RAMP2 influences glucagon receptor pharmacology via trafficking and signaling

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PRECIS: Please submit a brief description of your paper that will appear on the Table of Contents along with the title, should your paper be accepted. The description should be NO LONGER THAN 200 CHARACTERS and should serve to buttress the content of the title by simply stating what was done and what was concluded.	A combination of competition binding, functional assays assessing the Gαs and Gq pathways and β-arrestin recruitment, and siRNA knockdown are employed to examine the effect of RAMP2 on the GCGR
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Rebuttal for manuscript #EN-16-1755 entitled: "RAMP2 influences glucagon receptor pharmacology via trafficking and signalling".

Editor's comment

While the reviewers felt that your topic is important and interesting, and that your additions were excellent, they still have a few concerns that prevent acceptance of your manuscript in its present form. Their critiques indicate that additional data, experiments, and/or rewriting are required. All reviewer comments, appended below, require your careful consideration and appropriate response before the manuscript can be re-reviewed and considered for publication. ***Specifically, in reading over the paper and the comments, it seems that the major concern is that only one stable cell line was used for these studies, which always brings up concerns about specificity of results (is this due to some off-target vaguery of the clone, or is it really generalizable?). It should not be necessary to repeat every experiment, or use multiple cell lines - just performing the suggested binding study on one additional stable cell line, which you must have generated when deriving the line here, would go a long way toward ruling out a clonal-specific effect.***

Authors' response

We appreciate the editor's concern that only one stable cell line was used for the binding studies. Therefore, we have taken the editor's advice and performed binding experiments in an independent cell line stable expressing RAMP2. Briefly:

Methods:

To ensure that these findings were attributable to co-expression of RAMP2 with the GCGR, rather than artefactual, a second cell line with RAMP2 stably upregulated was investigated (CHO-K1-GCGR-CFP-RAMP2) and compared to a cell line transfected in parallel with a control (pcDNA3.1) plasmid. CHO-K1 cells expressing the human GCGR were co-transfected with C-terminally CFP-tagged RAMP2 (Tebu-bio Ltd, UK) and a plasmid conferring puromycin resistance using lipofectamine 2000 (Thermo Fisher). Forty-eight hours later, media was supplemented with puromycin 10 µg/ml to select cells containing the construct. Expression was confirmed by qPCR. Whole cell binding assay was performed with CFP-RAMP2 stably transfected cells and controls transfected with pcDNA. I^{125}glucagon was used as the competing peptide in all assays. Peptides were incubated with cells at room temperature for 90 minutes. Counts were measured over 240s, normalised to maximal of each cell line and normalised to protein content of cells as determined by BCA assay. Receptor density (Bmax) was calculated using GraphPad Prism 7.0b (GraphPad Software Inc., USA) using the following regression fit line:

Y=(Bmax*HotnM)/(HotnM + ColdNM + KdNM) + Bottom

Results:

Successful transfection of CHO-K1-GCGR cells with C-terminally CFP-tagged RAMP2 (Tebu-bio Ltd, UK) was confirmed by qPCR. H-RAMP2 was undetectable in the control cell line but expressed in the CHO-K1-GCGR-CFP-RAMP2 cells.

As with the first cell line (CHO-K1-GCGR-RAMP2), the binding affinity of glucagon for its receptor was not altered with the upregulation of RAMP2 (IC_{50} 4.377nM with CFP-RAMP2 vs 5.123nM without (p=0.16)) (see Supplemental Figure 2A), however the density of GCGR binding sites (Bmax) was significantly lower in the cell line with upregulated RAMP2 (p=0.0069) (Supplemental Figure 2B).

This data corroborates our initial binding data and we have now confirmed that the number of GCG

binding sites is reduced in the presence of RAMP2 in two independent stable cell lines.

Amendments to manuscript:
Methods: page 5 lines 131-134, page 6 lines 160-163
Results: page 10 lines 288-290, 304-310
Addition of Supplemental Figure 2

Reviewer's comment	**Authors' response**
Reviewer 1	
1. The authors need to alter the wording relating to "internalisation" throughout the manuscript. This has not actually been measured in this paper. They should use cellular distribution or similar instead. The confocal data for example show the presence of receptor/RAMP inside cells but this was measured at a single point in time and not in response to ligand. Therefore the authors cannot conclude that there is internalisation, rather than the receptor simply not reaching the cell surface. i.e. the authors could equally conclude that the receptor is trapped inside the cell by the RAMP.	As suggested, internalisation has been rephrased throughout the manuscript. **Amendments: Abstract page 2 line 55; Results page 12 line 368-369; Figure Legend page 21 line 706**
2. The binding data are now much clearer. However, the authors should not refer to binding data as "total binding", if in fact it is specific binding. The relevant text and figures need to be changed to deal with this.	As suggested, total binding has been corrected to specific binding. **Amendments: Methods page 6 line 150, line 154; Results page 10 lines 292-293, Discussion Page 13 line 389; Figure Legend page 20 line 663**
3. Page 3 line 92, reference 17 McLatchie does not report seven different receptors. Use Poyner et al 2002, Pharm Rev. instead. They have used 27 for 17 later (p15) as well. The references need to be carefully checked.	The references have been corrected and re-reviewed.
4. The wording for G(X) on page 5 is unclear. The motivation for using this ligand is less important than being clear on its actual sequence. Is this 1-15 glucagon together with 16-34 of exendin-4? Or is there more of a mix. Either reference the original sequence, explain more clearly or show the sequence in supplementary information. In order for any reader to reproduce the work, this is essential.	The sequence of G(X) has been further clarified, however, the actual sequence cannot be disclosed for intellectual property reasons. **Amendments: Methods page 5 line 112-119**
5. There still seems to be some confusion around curve fitting parameters and inconsistency in the paper. The data from signalling assays appear to have been fit with a variable slope (4 parameter) but the equation implies that this was 3 parameter with a fixed slope of 1. This needs to be clarified.	The curve fitting for the biphasic curves was done using a variable slope (four parameters) model. This has been clarified in the methods section. **Amendments: Methods page 7 lines 201-203**
6. All of the cell experiments use different conventions for reporting the cell number used, which is very confusing and does not allow	The reporting of cell numbers has now been harmonised and numbers for cells/ml have been

comparisons between assays. The actual cell number should be specified. Cells per mL is not acceptable if the volume pipetted into the well is not specified.	recalculated to give cells/well. **Amendments: Methods page 6 line 176, page 7 line 187**
7. Do not use "u" for micro. Use the correct symbol. This occurs in numerous places throughout the document.	This has been corrected throughout.
8. On page 14, the speculation about the work showing bias should be removed and left until the description on page 15, which is adequate. As it stands, the work does not show bias.	This has now been removed as suggested.
9. Table 1 should include statistical analysis.	Statistical analysis was performed however none of the comparisons were statistically significant. This is described further in the results section. **Amendments: Results Page 10 Lines 297-302**
10. Table 3. If some of these experiments are less than n=3 as is suggested by the legend, then there should not be statistical analysis on these data.	Statistical analysis was only carried out for experiments where n≥3.
Reviewer 2	
1. The additional data that has been added improves the work and helps somewhat to support the interpretations. It will still be very important to quantify the GCGR on the cell surface by a method other than "total binding relative to control". Since you have performed competition binding of GCG radioligand and included IC50 data in Table 1, the homologous competition curves should be analyzed for Ki and Bmax values. This will be much more important and informative than what is shown in Figure 1.	As explained above, Bmax has now been calculated for a second independent cell line. Ki is proportional to IC50 and therefore will be comparable with and without RAMP2. **Amendments:** **Methods: page 5 lines 131-134, page 6 lines 160-163** **Results: page 10 lines 288-290, 304-310** **Addition of Supplemental Figure 2**
2. Essentially all of the functional data presented can be explained by lower surface GCGR expression in the RAMP2 positive cell line than in the control cell line. It will be important to prepare and characterize several clonal lines with RAMP2 coexpression to be certain that the very low levels of GCGR in this line is reflective of the coexpression itself and not an artifact of the single clonal line studied.	To ensure that these findings were attributable to co-expression of RAMP2 with the GCGR, rather than artefactual, a second cell line with RAMP2 stably upregulated was investigated (CHO-K1-GCGR-CFP-RAMP2) and compared to a cell line transfected in parallel with a control (pcDNA3.1) plasmid. Importantly, these findings corroborate those described in the first cell line. **Amendments:** **Methods: page 5 lines 131-134, page 6 lines 160-163** **Results: page 10 lines 288-290, 304-310** **Addition of Supplemental Figure 2**
3. It would be quite interesting to start with a stable RAMP2-expressing CHO cell line and a non-RAMP-expressing CHO cell line and make stable GCGR-	The reviewer makes a good point, although we feel that this extensive work- not requested during the

expressing lines from both via independent transfection to try to achieve clonal lines with similar surface GCGR expression. That would be quite interesting to study functionally. I understand that this would represent a substantial new effort.	first round of review- is better served in another manuscript. Ideally, we would use CRISPR-Cas9 to delete/replace the endogenous loci in a beta or hepatocyte cell line, thus leading to stable and physiological GCGR expression levels in the presence or absence of RAMP. We have discussed this in the revised manuscript. **Amendments: Discussion page 15 line 471-474**
4. The data set that best seems to support a mechanism for the reduction in cell surface receptor when RAMP gets coexpressed is figure 6 in which morphology is done on transfected HEK cells, and the RAMP-transected cells clearly had fluorescent receptor internalized. I fear that there could be glucagon in the serum used to culture these cells and the agonist-stimulated internalization response could be amplified in the presence of the RAMP. This could be quite interesting, but would require additional experiments. It would be helpful to perform this and other experiments in the absence of serum that could contain glucagon or other agonist. Another way to achieve this would be to perform the work in the presence of glucagon antagonist.	The GCGR experiments performed in HEK cells were designed to corroborate the radioactive binding assays, rather than a complete work-up of receptor internalization mechanisms. In any case, we would not expect meaningful glucagon levels in FBS, since the fetus secretes high levels of insulin to counteract elevated maternal glucose concentration, and this would strongly inhibit alpha cell function. Additionally, glucagon-stimulated GCGR internalization is known to be a high dose phenomenon, occurring at ≥1 µM (Roed et al JBC 2015), far above the known glucagon concentration in calves of 30-40 pM (Bloom et al, J Physiol, 1974). Thus the results are likely due to the interaction between non-bound/non-activated receptor and RAMP. The reviewer makes a reasonable point, however, and we discuss the requirement for future experiments with a GCGR antagonist or serum-free medium, or alternatively excess glucagon, in the revised manuscript. **Amendments: Discussion page 15 line 451-454**
5. I am concerned that the differences being reported here from the literature may be a function of low level receptor expression that reflects clonal choice and/or hormone-stimulated internalization. I would like to see three additional sets of data to be convinced this is real. 1) independent clonal lines for the CHO-GCGR-RAMP2 expression to be sure the low level of surface receptor is real; 2) these lines need not only IC50 data for receptor binding, but also Bmax data to quantitatively determine receptor density; and 3) use of non-serum-containing medium or use of GCG antagonist to block agonist effect to downregulate the receptor.	As described above, we have completed additional experiments to address points 1) and 2). 1) Independent clonal lines for CHO-GCGR-RAMP2 expression confirm that the low level of surface receptor is real; 2) these lines provide IC50 data for receptor binding, but also Bmax data to quantitatively determine receptor density. In terms of 3), we have inserted a caveat into the discussion re. GCGR antagonist, although as explained in response to the point above, the glucagon levels in FBS are expected to be far below the levels which have been demonstrated to be necessary to observe agonist-downregulation of the glucagon receptor.

RAMP2 influences glucagon receptor pharmacology via trafficking and signaling

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ABSTRACT

Endogenous satiety hormones provide an attractive target for obesity drugs. Glucagon causes weight loss by reducing food intake and increasing energy expenditure. To further understand the cellular mechanisms by which glucagon and related ligands activate the glucagon receptor (GCGR), we have investigated the interaction of the GCGR with RAMP2, a member of the family of Receptor Activity Modifying Proteins.

We have used a combination of competition binding experiments, cell surface ELISA, functional assays assessing the Gαs and Gq pathways and β-arrestin recruitment, and siRNA knockdown to examine the effect of RAMP2 on the GCGR. Ligands tested were glucagon, glucagon-like peptide-1 (GLP-1), oxyntomodulin and analogue G(X), a GLP-1/glucagon co-agonist developed in-house. Confocal microscopy was employed to assess whether RAMP2 affects the subcellular distribution of GCGR.

Here we demonstrate that co-expression of RAMP2 and the GCGR results in reduced cell surface expression of the GCGR. This was confirmed by confocal microscopy, which demonstrated that RAMP2 co-localises with the GCGR and causes significant GCGR cellular redistribution. Furthermore, the presence of RAMP2 influences signalling through the Gαs and Gαq pathways, as well as recruitment of β-arrestin. This work suggests that RAMP2 may modify the agonist activity and trafficking of the GCGR, with potential relevance to production of new peptide analogues with selective agonist activities.

INTRODUCTION

Gut and pancreatic hormones involved in appetite regulation are an attractive target for the development of drugs that aim to cause effective weight loss with minimal side effects. Glucagon has been shown to potently increase satiety and acutely reduce food intake in humans (1). Additionally, glucagon significantly increases energy expenditure in man (2-4). This, in association with the anorectic effects of glucagon (1), enhances its usefulness as an anti-obesity therapy.

The glucagon receptor (GCGR) is a 7 transmembrane class B G-protein coupled receptor (GPCR). It classically activates adenylyl cyclase through Gαs with subsequent activation of protein kinase A (PKA) signalling (5,6). In hepatocytes, elevated PKA activity suppresses glycolysis and glycogen synthesis, and enhances gluconeogenesis and glycogenolysis (7,8). However activation of GCGR also stimulates the phospholipase C-inositol phosphate pathway in hepatocytes via Gq, inducing intracellular calcium (Ca^{2+}) signalling and stimulating glycogenolysis and gluconeogenesis (6,9). Although work to unpick glucagon signalling pathways has been underway since the 1970s, it has focussed primarily on understanding the interactions involved in the downstream effects in the liver and the pancreas. Less attention has been paid to the role of specific pathways in the extrahepatic roles of glucagon, namely in appetite regulation and control of energy expenditure. As a prototypical class B GPCR, the GCGR is desensitised and sequestered in the cytosol following activation (10-12). The internalised receptor is then either recycled to the cell surface or targeted for degradation. Krilov et al recently demonstrated that the GCGR recycles to the plasma membrane in a β-arrestin-dependent manner, and that downregulation of β-arrestins significantly reduces recycling (13,14).

Understanding the interaction of these pathways may allow 'biasing' of signalling to exploit desirable downstream effects (15,16). A particularly well characterised example of an accessory protein that clearly alters the pharmacology of GPCRs is a family of single transmembrane proteins known as Receptor Activity Modifying Proteins (RAMPs). RAMPs were discovered as proteins that interact with the calcitonin receptor–like receptor (CRLR) and calcitonin receptor (CTR) to give rise to receptors for different ligands (17). These four ligands (calcitonin, amylin, calcitonin gene-related peptide and adrenomedullin) bind to two receptors and in the presence of the three RAMPs give rise to seven different receptor types with distinct pharmacology (18). Additionally, RAMPs have a role in receptor trafficking including translocation from the endoplasmic reticulum (ER) to the Golgi, internalisation and recycling of the receptor (19-26). RAMPs have been shown to heterodimerise with a number of class B and C GPCRs, and influence their function and life-cycle (27,28). The ability of RAMPs to influence downstream signalling pathways is an exciting concept, as it may enable the creation of biased agonists that fully exploit the therapeutic potential of clinically important receptors.

The functional impact of RAMPs on GCGR pharmacology is not clearly understood. Over ten years ago, the Christopoulos group showed that the GCGR may interact with RAMP2 (27). Recently, one study has found that RAMP2 may alter GCGR ligand selectivity and G protein preference using yeast reporter systems (29). The work presented here is concerned with further understanding the effect of RAMP2 on the pharmacology of the GCGR in mammalian cells.

MATERIALS AND METHODS

Peptides

Human GCG, GLP-1 and OXM were purchased from Bachem, Ltd. (UK). GLP-1(7-36)NH_2 was the form used in all experiments, and will now be referred to simply as GLP-1. A dual glucagon/GLP-1 analogue, G(X), was designed in the Department of Investigative Medicine, Imperial College London and custom synthesised using solid-phase peptide synthesis (Bachem Ltd). G(X) contains identical amino acid sequences to glucagon from positions 1 to 15 as the N-terminal of glucagon has been shown to be critical for glucagon receptor binding and activation (30). To create a dual agonist that is also effective at the GLP-1 receptor, G(X) has been modified to resemble exendin-4. This peptide, first isolated from the venom of the lizard *Heloderma* species, has been found to be a potent agonist at the human GLP-1 receptor (31,32). Also favorable is its prolonged pharmacokinetic profile compared to native GLP-1. Therefore, from positions 16-34, amino acid substitutions have been made to resemble exendin-4.

Establishing a cellular co-expression system for RAMP2 and GCGR

Chinese hamster ovarian (CHO-K1 cells; GeneBLAzer® GCGR-CRE-*bla* CHO-K1 cells; K1855A)) (Invitrogen) cells expressing the GCGR were cultured in DMEM supplemented with 10% FBS, 0.1 mM non-essential amino acids, 25 mM HEPES (pH7.3), 100 IU/ml penicillin, 100 µg/ml streptomycin and 5 µg/ml blastocidin. This cell line expressed no background RAMP2, as confirmed using QPCR (CT values >32). The human RAMP2 DNA construct (pCMV6-AC-RAMP2) (Origene, USA) was transfected into CHO-K1 cells expressing the human GCGR using polyethylenimine (PEI, Sigma) (33). The cells were transfected with pCMV6-AC-RAMP2 (containing a neomycin resistance gene) and 9 nitrogen equivalents of PEI. Forty-eight hours later, media was supplemented with 800 µg/ml Geneticin to select cells containing the construct.

To establish a second independent cell line stably expressing RAMP2, CHO-K1 cells expressing the human GCGR were co-transfected with C-terminally CFP-tagged RAMP2 (Tebu-bio Ltd, UK) and a plasmid conferring puromycin resistance using lipofectamine 2000 (Thermo Fisher). Forty-eight hours later, media was supplemented with puromycin 10 µg/ml to select cells containing the construct.

Confirmation of gene expression

RNA was extracted from cells using a Purelink RNA Mini Kit and DNase set (Invitrogen, UK), reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, UK) and cDNA amplified by qPCR (probe Hs00359352_m1) (Life Technologies, UK) via a 7900HT Fast Real-Time PCR System (Applied Biosystems, UK).

Whole cell binding assays

Cells were grown up to 70% confluence and resuspended in 1.5 ml assay buffer (25 mM HEPES (pH 7.4), 2 mM $MgCl_2$, 1% BSA, 0.05% (w/v) Tween 20, 0.1 mM diprotin A and 0.2 mM PMSF). 50 µl of I^{125}-glucagon dissolved in assay buffer at 1000 counts per second (final concentration 5.6 nM), unlabelled peptide made up in 400 µl of assay buffer and 50 µl of the cell suspension was added to each microtube, vortexed and incubated at room temperature for 90 minutes. Microtubes were then centrifuged (15781 x g, 4°C, 3 minutes), supernatant removed, 500µl of assay buffer added, and then re-centrifuged. The supernatant was again discarded and the pellets measured for γ radiation for 240 seconds (Gamma counter NE1600, NE Technology Ltd, UK). The specific binding (maximal specific binding minus the non-specific binding) was calculated for each cell line. The binding data was normalised so that the maximal specific binding (i.e. when no unlabelled peptide was present) was 100%. The percentage specific binding was calculated for each peptide concentration as a percentage of the specific binding. The half-maximal inhibition concentrations (IC_{50}), a measure of binding affinity, were then calculated and compared for CHO-K1-GCGR and CHO-K1-GCGR-RAMP2 cells. IC50 values were calculated using the Graphpad Prism 5.01 (GraphPad Software Inc., USA) using the following regression fit line:

$$Y=Bottom + (Top-Bottom)/(1+10^{((LogEC_{50}-X))})$$

Where Y=% specific binding and X=concentration of the agonist.

To calculate receptor density (Bmax), binding data was normalised to protein content of the cell samples, as determined by a bicinchoninic acid assay (Sigma). Bmax was then calculated for using GraphPad Prism 7.0b (GraphPad Software Inc., USA) using the following regression fit line:

$$Y=(Bmax*HotnM)/(HotnM + ColdNM + KdNM) + Bottom$$

Cell surface expression experiment

CHO-K1 cells overexpressing the human GCGR (± RAMP2) were seeded overnight in 96 well plates (30,000/well). Following fixation (2% PFA), an in-cell ELISA was performed in non-permeabilised cells to detect surface GCGR expression. Antibodies used were rabbit primary vs. GCGR N-terminus (1:200, ab137649 (Abcam, UK) and anti-rabbit IgG HRP-conjugated secondary (1:2000, #15015) (Active Motif, UK), with 2% BSA block used during in all incubations. TMB substrate (Thermo Scientific, UK) was added and absorbance read at 450 nm after addition of 1M HCl. Surface GCGR expression was calculated as absorbance after subtraction of non-specific binding (determined in the absence of primary antibody) and normalisation to protein content (BCA assay).

cAMP accumulation assay for activation of adenylyl cyclase

CHO-K1 cells overexpressing the human GCGR (± RAMP2), plated onto 48 well plates at 40,000 cells/well, were incubated in serum-free media for 1 hour. Peptides were prepared in serum free DMEM containing 100 µM of IBMX (3-isobutyl-1-methylxanthine, Sigma-Aldrich, UK). The cells were incubated for 30 minutes with the test peptide after which media was replaced with 110 µl lysis

buffer (0.1M HCl with 0.5% Triton-X). The lysate was assayed using a direct cyclic AMP ELISA kit (Enzo Life Sciences, UK), as described in the assay manual. The cAMP response was corrected for well protein levels (Bradford reagent, Sigma) and expressed as a percentage of response to 10 µM forskolin.

Human hepatoma 7 cells overexpressing the human GCGR (Huh7-GCGR) were cultured in DMEM supplemented with 10% FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin and 10 µg/ml geneticin (standard maintenance media). They were plated onto 96 well plates at 20,000 cells/well in standard maintenance media with transfection reagents for gene silencing (see details below). After 72 hours, media was aspirated and replaced with 40µl of glucagon at different concentrations, prepared in serum-free DMEM. The cells were incubated for 30 minutes with the glucagon after which an equal volume of cAMP lysis buffer (CisBio cAMP Dynamic cell based assay kit) was added to each well. 25µl of lysate was transferred to a HTRF-compatible plate, and 12µl of 'D' reagent was added to each well, followed by 12µl of 'K' reagent in accordance with the manufacturer's instructions. The plate was read (i3x plate reader (Molecular Devices)) after 1 hour incubation at room temperature and cAMP response was expressed as a percentage of response to 10µM forskolin.

The maximal response (E_{max}) and the half-maximal effective concentrations (EC_{50}) were then calculated and compared for each peptide tested between CHO-K1-GCGR and CHO-K1-GCGR-RAMP2 cells, and for glucagon between Huh7-GCGR RAMP2 knock down and Huh7-GCGR control cells. EC_{50} values were calculated using the following regression fit line:

$$Y=Bottom + (Top-Bottom)/(1+10^{((LogEC_{50}-X)*HillSlope)})$$

where Y= cAMP response and X=agonist concentration. The curve fitting for the biphasic curves was done using a variable slope (four parameters) model.

Intracellular Ca^{2+} flux assay

The DiscoveRx Ca NW^{PLUS} Assay Kit (DiscoveRx Corporation Ltd, UK) was used as per the manufacturer's protocol to detect changes in intracellular Ca^{2+} in CHO-K1 cells overexpressing the human GCGR (± RAMP2) in response to glucagon, GLP-1, oxyntomodulin and analogue G(X). Cells, plated overnight onto 96 well plates at 50,000 cells/well were incubated in 75 µl Ca NW^{PLUS} working reagent for 1 hour at 37°C. 25 µl of glucagon (or related peptide) was applied from the reagent plate to the cell plate via the integrated transfer pipettor of a fluorescent microplate reader (NOVOstar, BMG Labtech Ltd, UK). Fluorescence signal was measured from 5 seconds prior to 30 seconds post injection of agonist. The Ca^{2+} response was expressed as a percentage of the ATP response (1 µM).

β-Arrestin recruitment assay

PathHunter™ CHO-K1 GCGR β-Arrestin GPCR assay (DiscoveRx Corporation Ltd, UK) was used to determine the effect of RAMP2 on the potency of GCGR ligands for recruitment of β-Arrestin-1 to the GCGR. The CHO-K1-βArr-GCGR cells are engineered to detect the interaction of β-arrestin with the activated GCGR using β-galactosidase fragment complementation. CHO-K1-βArr-GCGR cells were stably transfected ± RAMP2, as described above. Cells, plated at 100 µl/well into a 96-well plate were incubated with glucagon, GLP-1, oxyntomodulin, or G(X) (10 µl) for 90 minutes at 37°C and 5% CO_2. 55 µl of the PathHunter™ detection reagents was added to each well and the microplate was incubated at room temperature for 60 minutes.

SiRNA knockdown

SiRNA knockdown of RAMP2 in CHO-K1-GCGR-RAMP2 and CHO-K1-βArr-GCGR-RAMP2 cells was performed using pooled siRNA to RAMP2 previously validated by Albertin et al (34). SiRNA complexes (fully deprotected and desalted, Sigma, UK), added in a single pool (containing 4 duplexes) at final concentrations of 10 nM and 50 nM, were used for transfection with siPORT™ NeoFX™ (Ambion). siPORT NeoFX (diluted 1:20 into serum-free medium) and RNAs were combined (1:1) and incubated for 10 min at room temperature. The complexes (200 µl/well) were then dispensed into a 6-well plate and 2.3 ml of cell suspension containing 150,000 cells/well was added. The effects on RAMP2 gene expression were assessed 24 hours later. The effect of RAMP2 knockdown on GCGR signalling was carried out in a 96-well plate 24 hours later with volumes adjusted as follows: siRNA 10 µl/well, SiPORT NeoFX 10 µl/well, cell suspension 80 µl (6000 cells)/well.

In Huh7-GCGR cells, RAMP2 expression was transiently silenced using small interfering RNA against human RAMP2 (Ramp2 Silencer Select siRNA, Ambion). Lipofectamine 2000 reagent (Thermo Fisher) was diluted in Opti-MEM Reduced Serum medium (Thermo Fisher) (0.2µl/5µl) and then added to siRNA also diluted in Opti-MEM (0.5pmol/5µl) for an incubation period of 5 minutes. The siRNA-lipofectamine complex (final volume 10µl/well) was dispensed into the wells of a 96-well plate, and to each well 100µl of cell suspension at 150,000 cells/well was added. Cells were incubated for 72 hours. Control cells underwent exactly the same procedure except with siRNA with no gene target (Silencer Select Negative Control No.1 siRNA, Thermo Fisher).

Confocal microscopy

HEK293 cells were stably transfected with C-terminal GFP-tagged GCGR (Origene, USA) using Lipofectamine 2000 (Life Technologies Ltd, UK) as per the manufacturer's protocol. GFP-tagged GCGR-expressing HEK293 cells were seeded onto sterile coverslips coated with poly-L-lysine in a 6 well plate and transiently transfected ± C-terminally CFP-tagged RAMP2 (Tebu-bio Ltd, UK), non-tagged RAMP2 (Origene, USA) or empty vector (pcDNA3.1). The following day, cells were fixed

with 2% paraformaldehyde (PFA) (Sigma, UK) and mounted with Vectashield (Vector Laboratories Ltd, UK). A first set of experiments was carried out using a Crest X-Light spinning disk system coupled to a Nikon Eclipse Ti microscope and a 63x 1.4 NA oil immersion objective. GFP was excited using a solid-state at λ = 491 nm laser (Cobalt) and emitted signals collected at λ = 525/25 nm using a highly-sensitive Orca-Flash4.0 Digital CMOS camera. Due to bleedthrough of the intense GFP signal into the CFP channel at λ = 440 nm, the latter fluorophore was instead excited slightly off-peak using a solid-state 405 nm laser and emitted signals collected at λ = 525/25 nm. A second set of experiments was performed using a Zeiss LSM780 confocal microscope and a 63x 1.2 NA water immersion objective. GFP and CFP were excited using a λ = 488 nm argon laser and emitted signals collected at λ = 510 - 550 nm using a GaAsP spectral detector. CFP was excited using a λ = 405 nm diode laser and emitted signals collected at λ = 455-490. Images were post-processed using Zen software (Zeiss, UK) and subjected to Gaussian smoothing (1.3) to remove noise. Uniform linear adjustments were applied to contrast and brightness to improve image quality for analysis and presentation purposes, while preserving the pixel dynamic range and the intersample intensity differences. Cell surface expression of GCGR-GFP was calculated using the threshold plugin for ImageJ (NIH).

Statistical analysis

E_{max} and EC_{50} values, derived through 4 parameter curve fit were compared by paired t-test. Prism Version 5.01 (GraphPad Software Inc. San Diego, USA) was used for statistical analysis. $p<0.05$ was conventionally considered statistically significant. Zero concentration points were not included on the graphs in figures 2-6 for ease of viewing. Comparison of RAMP2 expression between two groups was performed using either unpaired or paired Student's t-test, or where multiple comparisons where required, one-way ANOVA followed by Bonferroni's multiple comparisons post hoc test. Controls with no peptide added were included in all experiments.

RESULTS

Confirmation of transfection of CHO-K1-GCGR and CHO-K1-βArr-GCGR cells with RAMP2

Successful transfection into CHO-K1-GCGR cells of the pCMV6-AC-RAMP2 plasmid was confirmed by qPCR. Subsequently, a second CHO-K1 cell line expressing the GCGR containing the β-arrestin recruitment reporter signal (CHO-K1-βArr-GCGR) was also transfected with RAMP2 (Supplementary Figures 1A and B).

Similarly, successful transfection of CHO-K1-GCGR cells with C-terminally CFP-tagged RAMP2 (Tebu-bio Ltd, UK) was confirmed by qPCR. H-RAMP2 was undetectable in the control cell line but expressed in the CHO-K1-GCGR-CFP-RAMP2 cells.

RAMP2 reduces specific glucagon binding at the GCGR

When specific glucagon binding to the GCGR was compared in RAMP2 positive and negative CHO-K1 cells, it was found to be 10-fold lower in the presence of RAMP2 (see Figure 1A). This was despite the protein content being similar in both groups (see Figure 1B).

Glucagon bound to the GCGR with an IC_{50} of 1.403 nM. This was not significantly altered when the GCGR was co-expressed with RAMP2 (Figure 1C, table 1). As expected, GLP-1 had poor affinity for the GCGR with an IC_{50} of >10000 nM (Figure 1D). Oxyntomodulin and analogue G(X) showed a 7-fold and 2.5 fold lower affinity for the GCGR than the native peptide, respectively (Figures 1E and F). Similar to glucagon, the presence of RAMP2 had no effect on the binding affinity at the GCGR for GLP-1, oxyntomodulin or analogue G(X).

To ensure that these findings were attributable to co-expression of RAMP2 with the GCGR, a second independent cell line with RAMP2 stably upregulated was investigated (CHO-K1-GCGR-CFP-RAMP2) and compared to a cell line transfected in parallel with a control (pcDNA3.1) plasmid. As with the first cell line (CHO-K1-GCGR-RAMP2), the binding affinity of glucagon for its receptor was not altered with the upregulation of RAMP2 (IC_{50} 4.377nM with CFP-RAMP2 vs 5.123nM without (p=0.16)) (Supplemental Figure 2A), however the density of GCGR binding sites (Bmax) was significantly lower in the cell line with upregulated RAMP2 (p=0.0069) (Supplemental Figure 2B).

RAMP2 reduces cell surface expression of the GCGR

Using an in-cell ELISA, surface GCGR expression was detected in non-permeabilised CHO-K1-GCGR cells (± RAMP2) (Supplementary Figure 3). GCGR cell surface expression was significantly reduced in cells expressing RAMP2.

RAMP2 reduces potency and increases efficacy of the Gαs pathway at the GCGR

To assess whether RAMP2 affected the Gαs pathway, cAMP accumulation was measured in its presence/absence in CHO-K1 cells (Figure 2, Table 2). In control cells, the highest concentrations of peptide resulted in cAMP accumulation lower than the E_{max}, which is a well described desensitisation effect (14). In the presence of RAMP2, glucagon, oxyntomodulin and analogue G(X) increased the EC_{50} i.e. RAMP2 reduced the potency of these ligands for GCGR Figure 2A, C and D). When the GCGR was stimulated by oxyntomodulin or analogue G(X), the E_{max} (efficacy) was increased in the presence of RAMP2. The EC_{50} and E_{max} were not calculable for GLP-1 response at the concentrations used (Figure 2B). There was no significant difference in cAMP responses to forskolin between control and RAMP2 expressing cells (0.136 (± 0.01) vs. 0.140 (± 0.01), relative absorbance units respectively; p=0.20).

To investigate whether changes in cAMP accumulation at the GCGR conferred by RAMP2 were generalizable to other cell types, cAMP accumulation in response to glucagon was measured in Huh7-GCGR cells with or without RAMP2 knockdown. Huh7-GCGR cells express a low level of endogenous RAMP2 and silencing conferred approximately 70% knock down. There was no statistically significant change in glucagon potency in Huh7-GCGR cells with RAMP2 knockdown and a trend towards a lower E_{max}, although this was not statistically significant (Supplementary Figure 4).

RAMP2 reduces efficacy of the Gq pathway at the GCGR

To assess the effect of RAMP2 on the Gαq pathway, intracellular Ca^{2+} flux was measured in real time in CHO-K1 cells. For glucagon and oxyntomodulin, the Ca^{2+} response was attenuated when cells expressing the glucagon receptor were co-expressed with RAMP2, as demonstrated by a significantly lower E_{max} (Figure 3A, B, C and E). RAMP2 also appeared to lower the response to G(X), however, as the maximal Ca^{2+} response was not achieved with cells expressing GCGR alone, and E_{max} could not be determined (Figure 3F). Similarly, the EC_{50} and E_{max} were not calculable for GLP-1 response at the concentrations used (Figure 3D). EC_{50} was unchanged in the presence of RAMP2 for all ligands (Table 2). There was no significant difference in Ca^{2+} responses to ATP between control and RAMP2 expressing cells (RFU fold increase from baseline 1.81 (± 0.08) vs. 1.84 (± 0.10) respectively; p=0.77) (Figures 3A and B).

RAMP2 abolishes β-Arrestin recruitment at the GCGR

For all ligands (glucagon, GLP-1, oxyntomodulin and analogue G(X)), β-arrestin recruitment did not occur in CHO-K1 cells expressing both GCGR and RAMP2 (Figure 4).

RAMP2 knockdown partially restores GCGR functioning for the Gαs and Gq pathways

Efficient siRNA knockdown of RAMP2 was achieved with both 10 nM and 50 nM siRNA pools (Figure 5A). siRNA knockdown of RAMP2 in CHO-K1-GCGR-RAMP2 cells resulted in a trend toward restoration of cAMP EC_{50} and E_{max} to levels seen with control cells (CHO-K1-GCGR cells), however, they were not significantly different to control or RAMP2 (without siRNA) cells (Figure 5B). A similar finding was demonstrated for Ca^{2+} fluxes (Figure 5C). The EC_{50} and E_{max} data is summarised in Table 3.

The GCGR and RAMP2 colocalise and the GCGR is internalised in the presence of RAMP2

High resolution confocal microscopy showed that GCGR-GFP and RAMP2-CFP co-localised as puncta within the cytosol of HEK293 (Figure 6A). In cells where RAMP2 was not overexpressed, GCGR-GFP remained predominantly at the cell surface/membrane (Figure 6B). This was not due to bleedthrough of GCGR-GFP fluorescence into the RAMP2-CFP channel, since signal could not be detected in RAMP2 negative/GCGR positive cells (Figure 6C). Overexpression of non-native protein (pcDNA3.1) did not interfere with the distribution of the GCGR-GFP, which remained almost exclusively at the membrane (Figure 6D), whereas non-tagged RAMP2 led to a significant decrease in receptor at the cell membrane (Fig. 6E). This demonstrates that protein expression *per se* is unlikely to interfere with GCGR localisation. Thus, overexpression of RAMP2-CFP or RAMP2 consistently leads to a decrease in cell surface GCGR-GFP (Fig. 6F).

DISCUSSION

It has previously been demonstrated by immunofluorescence confocal microscopy that RAMP2 may interact with the glucagon receptor. We have investigated the functional effect of this possible interaction by looking specifically at the effect of RAMP2 on: 1) ligand binding at the GCGR; 2) GCGR cell signalling; and 3) GCGR subcellular distribution. Co-expression of RAMP2 with GCGR did not alter the binding affinity of glucagon or its related peptides. However, the presence of RAMP2 had a marked effect on signalling via the Gαs and Gq pathways, as well as β-arrestin recruitment. Furthermore, RAMP2 appears to co-localise with the GCGR and influence its subcellular distribution.

Interaction between calcitonin family receptors and the individual RAMP proteins alters both ligand binding affinity and the intracellular signalling pathways engaged (17,35,36). By contrast, we found that expression of RAMP2 with the GCGR did not cause a significant alteration in the binding affinity of glucagon and its related peptides in whole cells. However, competition binding experiments using ^{125}I-glucagon as the radioligand revealed that co-expression of RAMP2 resulted in a ten-fold reduction in GCGR binding sites when compared with those determined in the absence of RAMP. This reduction in specific binding of glucagon may be due to reduced receptor expression at the cell surface. This could have been a direct effect of the interaction of RAMP2 and the GCGR resulting in internalisation. Alternatively, it might be an indirect effect if, for example, RAMP2 influences GCGR cell surface expression via its effect on β-arrestin recruitment.

The presence of RAMP2 completely abolished β-arrestin recruitment. This finding was consistent for glucagon as well as GLP-1, oxyntomodulin and G(X). One possible explanation is that RAMP2 interacts with the GCGR at the same site as β-arrestin binds or causes steric hindrance, thereby disrupting β-arrestin recruitment. Krilov et al have shown that β-arrestins are crucial for the recycling of the GCGR (13) and, therefore, loss of β-arrestin recruitment may result in reduced cell surface expression of the GCGR when RAMP2 is present. Alternatively, reduced cell surface expression of GCGR may be the primary effect of RAMP2 and this may in turn prevent β-arrestin recruitment.

Co-expression of RAMP2 with the GCGR also altered the intracellular signalling properties of the receptor in CHO-K1-GCGR cells, with the same effects seen for all agonists tested. With regards to the Gαs pathway, the presence of RAMP2 caused a reduction in potency and increase in efficacy. In Huh7-GCGR cells, the knockdown of RAMP2 resulted in no change in potency and a trend towards decreased efficacy. Whether this is a result of a change in availability of binding sites is yet to be determined. In contrast to our findings, Weston et al found that RAMP2 increases potency of the cAMP response at the GCGR (29). One possible explanation for these different findings could be the different cell lines used. Weston et al overexpressed RAMP2 in HEK cells that already express

endogenous RAMP2, whereas we overexpressed RAMP2 in CHO-K1 cells that do not express RAMP2. It has previously been shown that interaction of the CTR with RAMPs, especially RAMP2, is sensitive to the cellular background in which it is expressed, suggesting that other cellular components, such as G proteins, are likely to contribute to RAMP-receptor interactions (36).

The increase in efficacy of cAMP production observed with RAMP2 is intriguing. This enhancement in cAMP response is all the more striking as it is in the face of an apparent reduction of cell surface expression of GCGR. The simplest interpretation is that by some mechanism, RAMP2 increases the accessibility of the receptor to the G-protein (37). Alternatively, RAMP2 may inhibit the desensitisation response that is classically seen with the GCGR, involving phosphorylation of receptors by GPCR kinases (GRKs) and binding of β-arrestins, which uncouple receptors from G-proteins (38). We speculate that the GCGR-RAMP2 interaction causes loss of desensitisation, which may be driven by inhibition of β-arrestin recruitment. Indeed, RAMPs are crucial in the post-endocytic sorting of the CRLR, suggesting a broader regulatory role for RAMPs in receptor trafficking (24,25).

On examination of the Gq pathway, intracellular Ca^{2+} fluxes were found to be attenuated in the presence of RAMP2. Interestingly, preferential coupling to Gαs versus Gq has been reported for AMY1 and AMY3 receptors, but not AMY2 (39). The finding that cAMP signalling is specifically augmented and Ca^{2+} signalling attenuated by RAMP2 at the GCGR is important because the classic coupling pathway associated with GCGR activation has always been thought to be the stimulation of cAMP accumulation. Moreover, the presence or absence of endogenous RAMP2 may account for discrepancies in previous studies examining the signalling mechanisms engaged by the GCGR. Whether this is tissue-specific and dependent on the prevailing physiological conditions is yet to be seen.

Visualisation of RAMP2 and the non-ligand bound GCGR using confocal microscopy revealed two key findings. Firstly, it is demonstrated that RAMP2 and the GCGR show some co-localisation, although super-resolution approaches will be needed to confirm this, as well as delineate the compartment(s) involved. Secondly, in the presence of RAMP2, there was reduced GCGR cell surface expression. This is consistent with the competition binding and ELISA experiments, which found reduced binding of ^{125}I-GCG in the presence of RAMP2. These findings appear to be at odds with the work done by Christopoulos et al which reported that, when co-expressed with GCGR, RAMP2 translocates to the cell surface. A number of differences exist in the experimental approach between this current study and that of Christopoulos. Firstly, in their study only the RAMPs, and not the GCGR, were tagged so it was not possible to comment on where the receptor was trafficked to.

Secondly, in the Christopoulos study, RAMP2 was N-terminally tagged with haemagglutinin whereas here both C-terminally CFP-tagged and native RAMP2 was utilised. It is the N-terminal that contains the natural, predicted signal peptide sequence of RAMP2 and therefore this may have had a bearing on expression of RAMP2. In line with our findings, using C-terminal receptor-fluorescent protein fusion constructs and cell surface ELISAs of myc-tagged receptors, Weston et al found that expression of RAMP2 caused a non-significant decrease in cell surface expression of GCGR (29). To ensure that the agonist-stimulated internalization response is not due to glucagon in the serum used to culture these cells, with amplification in the presence of RAMP2, further experiments could be performed with a GCGR antagonist or serum-free medium, or alternatively excess glucagon.

Taken together, this work demonstrates that RAMP2 may affect the cell signalling pathways of the GCGR as well as its trafficking within the cell. There are two possible mechanisms by which RAMP2 could influence GCGR pharmacology. A direct on binding epitopes of the relevant ligands is possible. Alternatively, RAMP2 could act indirectly by altering the conformation of the GCGR.

This work has added to our understanding of GCGR's physiological function and how this may be modified by an allosteric modulator, RAMP2. This could be important in developing new therapeutic avenues for the treatment of obesity and diabetes. Allosteric modulation through the RAMP2 system may allow 'biasing' of the signalling pathways to exploit the desirable downstream effects, thus informing the construction of new peptide analogues with selective agonist activities. For example, these might incorporate therapeutically desirable properties such as appetite suppression and increase in energy expenditure, without unwanted properties such as increasing hepatic glucose output and hyperglycaemia.

The work conducted thus far has been in GCGR overexpressing cell lines. The logical next step would be to use primary cells in tissue relevant to glucagon receptor physiology. It would be interesting to use CRISPR-Cas9 to delete/replace the endogenous loci in a beta or hepatocyte cell line, thus leading to stable and physiological GCGR expression levels in the presence or absence of RAMP and study function. Additionally, endogenous tissue co-expression of RAMP2 and GCGR has not yet been investigated. RAMP mRNA tissue expression using northern blot analysis was reported initially by McLatchie et al on their first discovery of RAMPs (17). However, GCGR-relevant tissues such as brown adipose tissue, hypothalamus and the nodose ganglion were not specifically examined. An additional question is whether the RAMP2-GCGR interaction is controlled in a physiological setting. It would be important to determine what process controls this and what effect it has on glucagon signalling. Co-expression may occur in some tissues under certain conditions and not others as expression of RAMP2 may be controlled by the prevailing physiological conditions, for example, glucose and insulin levels.

In conclusion, RAMP2 can affect the cell signalling pathways of the GCGR as well as its trafficking within the cell. The effect that RAMP2 has on the GCGR and how this translates in vivo is yet to be determined.

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Author Contributions: JC, BJ, JG, TT and SRB designed the experiment; JC, BJ, JG, DJH, ERM and TM carried out the experiments; JC, BJ, JG, DJH, TT, ERM and SRB performed the analyses. JC, BJ, JG, DJH, ERM, TT and SRB wrote the manuscript. All authors contributed to editing the final manuscript and had final approval of the submitted article. JC is the guarantor of this work, had full access to all the data, and takes full responsibility for the integrity of data and the accuracy of data analysis.

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TABLE AND FIGURE LEGENDS

TABLE 1. Binding affinities of A) glucagon, B) GLP-1, C) oxyntomodulin and D) analogue G(X) to the human glucagon receptor. Whole CHO-K1-GCGR cells ± RAMP2 were used. I^{125}-glucagon was used as the competing peptide in all assays and IC_{50} values were calculated as a mean of four separate experiments (except for GLP-1 where n=2) with each peptide concentration performed in duplicate or triplicate during an individual experiment. Errors shown are ±SEM.

TABLE 2. Summary of cAMP accumulation and Ca^{2+} data for glucagon, GLP-1, oxyntomodulin and analogue G(X) at the glucagon receptor. EC_{50} is defined as the concentration of agonist required to cause 50% of the maximal possible effect of that agonist. E_{max} is the maximal response of the agonist expressed as a percentage of maximal positive control response. Values calculated as a mean from a minimum of four separate experiments (except for GLP-1 n=2). Values shown as mean ± SEM. * $p<0.05$ and ** $p<0.01$ comparing CHO-K1-GCGR cells ± RAMP2.

TABLE 3. The effect of siRNA knockdown of RAMP2 in CHO-K1-GCGR-RAMP2 cells on cAMP accumulation and Ca^{2+} flux for glucagon at the glucagon receptor. EC_{50} is defined as the concentration of agonist required to cause 50% of the maximal possible effect of that agonist. E_{max} is the maximal response of the agonist expressed as a percentage of maximal positive control response. Values shown as mean ± SEM. * $p<0.05$ and ** $p<0.01$ comparing CHO-K1-GCGR cells ± RAMP2. EC_{50} and E_{max} values for siRNA treated cells were not significantly different to control or RAMP2 positive cells. Values calculated as a mean from a minimum of two separate experiments. Errors shown are ±SEM.

FIGURE 1. A) Specific binding of I^{125}-glucagon to the GCGR in CHO-K1-GCGR cells ± RAMP2 ($p<0.0001$). B) The protein content was determined by Bradford assay (used here as a surrogate marker for the number of cells) for CHO-K1-GCGR cells ± RAMP2. Whole cell binding of C) glucagon, D) GLP-1, E) oxyntomodulin and F) analogue G(X) to the human glucagon receptor. Whole CHO-K1-GCGR cells ± RAMP2 were used. I^{125}-glucagon was used as the competing peptide in all assays and IC_{50} values were calculated as a mean of four separate experiments (except for GLP-1 where n=2), with each peptide concentration performed in duplicate or triplicate during an individual experiment. Values represent the mean ±SEM.

FIGURE 2. Human glucagon receptor-mediated cAMP accumulation in CHO-K1-GCGR cells ± RAMP2 by ligands A) glucagon, B) GLP-1, C) oxyntomodulin and D) analogue G(X). Each peptide concentration was tested in duplicate or triplicate in each experiment. Values calculated as a mean from a minimum of four separate experiments (unless stated otherwise). * $p<0.05$ comparing the E_{max} for CHO-K1-GCGR cells ± RAMP2. Values represent the mean ±SEM.

FIGURE 3. Intracellular Ca^{2+} flux in response to varying doses of glucagon in real-time in CHO-K1-GCGR cells A) without RAMP2 and B) with RAMP2 (measured in Relative Fluorescence Unit (RFU) fold increase from baseline RFU). Human glucagon receptor-mediated Ca^{2+} flux in CHO-K1-GCGR cells ± RAMP2 by ligands C) glucagon, D) GLP-1 and E) oxyntomodulin and F) analogue G(X). Each peptide concentration was tested in duplicate or triplicate in each experiment. Values calculated as a mean from a minimum of four separate experiments (unless stated otherwise). ** $p<0.01$ comparing the E_{max} for CHO-K1-GCGR cells ± RAMP2. Values represent the mean ±SEM.

FIGURE 4. Human glucagon receptor-mediated β-arrestin recruitment in CHO-K1- βArr-GCGR cells ± RAMP2 by endogenous ligands A) glucagon, B) GLP-1 and C) oxyntomodulin and D) analogue G(X). Each peptide concentration was tested in duplicate or triplicate in each experiment. Results are expressed as a percentage of maximal glucagon-mediated β-arrestin recruitment. Values calculated as a mean from a minimum of four separate experiments. **** $p<0.0001$ comparing the E_{max} for CHO-K1-βArr-GCGR cells ± RAMP2. Values represent the mean ±SEM.

FIGURE 5. Effect of siRNA knockdown of RAMP2 in CHO-K1-GCGR-RAMP2 cells on A) hRAMP2 expression by qPCR, B) cAMP accumulation and C) Ca^{2+} flux in response to glucagon.

Each peptide concentration was tested in duplicate or triplicate in each experiment. Values calculated as a mean from a minimum of two separate experiments. **p<0.01 ***p<0.001. Values shown are ±SEM.

FIGURE 6. Effect of RAMP2 on GCGR localisation. A) Representative image showing that GCGR-GFP (green) and RAMP2-CFP (red) can colocalise (yellow) within the cytosolic compartment following their overexpression (n = 15 cells) (zooms are inset to the right). B) Representative image showing that GCGR-GFP expression is predominantly at the membrane/surface in HEK cells without RAMP2 overexpression (n = 8 cells). C) A negative control indicating that the 405 nm laser does not excite GCGR-GFP (n = 3 cells). D) Overexpression of non-native protein (pcDNA3.1) does not interfere with the distribution of the GCGR-GFP, which remains at the membrane (n = 7 cells). E) Overexpression of non-tagged RAMP2 leads to redistribution of GCGR-GFP into the cell (n = 7 cells) (zooms are inset to the right). F) Bar graph showing that overexpression of either RAMP2-CFP or non-tagged RAMP2, but not pcDNA3.1, leads to a significant reduction in cell surface GCGR-GFP expression (**p<0.01) (n = 8-14 cells from at least three independent experiments). Scale bar = 10 µm. Values represent the mean ± SEM.

SUPPLEMENTARY FIGURE 1. Expression of RAMP2 in control cells and cells transfected with the pCMV6-AC-RAMP2 plasmid in two different cell lines A) CHO-K1-GCGR cells and B) CHO-K1-βArr-GCGR cells. CHO-K1-GCGR±RAMP2 cells were used for cAMP accumulation and Ca^{2+} flux experiments while CHO-K1-βArr-GCGR±RAMP2 cells, containing the β-Arrestin recruitment reporter signal, were used in the β-Arrestin recruitment assays. Values calculated as a mean from a minimum of two separate experiments. ****p<0.0001. Errors shown are ±SEM.

SUPPLEMENTARY FIGURE 2. A) Whole cell binding of glucagon to the human glucagon receptor, with I^{125}-glucagon used as the competing peptide. Whole CHO-K1-GCGR cells ± CFP-RAMP2 were used. Data is presented as mean and standard error of mean of four separate experiments with each peptide concentration performed in duplicate or triplicate during an individual experiment. B) Whole cell binding of glucagon to the human glucagon receptor, with I^{125}-glucagon used as the competing peptide. Whole CHO-K1-GCGR cells ± CFP-RAMP2 were used. Data is presented as mean and standard error of mean of Bmax as calculated from three separate experiments with each peptide concentration performed in duplicate or triplicate during an individual experiment.**p<0.01)

SUPPLEMENTARY FIGURE 3. Cell surface GCGR expression in CHO-K1-GCGR cells ± RAMP2. Surface GCGR expression was calculated as absorbance after subtraction of non-specific binding (determined in absence of primary antibody) and normalisation to protein content (BCA assay). Values expressed in arbitrary units (the signal divided by the protein content in mg/ml). Values calculated as a mean from five separate experiments. * p<0.05 comparing CHO-K1-GCGR cells ± RAMP2. Values represent the mean ±SEM.

SUPPLEMENTARY FIGURE 4. Human glucagon receptor-mediated cAMP accumulation in Huh7-GCGR cells by glucagon ± RAMP2 knockdown. Each peptide concentration was tested in duplicate or triplicate in each experiment. Summary of EC_{50} and E_{max} data is shown in the accompanying table. Values calculated as a mean from three separate experiments. Values shown as mean ± SEM.

Table 1

IC_{50}	GCGR Control	GCGR + RAMP2
Glucagon	**1.403 nM** ±0.21	**0.768 nM** ±0.15
GLP-1	**>10000 nM**	**>10000 nM**
OXM	**10.43 nM** ±2.59	**3.873 nM** ±0.93
G(X)	**3.381 nM** ±1.07	**3.984 nM** ±1.81

TABLE 1. Binding affinities of A) glucagon, B) GLP-1, C) oxyntomodulin and D) analogue G(X) to the human glucagon receptor. Whole CHO-K1-GCGR cells ± RAMP2 were used. I^{125}-glucagon was used as the competing peptide in all assays and IC_{50} values were calculated as a mean of four separate experiments (except for GLP-1 where n=2) with each peptide concentration performed in duplicate or triplicate during an individual experiment. Errors shown are ±SEM.

Table 2

		cAMP accumulation		intracellular Ca^{2+} flux	
		CHO-K1-GCGR cells	**CHO-K1-GCGR cells +RAMP2**	**CHO-K1-GCGR cells**	**CHO-K1-GCGR cells +RAMP2**
Glucagon	**EC_{50} (nM)**	**0.161** ± 0.063	**1.263** ± 0.289*	**256.5** ± 27.46	**314.1** ± 37.03
	E_{max} (%)	**34.04** ± 6.897	**54.50** ± 9.781	**109.0** ± 2.215	**57.7** ± 1.313**
GLP-1	**EC_{50} (nM)**	**N/A**	**N/A**	**N/A**	**N/A**
	E_{max} (%)	**N/A**	**N/A**	**N/A**	**N/A**
Oxyntomodulin	**EC_{50} (nM)**	**1.089** ± 0.382	**12.97** ± 8.544*	**109.6** ± 11.5	**156.8** ± 43.21
	E_{max} (%)	**34.69** ± 6.815	**46.23** ± 7.409*	**108** ± 9.28	**64** ± 12.3**
Analogue G(X)	**EC_{50} (nM)**	**0.074** ± 0.056	**0.538** ± 0.065*	**N/A**	**656.6** ± 35.0
	E_{max} (%)	**31.11** ± 3.578	**65.43** ± 7.027*	**N/A**	**62.5** ± 6.06

TABLE 2. Summary of cAMP accumulation and Ca^{2+} data for glucagon, GLP-1, oxyntomodulin and analogue G(X) at the glucagon receptor. EC_{50} is defined as the concentration of agonist required to cause 50% of the maximal possible effect of that agonist. E_{max} is the maximal response of the agonist expressed as a percentage of maximal positive control response. Values calculated as a mean from a minimum of four separate experiments (except for GLP-1 n=2). Values shown as mean ± SEM. * $p<0.05$ and ** $p<0.01$ comparing CHO-K1-GCGR cells ± RAMP2.

Table 3

		CHO-K1-GCGR cells	CHO-K1-GCGR cells +RAMP2	CHO-K1-GCGR cells +RAMP2 SiRNA 10nM	CHO-K1-GCGR cells +RAMP2 SiRNA 50nM
cAMP accumulation	**EC_{50} (nM)**	**0.161** ± 0.063	**1.263** ± 0.289*	**0.778** ± 0.018	**0.846** ± 0.018
	E_{max} (%)	**34.04** ± 6.897	**54.50** ± 9.781	**50.46** ± 0.96	**51.26** ± 0.11
intracellular Ca^{2+} flux	**EC_{50} (nM)**	**256.5** ± 27.46	**314.1** ± 37.03	**387.5** ± 108.5	**295.3** ± 54.2
	E_{max} (%)	**109.0** ± 2.215	**57.7** ± 1.313**	**74.13** ± 2.5	**70.23** ± 1.5

TABLE 3. The effect of siRNA knockdown of RAMP2 in CHO-K1-GCGR-RAMP2 cells on cAMP accumulation and Ca^{2+} flux for glucagon at the glucagon receptor. EC_{50} is defined as the concentration of agonist required to cause 50% of the maximal possible effect of that agonist. E_{max} is the maximal response of the agonist expressed as a percentage of maximal positive control response. Values shown as mean ± SEM. * $p<0.05$ and ** $p<0.01$ comparing CHO-K1-GCGR cells ± RAMP2. EC_{50} and E_{max} values for siRNA treated cells were not significantly different to control or RAMP2 positive cells. Values calculated as a mean from a minimum of two separate experiments. Errors shown are ±SEM.

Figure 1

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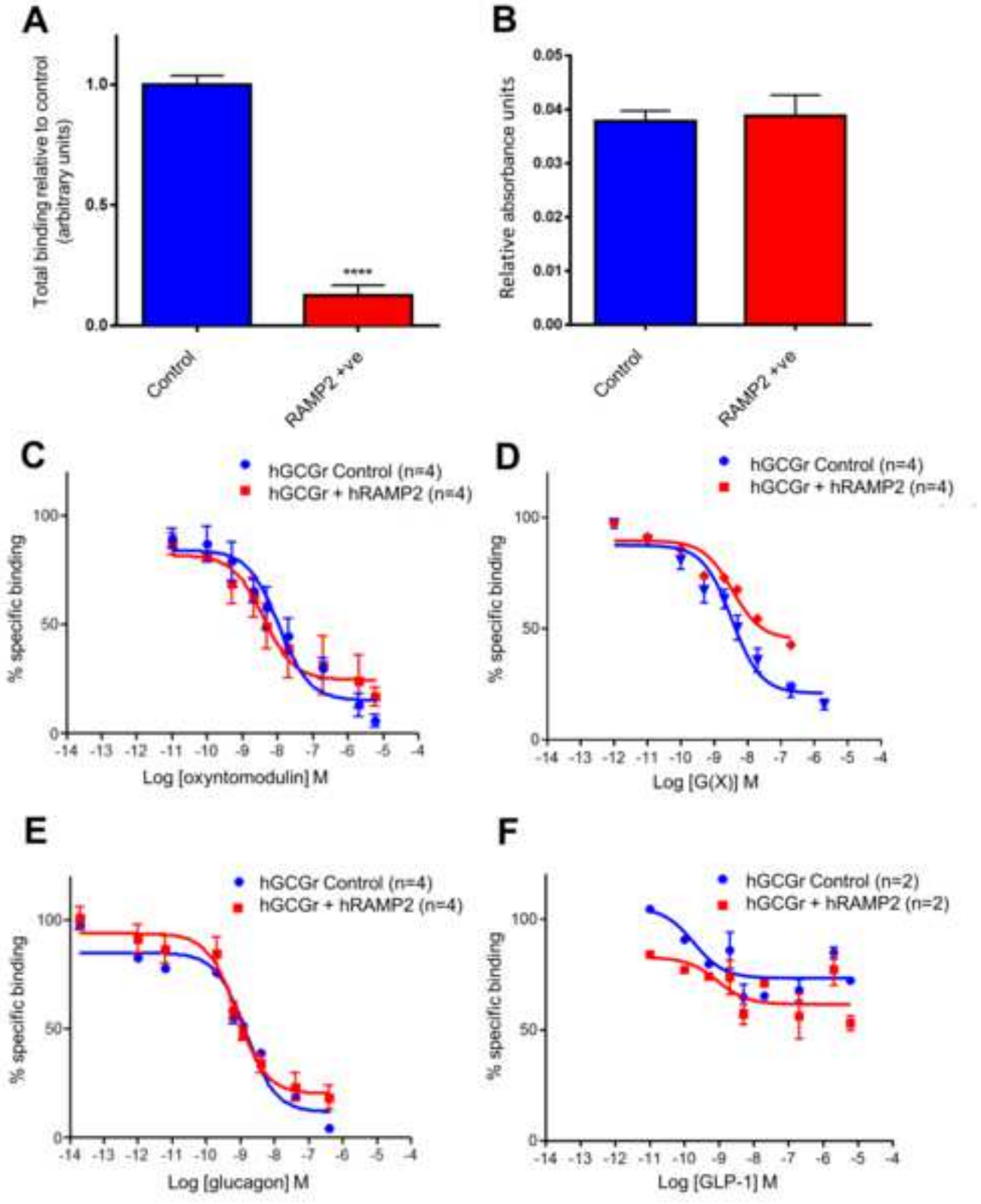

Figure 2

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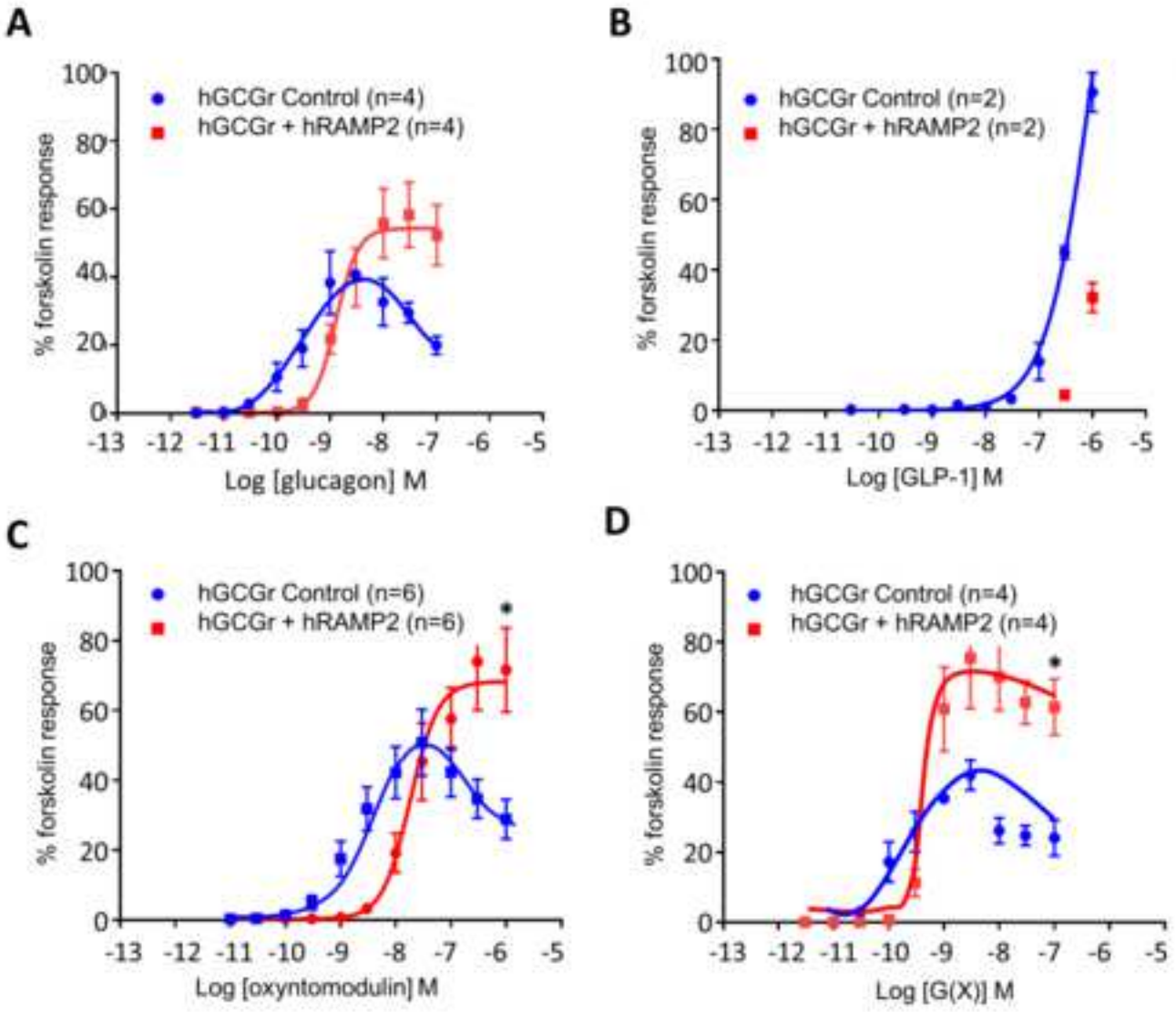

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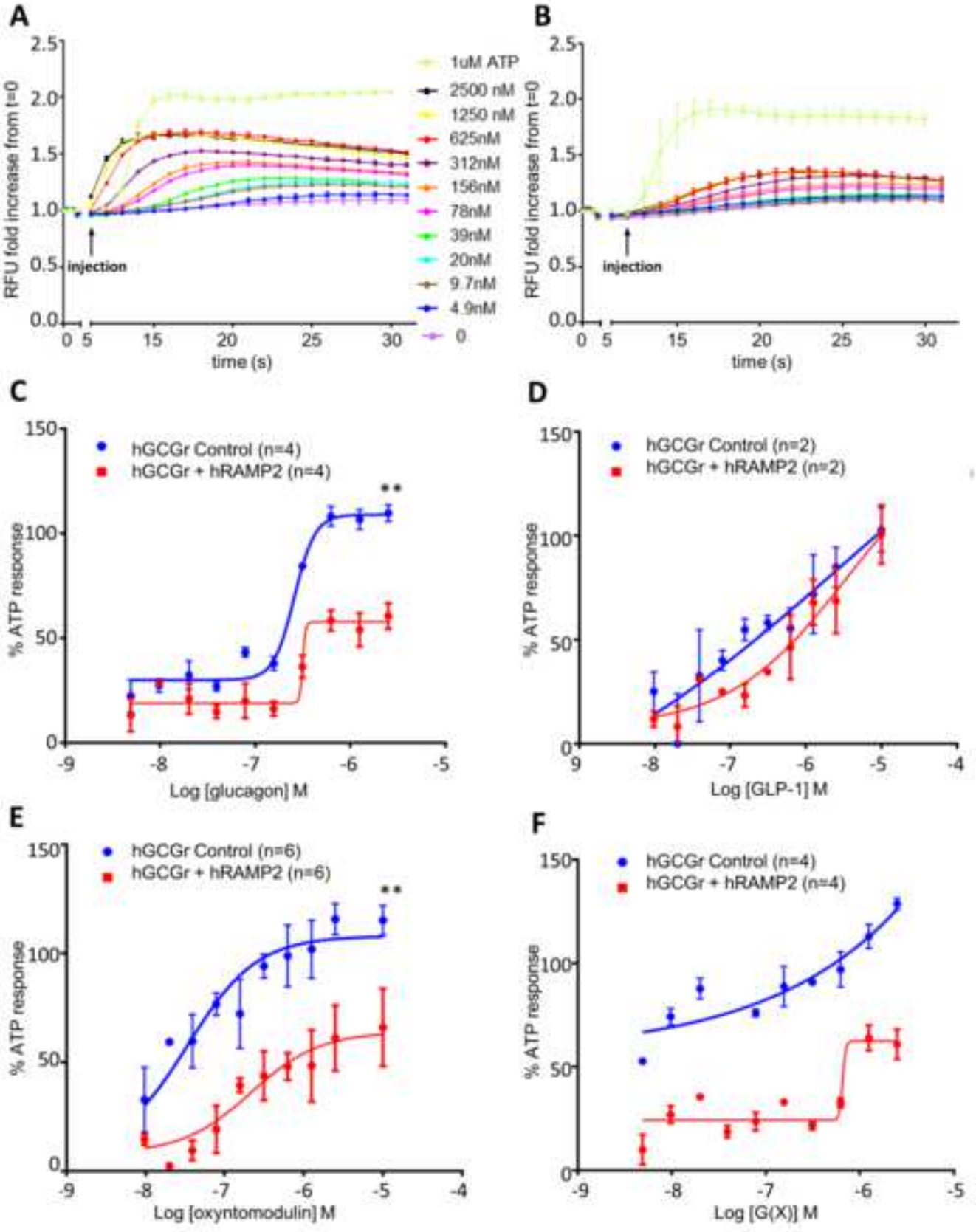

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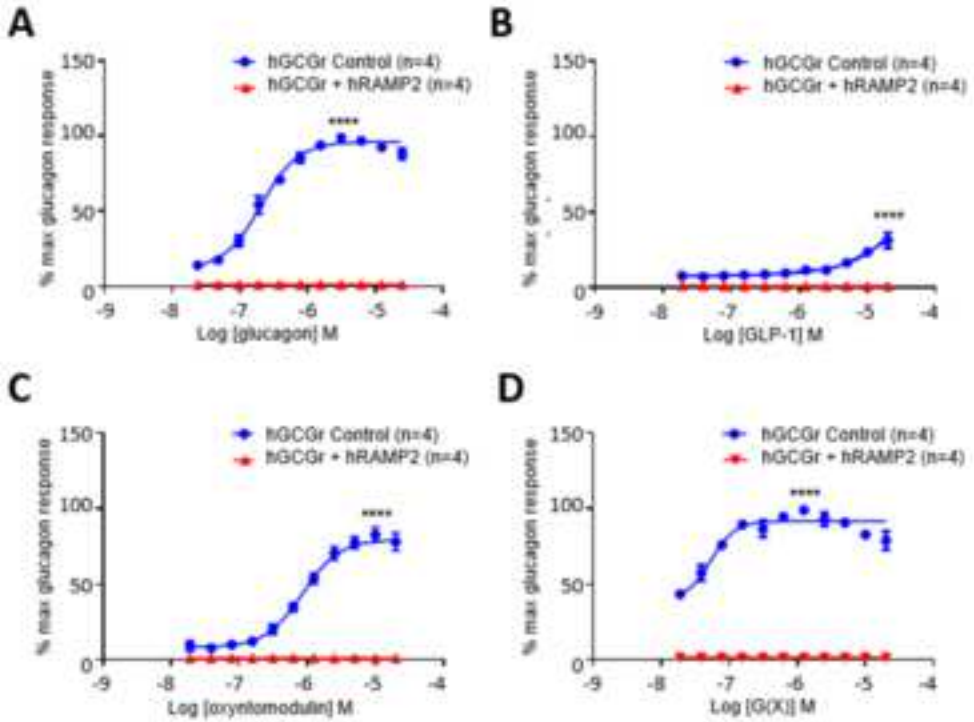

Figure 5

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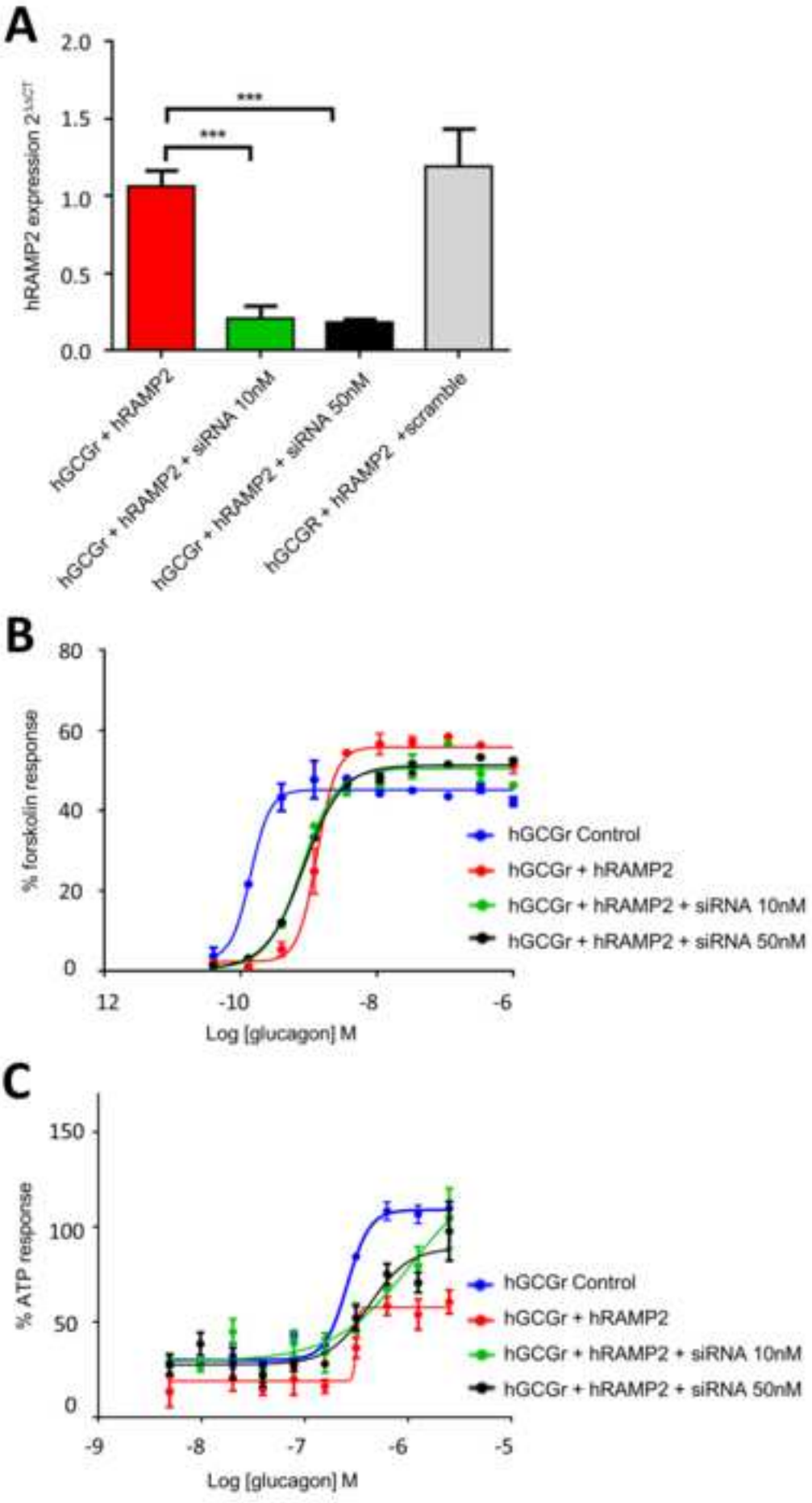

Figure 6

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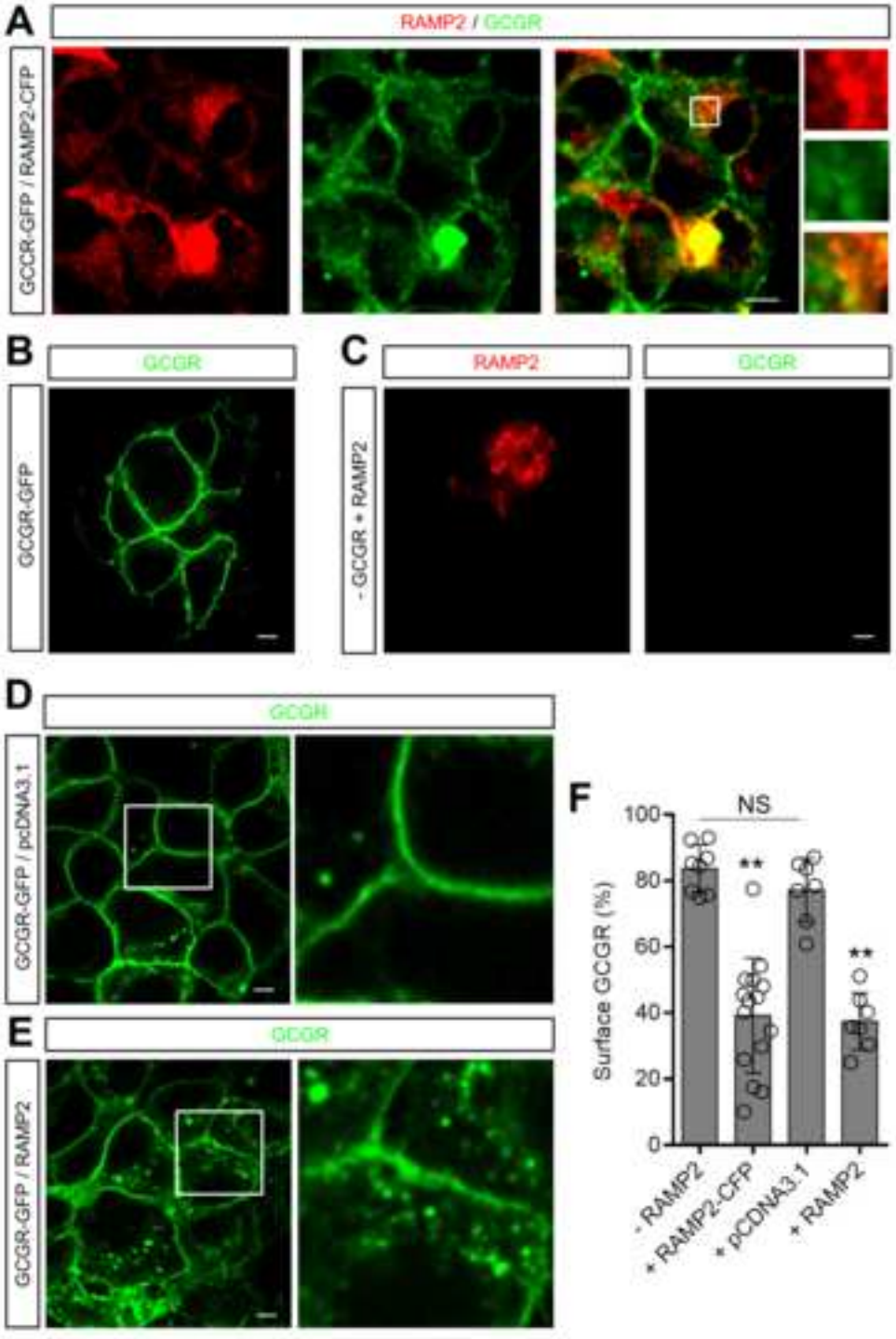

Supplemental Figure 1

Supplemental Figure 2

Supplemental Figure 3

Supplemental Figure 4