S. (2000) Increased gene expression of adrenomedullin and adrenomedullin-receptor complexes, receptor-activity modifying protein (RAMP) 2 and calcitonin-receptor-like receptor (CRLR) in the hearts of rats with congestive heart failure. *Clin Sci* **99,** 541-546.
39. Ban, K., Noyan-Ashraf, M.H., Hoefer, J., Bolz, S.S., Drucker, D.J., and Husain, M. (2008) Cardioprotective and vasodilatory actions of glucagon-like peptide 1 receptor are mediated through both glucagon-like peptide 1 receptor–dependent and–independent pathways. *Circulation* **117**, 2340-2350.
40. Sonne, D.P., Engstrøm, T. and Treiman, M. (2008) Protective effects of GLP-1 analogues exendin-4 and GLP-1 (9–36) amide against ischemia–reperfusion injury in rat heart. *Regulatory peptides* **146**, 243-249.
41. Cho, Y.M., Merchant, C.E. and Kieffer, T.J. (2012) Targeting the glucagon receptor family for diabetes and obesity therapy. *Pharmacol Ther* **135**, 247-278.
42. Underwood, C.R., Garibay, P., Knudsen, L.B., Hastrup, S., Peters, G.H., Rudolph, R. and Reedtz-Runge, S. (2010) Crystal structure of glucagon-like peptide-1 in complex with the extracellular domain of the glucagon-like peptide-1 receptor. *J Biol Chem* **285**, 723-730.
43. Watkins, H.A., Walker, C.S., Ly, K.N., Bailey, R.J., Barwell, J., Poyner, D.R. and Hay, D.L. (2014) Receptor activity-modifying protein-dependent effects of mutations in the calcitonin receptor-like receptor: implications for adrenomedullin and calcitonin gene‐related peptide pharmacology. *Br J. Pharmacol* **171**, 772-788.
44. Siu, F.Y., He, M., de Graaf, C., Han, G.W., Yang, D., Zhang, Z., Zhou, C., Xu, Q., Wacker, D., Joseph, J.S., Liu, W., Lau, J., Cherezov, V., Katritch, V., Wang, M.W. and Stevens, R.C. (2013) Structure of the human glucagon class B G-protein-coupled receptor. *Nature* **499**, 444-449.
45. Koth, C. M., Murray, J. M., Mukund, S., Madjidi, A., Minn, A., Clarke, H. J., Wong, T., Chiang, V., Luis, E., Estevez, A., Rondon, J., Zhang, Y., Hoetzel, I. and Allan, B. B. Molecular basis for negative regulation of the glucagon receptor. (2012) *Proc Natl Acad Sci USA* **109**, 14393-14398.
46. Booe, J.M., Walker, C.S., Barwell, J., Kuteyi, G., Simms, J., Jamaluddin, M.A., Warner, M.L., Bill, R.M., Harris, P.W., Brimble, M.A., Poyner, D.R., Hay, D.L. and Pioszak, A.A. (2015) Structural

Basis for Receptor Activity-Modifying Protein-Dependent Selective Peptide Recognition by a G Protein-Coupled Receptor. *Mol Cell* **58**, 1040-1052.

47. Hulme, E.C. (2013) GPCR activation: a mutagenic spotlight on crystal structures. *Trends Pharmacol Sci* **34**, 67-84.
48. Kruse, A.C., Hu, J., Pan, A.C., Arlow, D.H., Rosenbaum, D.M., Rosemond, E., Green, H.F., Liu, T., Chae, P.S., Dror, R.O., Shaw, D.E., Weis, W.I., Wess, J. and Kobilka, B.K. (2012) Structure and dynamics of the M3 muscarinic acetylcholine receptor. *Nature* **482**, 552-556.
49. Udawela, M., Christopoulos, G., Tilakaratne, N., Christopoulos, A., Albiston, A., and Sexton, P.M. (2006) Distinct receptor activity-modifying protein domains differentially modulate interaction with calcitonin receptors. *Mol pharmacol* **69,** 1984-1989.
50. Quesada, I., Tuduri, E., Ripoll, C. and Nadal, A. (2008) Physiology of the pancreatic α-cell and glucagon secretion: role in glucose homeostasis and diabetes. *J Endocrinol* **199**, 5-19.
51. Husmann, K., Sexton, P.M., Fischer, J.A. and Born, W. (2000) Mouse receptor-activity-modifying proteins 1,-2 and-3: amino acid sequence, expression and function. *Mol Cell Endo* **162**, 35-43.

FIGURE 1: RAMP2 interacts with the GCGR. A, Plasma membrane expression of ^{FLAG}RAMPs in cells transfected with vector control, CLR, GCGR or GLP-1 receptor determined by ELISA. **B**, HEK-293 cells transfected with RAMP-GFP fusion constructs in the presence and absence of various GPCRs as indicated. Arrows indicate plasma membrane localisation. **C**, cAMP accumulation was determined in HEK-293 cells transiently transfected with the GCGR in the presence of vector control, RAMP1, RAMP2 or RAMP3 following 30 min stimulation with glucagon. **D**, Plasma membrane expression of myc-CLR or myc-GLP-1R (as a positive control) determined by ELISA in HEK-293 cells co-transfected with vector control, or RAMP2. **E**, cAMP accumulation, measured following 30 min stimulation of HEK-293 cells with CGRP or AM expressing CLR in the presence of vector control, RAMP1 or RAMP2. All values are mean of at least 5 independent experiments ± SEM. Statistical significance was determined using one-way ANOVA with Dunnett's post-test where each data set was compared with vector control (***$P < 0.001$, ****$P < 0.0001$).

FIGURE 2: Co-expression of RAMP2 does not affect cell surface expression of the GCGR or glucagon binding. A, (i) HEK-293 cells were transfected with fluorescently labelled receptors in the presence or absence of RAMP2. Arrows indicate plasma membrane localisation. (ii) The percentage of plasma membrane fluorescence was quantified using the QUIMP plugin for ImageJ for at least 20 cells in each population and mean data ± SEM represented in the bar chart. **B**, Plasma membrane expression of myc-tagged-GCGR in cells transfected with vector control, RAMP1 or RAMP2 determined by ELISA. **C**, Plasma membrane expression of (i) FLAG-tagged-RAMP2 and (ii) myc-tagged-GCGR determined by ELISA in HEK-293 cells transfected with indicated ratios of FLAG-tagged-RAMP2 to myc-tagged-GCGR. **D**, HEK-293 cells expressing the GCGR and indicated ratios of RAMP2 were assayed for cAMP accumulation following 30 min stimulation with glucagon. **E**, Whole cell competition binding analysis of glucagon at the GCGR with [I^{125}]glucagon tracer in the presence or absence of RAMP2. Values were corrected for non-specific binding (NSB) and normalized to maximum [I^{125}]glucagon binding. All values are mean of at least 5 independent experiments ± SEM. Statistical significance was determined using one-way ANOVA with Dunnett's post-test where each data set was compared with vector control (**$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

FIGURE 3: RAMP2-mediated modulation of the GCGR is G protein-dependent. Glucagon concentration-response curves in yeast strains containing either **A**, GPA1/$G\alpha_s$ or **B**, GPA1/$G\alpha_i$ chimeras expressing the GCGR in the presence of a vector control, RAMP1 or RAMP2. Activation of the reporter gene was calculated as a percentage of the maximum response observed in the absence of RAMP2. SPAs to determine **C**, $G\alpha_s$ and **D**, $G\alpha_i$ activation upon stimulation of cell membranes, containing the GCGR in the presence or absence of RAMP2, with increasing concentrations of glucagon. **E**, HEK-293 cells containing the GCGR and RAMP2 or a vector control were incubated with 200 ng/ml PTX or a vehicle control for 16 h before stimulation with glucagon and determination of cAMP accumulation. All values are mean of at least 3 independent experiments ± SEM.

FIGURE 4: RAMP2-mediated modulation of the GCGR is ligand-dependent. A, HEK-293 cells co-transfected with the GCGR and either a vector control or RAMP2 were incubated with 200 ng/ml PTX (or vehicle control) for 16 h before stimulation with oxyntomodulin for 30 min and measurement of cAMP accumulation. **B**, Whole cell competition binding analysis of oxyntomodulin with [I^{125}]glucagon tracer at the GCGR in the presence or absence of RAMP2. Values were corrected for non-specific binding (NSB) and normalized to maximum [I^{125}]glucagon binding. **C**, GPA1/$G\alpha_s$ or **D**, GPA1/$G\alpha_i$ chimeric yeast strains transformed with the GCGR and either a vector control or RAMP2 were stimulated with oxyntomodulin and receptor activity determined. All values are expressed as a percentage of the maximum response observed to glucagon in the absence of RAMP2 and are the mean of at least 5 independent experiments ± SEM.

FIGURE 5: GLP-1 receptor agonists are functional at the GCGR in the absence of RAMP2. A, Sequences of the various peptide ligands used in this study aligned to the natural agonist, glucagon. Amino acids differing from those in glucagon are highlighted in grey. Red boxes highlight partially conserved

hydrophobic regions required for GCGR binding (31). **B**, GPA1/Gα_s or **C**, GPA1/Gα_i chimera yeast strains containing GCGR in the presence or absence of RAMP2 were used to determine reporter gene activity following stimulation with various GLP-1 receptor agonists. All data are expressed as a percentage of the maximum response observed to glucagon in the absence of RAMP2 and are the mean of at least 5 independent experiments ± SEM.

FIGURE 6: RAMP2 co-expression does not affect GLP-1 receptor pharmacology. The GLP-1 receptor was expressed in the presence and absence of RAMP2 in yeast strains containing **A**, GPA1/Gαs or **B**, GPA1/Gαi chimeras and reporter gene activity was determined following stimulation with the natural agonist, GLP-1. **C**, cAMP accumulation was measured following 30 min stimulation of un-transfected HEK-293 cells and those expressing the GLP-1 receptor in either the presence or absence of RAMP2. All data are expressed as a percentage of the maximum response observed to GLP-1 in the absence of RAMP2 and are mean of at least 5 independent experiments ± SEM.

FIGURE 7: GLP-1 specifically binds the GCGR only in the absence of RAMP2. cAMP accumulation was measured in HEK-293 cells co-transfected with the GCGR and either a vector control or RAMP2 following stimulation with **A**, GLP-1 and **B**, liraglutide in the presence and absence of a GCGR antagonist, des-His^{1}-[Glu9]-glucagon (1-29) amide. **C**, Whole-cell competition binding analysis of GLP-1 and liraglutide with [I^{125}]glucagon tracer at the GCGR in the presence or absence of RAMP2. Values were corrected for non-specific binding (NSB) and normalized to maximum [I^{125}]glucagon binding. **D,** HEK-293 cells expressing the GCGR and indicated ratios of RAMP2 were assayed for cAMP accumulation following 30 min stimulation with GLP-1. All data are expressed as a percentage of the maximum response observed to glucagon in the absence of RAMP2 and are the mean of at least 5 independent experiments ± SEM.

TABLE 1

Potency (pEC_{50}) and maximal response (E_{max}) to glucagon in cAMP assay performed on HEK-293 cells expressing the GCGR ± indicated RAMPs

	Vector		RAMP1	RAMP2		RAMP3
	-PTX	+PTX	-PTX	-PTX	+PTX[z]	-PTX
pEC_{50}[a]	10.7 ±0.4	12.9 ±0.5	10.5 ±0.6	12.1 ±0.6	12.4 ±0.4	10.9 ±0.2
E_{max}[b]	100 ±5.1	192 ±10.1***	88.1 ±6.1	172 ±7.1***	164 ±8.7	105 ±8.8

Values generated through fitting of a three-parameter logistic equation and represent the mean ± SEM from 5 independent experimental repeats.

[a] The negative logarithm of the agonist concentration required to generate half the maximal response.

[b] The maximal response to the ligand expressed as a percentage of that obtained in the absence of pertussis toxin in cells expressing empty vector control.

Statistical significance compared to vector –PTX (RAMP2 –PTX for [z]) (***, $p < 0.001$) was determined by one-way ANOVA with Dunnett's post-test.

TABLE 2

Potency (pEC_{50}) and maximal response (E_{max}) to glucagon at the GCGR expressed ± RAMP2 measured in yeast cell and scintillation proximity assays.

Yeast	Vector		RAMP2	
	GPA1/Gαs	GPA1/Gαi	GPA1/Gαs	GPA1/Gαi
pEC_{50}[a]	7.9 ±0.1	7.8 ±0.3	8.4 ±0.2	9.3 ±1.2
E_{max}[b]	100 ±7.1	97.4 ±8.1	189.7 ±8.7***	32.7 ±8.5**
SPA	**Vector**		**RAMP2**	
	Gαs	**Gαi**	**Gαs**	**Gαi**
pEC_{50}[a]	11.0 ±0.1	9.2 ±0.1	10.7 ±0.1	9.0 ±0.1
E_{max}[b]	103.3 ±3.4	99.4 ±1.8	92.1 ±2.4	59.9 ±1.9***

Values generated through fitting of a three-parameter logistic equation and represent the mean ± SEM from at least 3 independent experimental repeats.

[a] The negative logarithm of the agonist concentration required to generate half the maximal response.

[b] The maximal response to the ligand expressed as a percentage of that obtained in the absence of RAMP expression.

Statistical significance compared to vector (**, $p < 0.01$, ***, $p < 0.001$) was determined by one-way ANOVA with Dunnett's post-test.

TABLE 3

Potency (pEC_{50}), affinity (pK_A) and coupling efficacy (log τ) values for various glucagon receptor agonists measured in HEK-293 cells expressing the GCGR ± RAMP2 using a cAMP assay.

Ligand	Vector		RAMP2	
	pEC_{50}[a]	**E_{max}[b]**	**pEC_{50}[a]**	**E_{max}[b]**
Glucagon	10.6 ±0.4	100 ±5.1	12.1 ±0.6	172.9 ±7.1***
Oxyntomodulin	8.9 ±0.2	66.8 ±4.3	11.0 ±0.4*	200 ±18.3***
GLP-1	9.4 ±0.6	42.1 ±5.1	N.R	N.R
Liraglutide	8.0 ±0.5	60.8 ±11.1	N.R	N.R
Exenatide	N.R	N.R	N.R	N.R
Lixisenatide	N.R	N.R	N.R	N.R
	Vector		**RAMP2**	
	pK_A[c]	**log τ[d]**	**pK_A[c]**	**log τ[d]**
Glucagon				
Oxyntomodulin	8.4 ±0.2	0.4 ±0.1	10.8 ±0.3*	-0.4 ±0.1
GLP-1	8.9 ±0.1	-0.2 ±0.1	N.R	N.R
Liraglutide	7.3 ±0.2	0.3 ±0.1	N.R	N.R
Exenatide	N.R	N.R	N.R	N.R
Lixisenatide	N.R	N.R	N.R	N.R

All values are mean ± SEM of five independent experimental repeats.

[a] The negative logarithm of the agonist concentration required to produce a half-maximal response

[b] The maximal response to the ligand expressed as a percentage of that obtained to glucagon in the absence of RAMP co-transfection

[c] The negative logarithm of the relative equilibrium disassociation constant for each ligand generated through use of the operational model for partial agonism relative to glucagon.

[d] τ is the coupling efficiency parameter, generated by comparison to the natural ligand, glucagon using the operational model for partial agonism.

Statistical significance compared to vector (*, $p < 0.05$, ***, $p < 0.001$) was determined by one-way ANOVA with Dunnett's post-test.

N.R signifies no response.

TABLE 4

Potency (pEC_{50}), affinity (pK_A) and coupling efficacy (log τ) values for various glucagon receptor agonists measured in yeast strains containing the GPA1/Gαs chimera and expressing the GCGR ± RAMP2.

Ligand	Vector		RAMP2	
	pEC_{50}[a]	**E_{max}[b]**	**pEC_{50}[a]**	**E_{max}[b]**
Glucagon	7.9 ±0.1	100 ±7.1	8.4 ±0.2	189.7 ±8.7***
Oxyntomodulin	7.4 ±0.2	100 ±5.2	8.1 ±0.2	211.3 ±16.1***
GLP-1	6.6 ±0.2	60.0 ±5.2	N.R	N.R
Liraglutide	5.5 ±0.1	80.1 ±7.5	N.R	N.R
Exenatide	N.R	N.R	N.R	N.R
Lixisenatide	N.R	N.R	N.R	N.R
	Vector		**RAMP2**	
	pK_A[c]	**log τ[d]**	**pK_A[c]**	**log τ[d]**
Glucagon				
Oxyntomodulin	6.8 ±0.2	1.3 ±0.1	7.6 ±3.4	0.6 ±0.2
GLP-1	6.2 ±0.3	0.04 ±0.1	N.R	N.R
Liraglutide	4.8 ±0.1	0.5 ±0.2	N.R	N.R
Exenatide	N.R	N.R	N.R	N.R
Lixisenatide	N.R	N.R	N.R	N.R

All values are mean ± SEM of five independent experimental repeats.

[a] The negative logarithm of the agonist concentration required to produce a half-maximal response

[b] The maximal response to the ligand expressed as a percentage of that obtained to glucagon in the absence of RAMP co-expression.

[c] The negative logarithm of the relative equilibrium disassociation constant for each ligand generated through use of the operational model for partial agonism relative to glucagon.

[d] τ is the coupling efficiency parameter, generated by comparison to the natural ligand, glucagon using the operational model for partial agonism.

Statistical significance compared to vector (***, $p < 0.001$) was determined by one-way ANOVA with Dunnett's post-test.

N.R signifies no response.

TABLE 5

Potency (pEC_{50}), affinity (pK_A) and coupling efficacy (log τ) values for various glucagon receptor agonists measured in yeast strains containing the GPA1/Gαi chimera and expressing the GCGR ± RAMP2.

Ligand	Vector		RAMP2	
	pEC_{50}[a]	E_{max}[b]	pEC_{50}[a]	E_{max}[b]
Glucagon	7.8 ±0.3	97.4 ±8.1	9.3 ±1.2	32.7 ±8.5***
Oxyntomodulin	7.6 ±0.2	82.0 ±7.4	8.0 ±0.6	85.1 ±10.1
GLP-1	7.9 ±0.3	35.1 ±3.4	N.R	N.R
Liraglutide	7.7 ±0.2	84.0 ±9.4	N.R	N.R
Exenatide	N.R	N.R	N.R	N.R
Lixisenatide	N.R	N.R	N.R	N.R
	Vector		**RAMP2**	
	pK_A[c]	log τ[d]	pK_A[c]	log τ[d]
Glucagon				
Oxyntomodulin	6.8 ±0.3	0.8 ±0.2	N.D	N.D
GLP-1	7.6 ±0.4	-0.3 ±0.1	N.R	N.R
Liraglutide	6.8 ±0.7	0.7 ±0.5	N.R	N.R
Exenatide	N.R	N.R	N.R	N.R
Lixisenatide	N.R	N.R	N.R	N.R

All values are mean ± SEM of five independent experimental repeats.

[a] The negative logarithm of the agonist concentration required to produce a half-maximal response

[b] The maximal response to the ligand expressed as a percentage of that obtained to glucagon in the absence of RAMP co-expression.

[c] The negative logarithm of the relative equilibrium disassociation constant for each ligand generated through use of the operational model for partial agonism relative to glucagon.

[d] τ is the coupling efficiency parameter, generated by comparison to the natural ligand, glucagon using the operational model for partial agonism.

N.R signifies no response.

N.D represents not determined

Figure 1

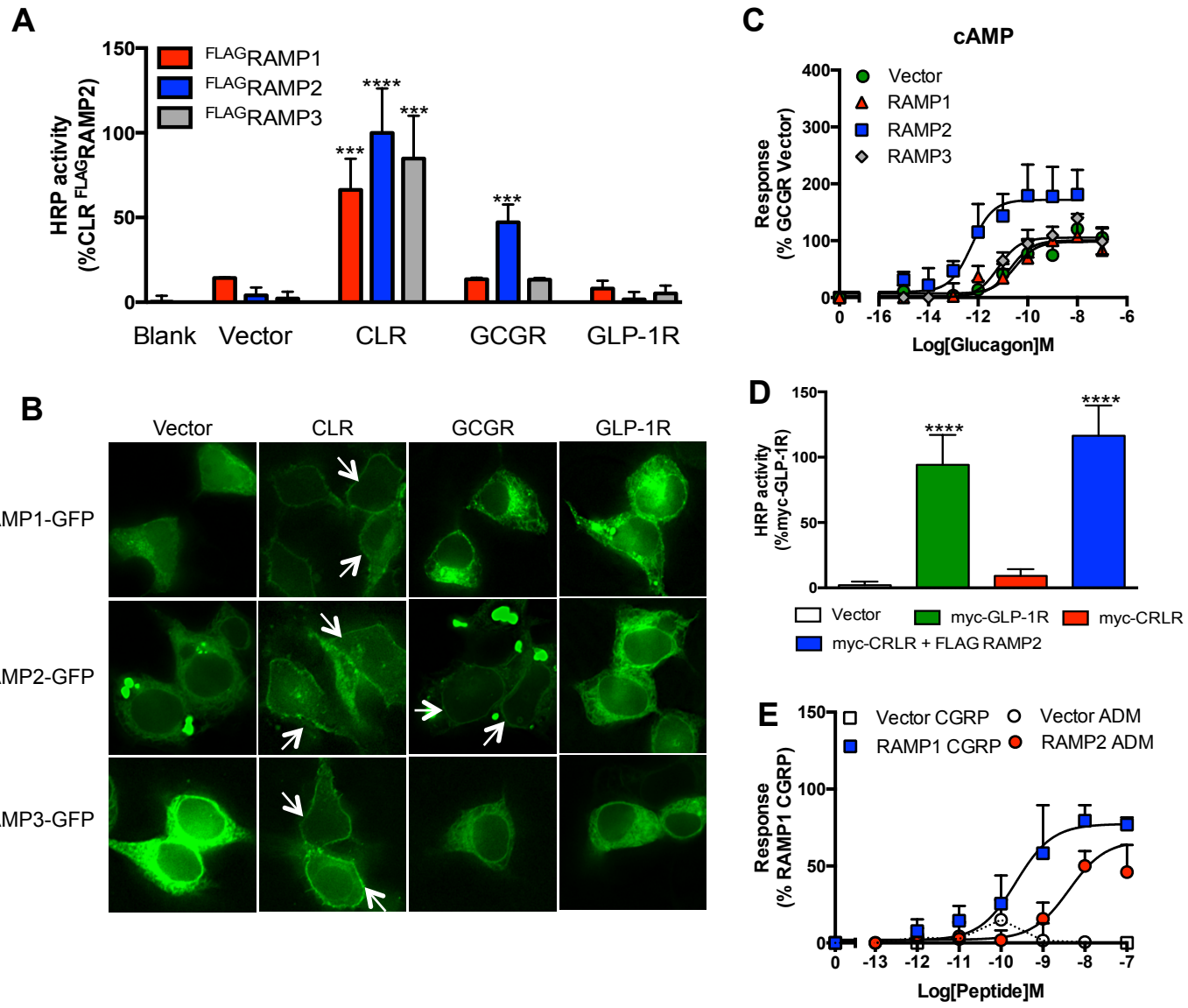

A
HRP activity (%CLR FLAGRAMP2)
FLAGRAMP1
FLAGRAMP2
FLAGRAMP3
Blank
Vector
CLR
GCGR
GLP-1R
B
Vector
CLR
GCGR
GLP-1R
RAMP1-GFP
RAMP2-GFP
RAMP3-GFP
C
cAMP
Response (% GCGR Vector)
Vector
RAMP1
RAMP2
RAMP3
Log[Glucagon]M
D
HRP activity (%myc-GLP-1R)
Vector
myc-GLP-1R
myc-CRLR
myc-CRLR + FLAG RAMP2
E
Vector CGRP
Vector ADM
RAMP1 CGRP
RAMP2 ADM
Response (% RAMP1 CGRP)
Log[Peptide]M

Figure 2

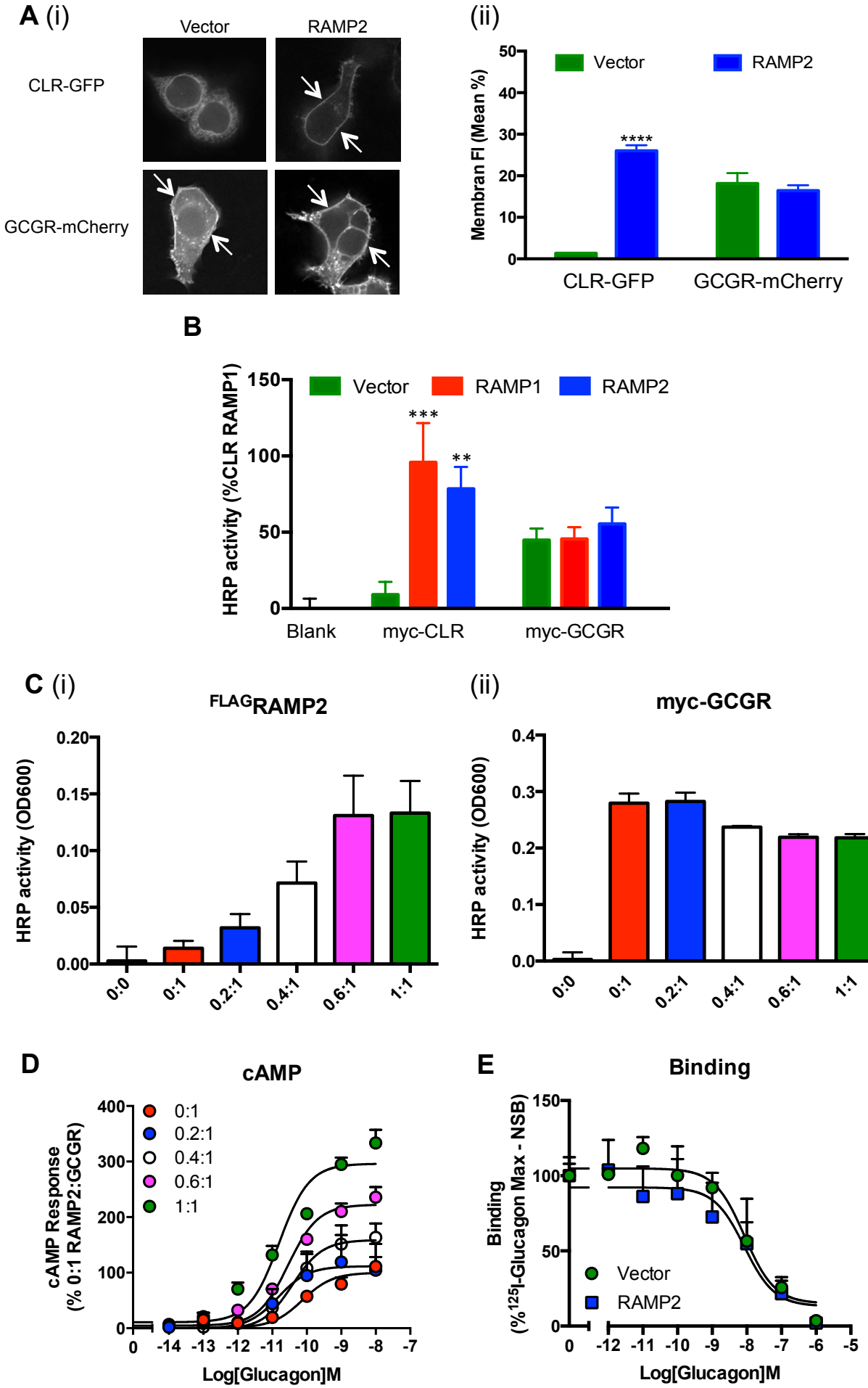

A (i)
Vector
RAMP2
CLR-GFP
GCGR-mCherry
(ii)
Membran FI (Mean %)
Vector
RAMP2
****
CLR-GFP
GCGR-mCherry
B
HRP activity (%CLR RAMP1)
Vector
RAMP1
RAMP2
***
**
Blank
myc-CLR
myc-GCGR
C (i)
FLAGRAMP2
HRP activity (OD600)
0:0
0:1
0.2:1
0.4:1
0.6:1
1:1
(ii)
myc-GCGR
HRP activity (OD600)
D
cAMP
cAMP Response (% 0:1 RAMP2:GCGR)
0:1
0.2:1
0.4:1
0.6:1
1:1
Log[Glucagon]M
E
Binding
Binding (%125I-Glucagon Max - NSB)
Vector
RAMP2
Log[Glucagon]M

Figure 3

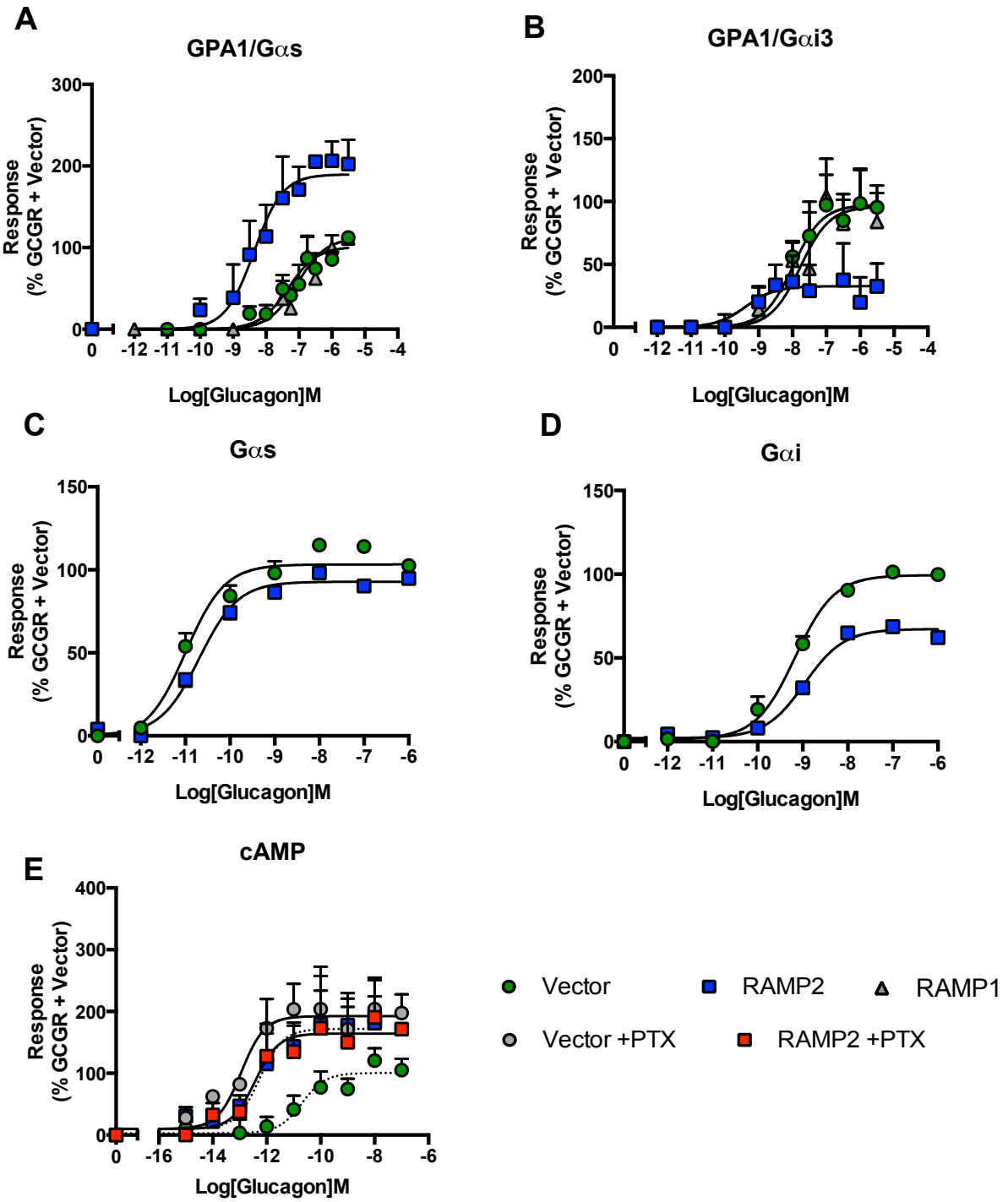

A
GPA1/Gαs
Response (% GCGR + Vector)
Log[Glucagon]M
B
GPA1/Gαi3
Response (% GCGR + Vector)
Log[Glucagon]M
C
Gαs
Response (% GCGR + Vector)
Log[Glucagon]M
D
Gαi
Response (% GCGR + Vector)
Log[Glucagon]M
E
cAMP
Response (% GCGR + Vector)
Log[Glucagon]M
Vector
RAMP2
RAMP1
Vector +PTX
RAMP2 +PTX

Figure 4

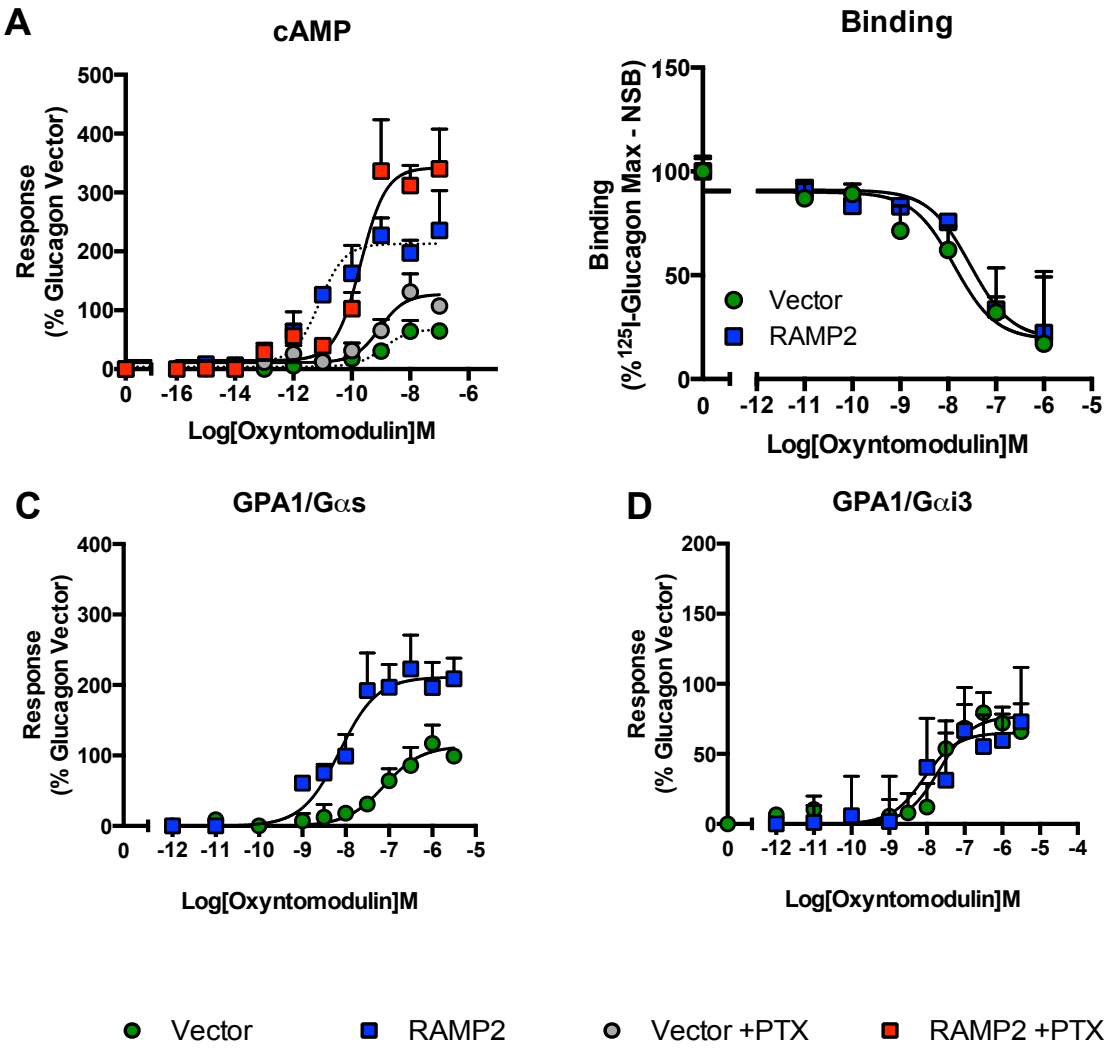

A
cAMP
Response (% Glucagon Vector)
Log[Oxyntomodulin]M
Binding
Binding (%125I-Glucagon Max - NSB)
Vector
RAMP2
Log[Oxyntomodulin]M
C
GPA1/Gαs
Response (% Glucagon Vector)
Log[Oxyntomodulin]M
D
GPA1/Gαi3
Response (% Glucagon Vector)
Log[Oxyntomodulin]M
Vector
RAMP2
Vector +PTX
RAMP2 +PTX

Figure 5

A

Ligand	Sequence
Glucagon	HSQGTFTSDYSKYLDSRRAQDFVQWLMNT
Oxyntomodulin	HSQGTFTSDYSKYLDSRRAQDFVQWLMNTKRNKNNIA
GLP-1	HAEGTFTSDVSSYLEGQAAKEFIAWLVKGR
Liraglutide	HAEGTFTSDVSSYLEGQAAKEFIAWLVRGR
Exenatide	HGEGTFTSDLSKQMEEEAVRLFIETLKNGGPSSGAPPPS
Lixisenatide	HGEGTFTSDLSKQMEEEAVRLFIETLKNGGPSSGAPPSKKKKKK

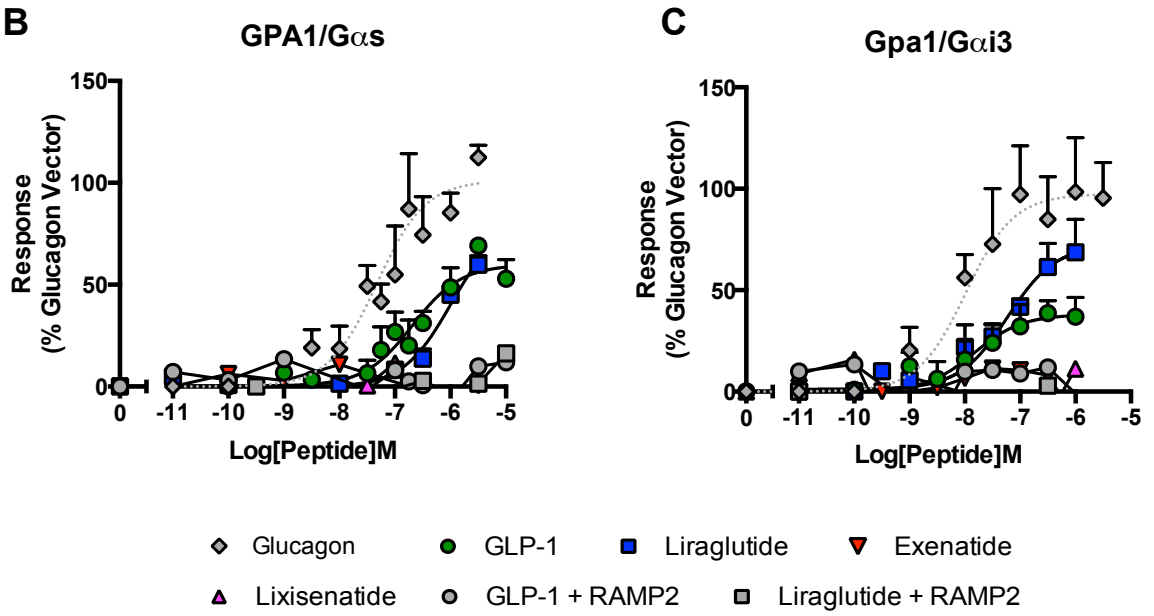


Figure 6

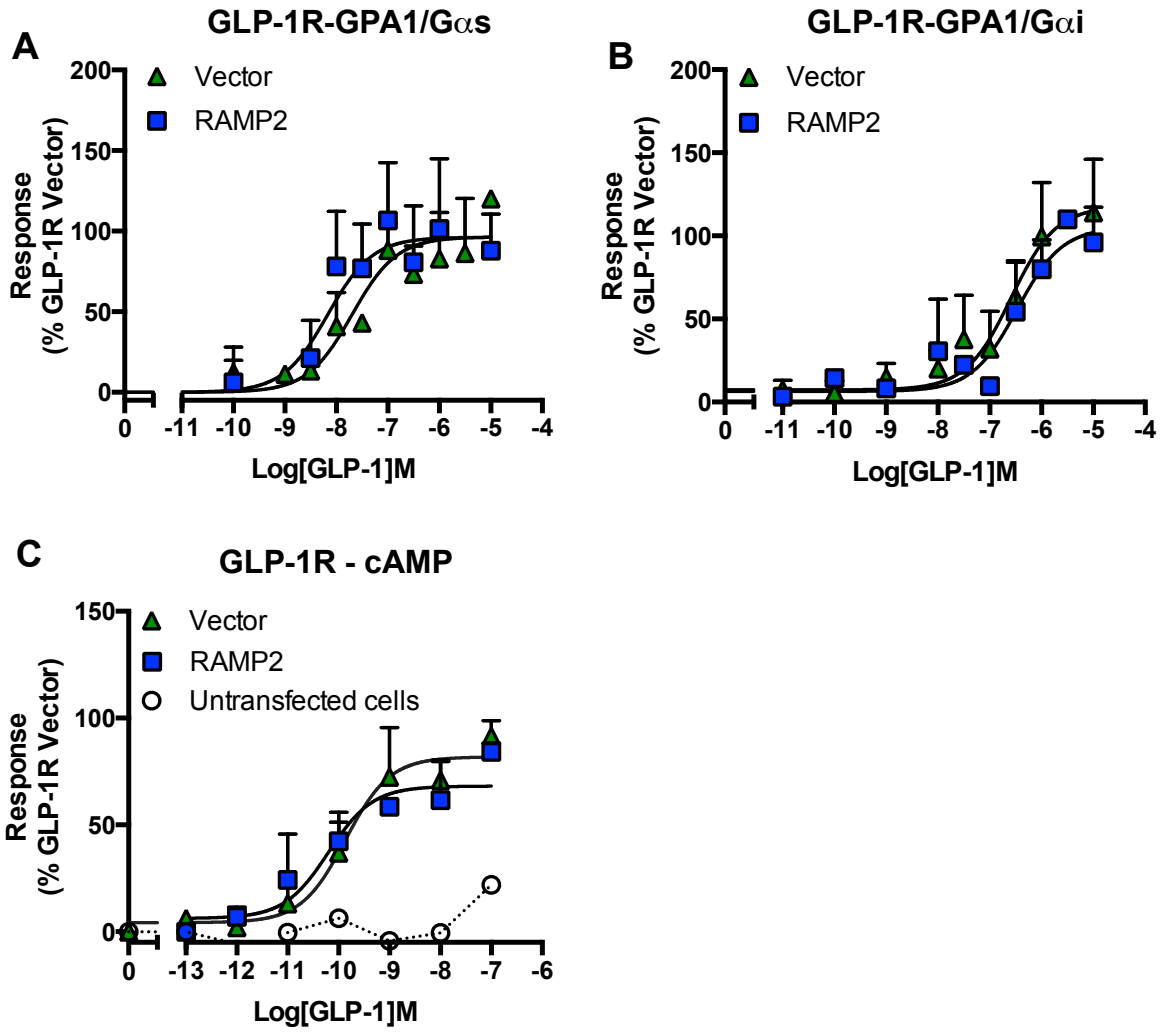
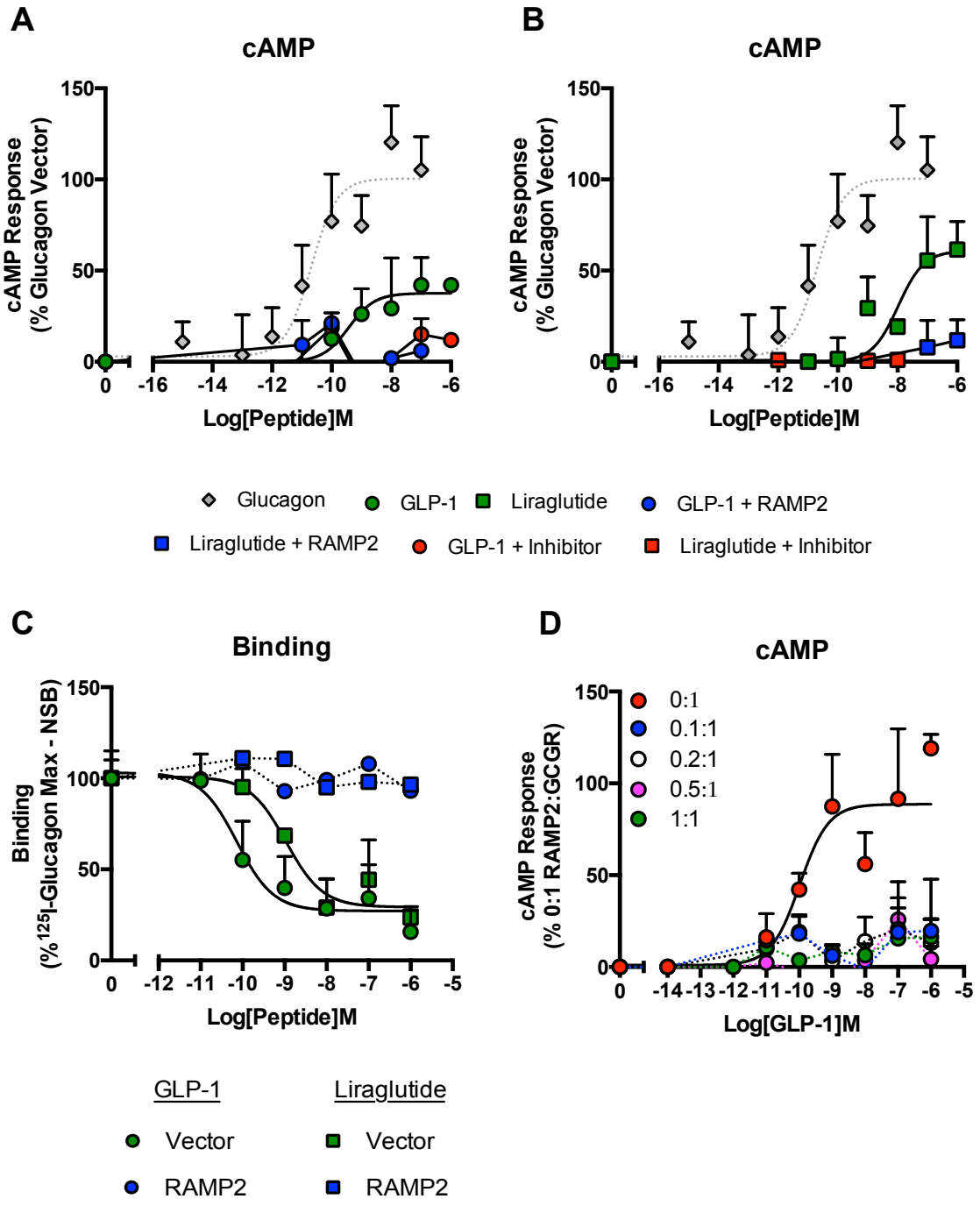

A
GLP-1R-GPA1/Gαs
Vector
RAMP2
Response (% GLP-1R Vector)
200
150
100
50
0
0 -11 -10 -9 -8 -7 -6 -5 -4
Log[GLP-1]M
B
GLP-1R-GPA1/Gαi
Vector
RAMP2
Response (% GLP-1R Vector)
200
150
100
50
0
0 -11 -10 -9 -8 -7 -6 -5 -4
Log[GLP-1]M
C
GLP-1R - cAMP
Vector
RAMP2
Untransfected cells
Response (% GLP-1R Vector)
150
100
50
0
0 -13 -12 -11 -10 -9 -8 -7 -6
Log[GLP-1]M

Figure 7


A
cAMP
cAMP Response (% Glucagon Vector)
Log[Peptide]M
B
cAMP
cAMP Response (% Glucagon Vector)
Log[Peptide]M
Glucagon
GLP-1
Liraglutide
GLP-1 + RAMP2
Liraglutide + RAMP2
GLP-1 + Inhibitor
Liraglutide + Inhibitor
C
Binding
Binding (% 125I-Glucagon Max - NSB)
Log[Peptide]M
GLP-1
Vector
RAMP2
Liraglutide
Vector
RAMP2
D
cAMP
cAMP Response (% 0:1 RAMP2:GCGR)
Log[GLP-1]M
0:1
0.1:1
0.2:1
0.5:1
1:1