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ORIGINAL ARTICLE

Differential effects of glucose-dependent insulinotropic polypeptide receptor/glucagon-like peptide-1 receptor heteromerization on cell signaling when expressed in HEK-293 cells

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Abstract

The incretin hormones: glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are important regulators of many aspects of metabolism including insulin secretion. Their receptors (GIPR and GLP-1R) are closely related members of the secretin class of G-protein-coupled receptors. As both receptors are expressed on pancreatic β-cells there is at least the hypothetical possibility that they may form heteromers. In the present study, we investigated GIPR/GLP-1R heteromerization and the impact of GIPR on GLP-1R-mediated signaling and vice versa in HEK-293 cells. Real-time fluorescence resonance energy transfer (FRET) and bioluminescence resonance energy transfer (BRET) saturation experiments confirm that GLP-1R and GIPR form heteromers. Stimulation with 1 μM GLP-1 caused an increase in both FRET and BRET ratio, whereas stimulation with 1 μM GIP caused a decrease. The only other ligand tested to cause a significant change in BRET signal was the GLP-1 metabolite, GLP-1 (9–36). GIPR expression had no significant effect on mini-G_s recruitment to GLP-1R but significantly inhibited GLP-1 stimulated mini-G_q and arrestin recruitment. In contrast, the presence of GLP-1R improved GIP stimulated mini-G_s and mini-G_q recruitment to GIPR. These data support the hypothesis that GIPR and GLP-1R form heteromers with differential consequences on cell signaling.

KEYWORDS
$BRET_4$, $FRET_3$, G-protein-coupled $receptor_1$, GIP_6, $GLP\text{-}1_5$, $heteromerization_2$

Abbreviations: BRET, bioluminescence resonance energy transfer; cAMP, cyclic adenosine monophosphate; FRET, fluorescence resonance energy transfer; GIP, glucose-dependent insulinotropic polypeptide; GLP-1, glucagon-like peptide-1; GLP-1R, GLP-1 receptor; GPCRs, G-protein-coupled receptors; HBSS, Hank's Balance Salt Solution; T2DM, type-2 diabetes mellitus; TIRF, Total internal reflection fluorescence.

1 | INTRODUCTION

The incretin hormones; glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) are secreted from the intestines upon nutrient ingestion and potentiate insulin secretion by acting on their respective receptors expressed on pancreatic β-cells.[1] In patients with type-2 diabetes mellitus (T2DM) the incretin effect is severely impaired and this may contribute to the pathology of the disease.[2] While pharmacological doses of GLP-1 are capable of potentiating insulin secretion in patients with T2DM, GIP is almost completely inactive in this regard.[3] As a result, several long-acting GLP-1 receptor (GLP-1R) agonists are used clinically to treat T2DM.[4] GLP-1R agonists also inhibit appetite and reduce food intake which often results in significant weight loss, an added benefit when treating T2DM.[5] In contrast, both GIP receptor (GIPR) agonists and antagonists have been investigated as potential treatments for both T2DM and obesity, however, neither are used clinically.[6,7] A recently employed strategy has been the development of unimolecular peptides that activate both the GLP-1 and GIP receptor.[8] The results of clinical trials for the dual GLP-1/GIP receptor agonist tirzepatide have been very successful in terms of lowering glycated hemoglobin levels and weight loss.[9,10] However, the degree to which GIP receptor activation contributes to these results is still unclear.[11] Confusingly, simultaneous blockade of the GIP receptor and activation of the GLP-1 receptor has also shown promise in nonhuman primate trails.[12] It is unclear at present why both GIPR agonists and antagonists should produce similar results in terms of glucose control and weight loss. One possibility is that long-term exposure to GIPR agonists results in receptor desensitization and downregulation, effectively acting as an antagonist.[13] Alternatively, treatment with GIPR antagonists has been demonstrated to increase cell surface expression of GIPR, potentially restoring sensitivity to GIP.[14] Furthermore, some dual GLP-1/GIP receptor agonists have been shown to act quite differently from the GLP-1 receptor agonists currently in clinical use, showing preference for some signaling pathways over others.[15] This phenomenon is termed biased signaling or functional selectivity.[16]

The GLP-1 and GIP receptors are closely related members of the secretin family of G-protein-coupled receptors (GPCRs) and share a high degree of sequence homology.[17,18] Nonetheless, both receptors are highly selective for the endogenous ligands. GLP-1R and GIPR couple primarily to $G\alpha_s$, resulting in the activation of adenylate cyclase and an increase in intracellular cyclic adenosine monophosphate (cAMP).[19] Both receptors have also been reported to continue to generate cAMP following endocytosis.[20,21] In an effort to understand why GLP-1 (albeit at pharmacological levels) but not GIP, remains insulinotropic in T2DM, differences in their signaling mechanisms and the behavior of their receptors have been investigated.[22] Unlike GIPR, GLP-1R also couples to $G\alpha_q$ relatively well and a mechanism for how this can preserve signaling under hyperglycemic conditions has been proposed.[23] Activation of GLP-1R results in the recruitment of arrestins, however agonist-induced internalization of GLP-1R appears to be an arrestin-independent process.[24,25] In contrast, arrestin recruitment to GIPR remains controversial and when expressed in adipocytes, GIPR undergoes constitutive internalization and recycling to the plasma membrane.[13,14,26–28] GPCRs have been shown to function as monomers, dimers, higher-order oligomers, and heteromers.[29,30] As GLP-1R and GIPR are both expressed on pancreatic β-cells, there exists the possibility that they may function as heteromers. In this study, we investigate the impact of GIPR on GLP-1R-mediated signaling and vice versa using resonance energy transfer techniques in order to further understand the pharmacology of these clinically important receptors.

2 | MATERIALS AND METHODS

2.1 | Materials

All peptide ligands were purchased from Bachem, with the exception of (Pro3)GIP and P18, which were custom synthesized by Pepceuticals Ltd. Cell culture reagents were purchased from Gibco-Invitrogen (Paisley) and Sigma-Aldrich. General chemicals were purchased from Sigma-Aldrich.

2.2 | Construction of cDNA

cDNA encoding the following constructs have previously been described; wild-type and C-terminally super yellow fluorescent protein 2 (SYFP2) labeled human GLP-1R and GIPR, arrestin3-Nano Luciferase (Nluc) arrestin3-YFP β2AR-YFP, βrAR-mTurq. mCherry-CAAX.[26,28,29,31] To generate GLP-1R and GIPR labeled at the C-terminus with Nluc the open reading frame of the receptor was amplified using primers which added a *HindIII* restriction site directly upstream of the start codon and replaced the stop codon with an *XbaI* restriction site. The resulting PCR product was ligated into Arr3-Nluc that had previously been digested with *HindIII* and *XbaI*, replacing the Arr3 open reading frame with that of the receptor. GLP-1R-Nluc was subsequently cloned into pcDNA5-FRT (Invitrogen) in order to generate a stable isogenic cell line. A similar strategy was used to generate GLP-1R and GIPR-mTurquoise, except that in this case the resulting PCR product was cloned into GRK2-mTurquoise[32] that had previously been digested with *HindIII* and *XbaI*. NES-Venus-m$G\alpha_s$ and NES-Venus-m$G\alpha_q$[33] were a kind gift from Mohammed Ayoub (United Arab Emirates University). Both GLP-1R and GIPR contain a N-terminal signal peptide that is cleaved during receptor processing and trafficking to the plasma membrane.[34,35] Therefore, to label GLP-1R and GIPR with SYFP2 at their N-termini the fluorescent protein was introduced immediately downstream of the predicted signal peptide. This was achieved by amplifying the open reading from SYFP2 with primers that introduced the influenza hemagglutinin signal peptide (MKTIIALSYIFCLVFAA) to the N-terminus of the fluorescence

protein and which also flanked the PCR product with a *Kpn-1* and *Not-1* site. The resulting PCR product was cloned into the previously described myc-GLP-1R construct[26] that had previously been digested with *Kpn-1* and *Not-1*. myc-GLP-1R contains a *Kpn-1* site immediately upstream of the signal peptide and a *Not-1* and myc-tag (EQKLISEEDL) immediately downstream of the predicted signal peptide of GLP-1R.

All constructs were verified through Sanger sequencing.

2.3 | Cell culture and transfection of cells

HEK-293 and Flip-In HEK-293 cells (Invitrogen) were cultured in Dulbecco's modified Eagle's media supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained at 37°C in a humidified environment containing 5% CO_2. HEK-293 cells were transiently transfected using Effectene (Qiagen), following the manufacturer's protocol. In order to generate stable cell lines, Flp-In HEK-293 cells were transfected with the pcDNA5.FRT vector and pOG44 using Effectene. Stable isogenic clones were selected by the addition of hygromycin (200 μg/ml).

2.4 | Bioluminescence resonance energy transfer assays

For bioluminescence resonance energy transfer (BRET) saturation assays Flp-In HEK-293 cells stably expressing GLP-1R-Nluc were transiently transfected with increasing amounts of GIPR-SYFP2 DNA (0–2 μg). For dose–response curves, HEK-293 cells were transiently transfected with equal amounts of Nluc-labeled receptor, YFP-labeled G protein/arrestin, and unlabeled receptor. Forty-eight hours post-transfection cells were detached and washed with Hank's Balance Salt Solution (HBSS). Cells were re-suspended in HBSS and plated onto white 96-well plates (PerkinElmer) in suspension at a density of 180000 cells/well. Cells were incubated with agonist for 15 min and BRET measurements were taken using a Victor X4 (PerkinElmer) plate reader immediately after the addition of coelenterazine *h* (final conc. 1 μM). Nluc emission was measured through a 460/40 nm filter and the resulting SYFP2 emission was read through a 535/25 nm filter. Expression levels of Nluc and SYFP2-labeled constructs were monitored by measuring luminescence and fluorescence, respectively. Luminescence was measured using a Victor X4 and factory settings for luminescence. For fluorescence measurements, cells from the same transfection were plated onto black 96-well plates and after a 1-h incubation period in darkness, total fluorescence was measured with excitation filters at 490/6 nm and an emission filters at 535/25 nm. For BRET saturation assays, raw data were corrected by subtracting the BRET ratio determined from cells expressing Nluc only. Data were then plotted as BRET ratio versus fluorescence/luminescence and curves were fitted using "one site-specific binding" function (GraphPad 8.0). In the case of dose–response curves the BRET ratio was expressed as fold-change from non-stimulated and curves were fitted using a 'sigmoidal dose-response curve' function.

2.5 | Fluorescence resonance energy transfer measurements

HEK293T cells were transiently transfected with 0.8 μg GLP1R-SYFP2 and 1 μg GIPR-mTurquoise or 1 μg GLP1R-mTurquoise and 0.8 μg GIPR-SYFP2 using Effectene (Qiagen). The amount of DNA used for the mTurquoise-containing plasmid was higher to compensate for the lower brightness of the mTurquoise. On the day after transfection, cells were plated on round (25 mm diameter) glass coverslips that had been coated with poly-L-lysine. One day later, fluorescence resonance energy transfer (FRET) measurements were performed as previously described.[26] Images were acquired every 250 ms. Cells were either stimulated with 1 μM GLP1 or 1 μM GIP.

2.6 | Confocal microscopy

HEK-293 cells transiently expressing SYFP2- and mTurquoise-labeled receptors and mCherry-CAAX were plated on to a poly-D-lysin-coated coverslip and mounted on to an "Attofluor" holder (Molecular Probes). The cellular location of the labeled receptors was monitored by live cell confocal microscopy performed on a Zeiss LSM 800 meta system (Carl Zeiss). Zeiss Zen Blue 2 software (2.1) was used for data acquisition and analysis. Images were taken with an oil-immersion 63× lens using the factory settings for mCherry, YFP, and CFP.

2.7 | Microcontact printing

Protein micropatterning was performed as described previously.[36,37] In short, a large-area PFPE elastomeric stamp (carrying 1 μm grid features; obtained from EV-Group) was incubated with a BSA solution (1 mg/ml) for 30 min, followed by a washing step with PBS and dH_2O. The stamp was dried under a stream of nitrogen and placed with homogenous pressure onto a clean epoxysilane-coated glass bottom of a 96-well plate and incubated overnight at 4°C. On the next day, the stamp was stripped from the glass, and the patterned substrate was bonded to the 96-well plastic casting using an adhesive tape (3 M).

2.8 | Subcellular micropatterning experiments

For live cell experiments, respective wells were incubated with 100 μl streptavidin solution (50 μg/ml; Sigma Aldrich) for 30 min at room temperature, followed by rigorous washing with PBS. Subsequently, biotinylated anti-GFP antibody (10 μg/ml; antibodies-online) was incubated for a further 30 min. Prior to cell seeding, wells were washed

again with PBS. Cells were allowed to attach to the antibody-patterned surface for at least 3–4 h. Total internal reflection fluorescence (TIRF) microscopy was carried out on an epi-fluorescence microscope (Nikon Eclipse Ti2), where the samples were illuminated in TIR configuration (Nikon Ti-LAPP) using a 60× oil immersion objective (NA = 1.49, APON 60XO TIRF). A multi-laser engine (Toptica Photonics) was used for selective fluorescence excitation of CFP and YFP at 405 and 516 nm, respectively. After appropriate filtering using standard filter sets, the fluorescence was imaged onto a sCMOS camera (Zyla 4.2, Andor). The samples were mounted on an x-y-stage (CMR-STG-MHIX2-motorized table, Märzhäuser), and scanning of the larger areas was supported by a laser-guided automated Perfect Focus System (Nikon PFS). Quantitation of fluorescence contrast was carried out as previously.[38]

2.9 | Data analyses

Dose–response data were fitted to a sigmoidal curve and BRET saturation experiments were fitted to one site-specific binding curve using GraphPad 8.0 (GraphPad). The values are expressed as the mean ± standard error of the mean; n = number of independent experiments. Statistical analysis of significance was calculated with GraphPad 8.0 using a two-tailed, unpaired Student's t-test or ANOVA where appropriate.

2.10 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY[39] and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20.[40]

3 | RESULTS

3.1 | Heteromerization of GLP-1R and GIPR

Heteromerization of GLP-1R and GIPR was assessed using BRET saturation experiments. Increasing amounts of SYFP2-labeled GIPR were transiently expressed in Flp-In HEK-293 cells stably expressing Nluc-labeled GLP-1R. The plot of the ratio of GIPR-SYFP2 fluorescence and GLP-1R-Nluc luminescence against BRET ratio produced an exponential curve which reached an asymptote, consistent with a specific BRET signal (Figure 1A). The presence of 1 μM GLP-1 resulted in a significant ($p < .05$) increase in $BRET_{max}$. Treatment with 1 μM GIP decreased $BRET_{max}$, however, it was not significantly different from that of untreated cells. Heteromerization of GLP-1R and GIPR was further investigated using real-time FRET microscopy (Figure 1B). HEK-293 cells were transiently transfected with GIPR tagged with SYFP2 and mTurquoise-labeled GLP-1R. Perfusion with 1 μM GLP-1 resulted in an increase in the FRET signal, whereas treatment with 1 μM GIP decreased the FRET signal. GLP-1-dependent GIPR/GLP-1R heteromerization could be further proved by subcellular micropatterning experiments (Figure S1). Upon the addition of GLP-1, GIPR-mTurquoise was redistributed in YFP-GLP-1R micropatterned areas, indicating heteromerization processes.

BRET saturation experiments also show a specific BRET signal between GLP-1R-Nluc and GLP-1R-SYFP2, suggesting that GLP-1R also forms homomers (Figure S2A). In contrast, the results from GIPR-Nluc/GIPR-SYFP2 BRET saturation experiments were ambiguous (Figure S2B). These findings were supported by real-time FRET microscopy experiments (Figure S2C), which show that perfusion with 1 μM GLP-1 caused a decrease in FRET signal between SYFP2 and mTurquoise-labeled GLP-1R. Subcellular micropatterning experiments show that the addition of 1 μM GLP-1 resulted in GLP-1R-mTurquoise being redistributed in SYFP2-GLP-1R micropatterned areas, supporting both BRET and FRET experiments (Figure S3). As

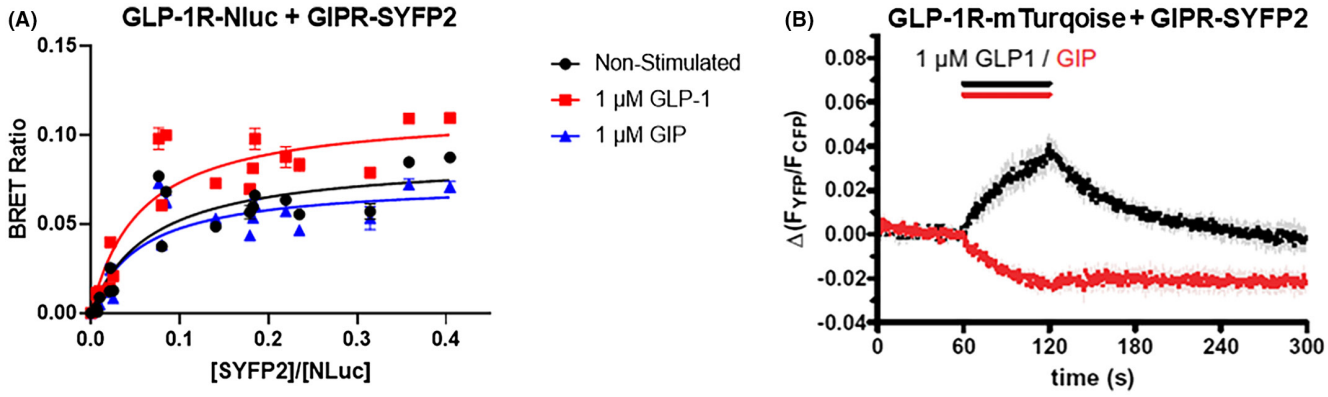


FIGURE 1 GLP-1R and GIPR heteromerization. (A) BRET saturation experiments between GLP-1R-Nluc and GIPR-SYFP2 in the absence of ligand (black) or presence of 1 μM GLP-1 (red) or 1 μM GIP (blue). Data are pooled results from at least three independent experiments performed in triplicate. (B). Agonist-induced changes in FRET between GLP-1R-mTurquoise and GIPR-SYFP2. Perfusion with 1 μM GLP-1 (black) resulted in an increase in the FRET signal, whereas treatment with 1 μM GIP (red) decreased the FRET signal. Similar results were obtained in cells transfected with GLP-1R-SYFP2 and GIPR-mTurquoise. Traces are the mean ± SEM displayed as error bars of at least three independent experiments.

with the BRET experiments, FRET and subcellular micropatterning experiments investigating GIPR homerization were inconclusive (Figure S4). The specificity of these interactions was tested using the β2-adrenoceptor as a negative control. BRET saturation experiments using GLP-1R-Nluc and β2-AR produced a straight line indicating a non-specific interaction (Figure S5). This finding was supported by micropatterning experiments using GLP-1R-SYFP2 and β2AR-mTurq (Figure S6).

3.2 | Agonist-induced changes in BRET between GLP-1R and GIPR

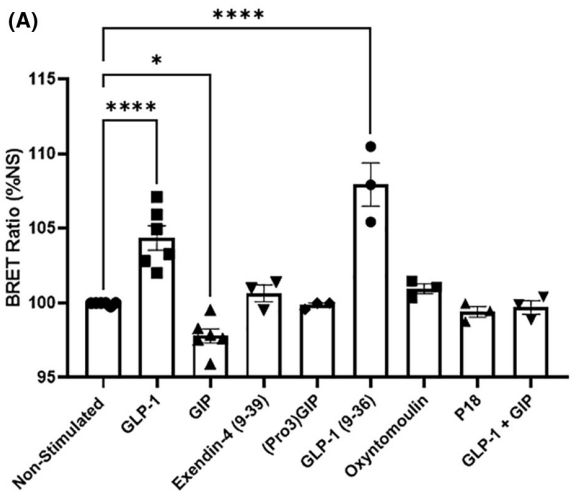
The effect of different GLP-1R and GIPR ligands on the BRET signal between their receptors was investigated in HEK-293 cells expressing a fixed ratio of GLP-1R-Nluc and GIPR-SYFP2 (Figure 2). Treatment with 1 μM GLP-1 resulted in a significant ($p < .001$) increase in BRET ratio compared to non-stimulated cells, whereas treatment with 1 μM GIP resulted in a significant ($p < .05$) decrease in BRET ratio. The only other ligand tested that caused a significant change in BRET ratio was the GLP-1 metabolite, GLP-1 (9-36), which significantly increased the BRET ratio ($p < .001$).

3.3 | Impact of GIPR/GLP-1R heterodimerization on cell signaling

In order to investigate the effect of the presence of GIPR on GLP-1R-mediated signaling and vice-versa, HEK-293 cells were transiently transfected with Nluc-labeled receptor and Venus-labeled mini-G protein or YFP-labeled arrestin3 in the presence or absence of and either unlabeled GLP-1R or GIPR. GIP stimulated Venus-mG$_s$ recruitment to GIPR with significantly greater potency than GLP-1 stimulated Venus-mG$_s$ recruitment to GLP-1R (Table 1; Figure 3A). However, GLP-1 recruited Venus-mG$_s$ to GLP-1R with a significantly higher E_{MAX} than GIP could recruit Venus-mG$_s$ to GIPR (Figure 3B). Neither GLP-1 nor GIP displayed any detectable activity at the other receptor (Table 1; Figure 3A,B). The presence of either unlabeled GLP-1R or GIPR had no significant effect on GLP-1 stimulated recruitment of Venus-mG$_s$ to GLP-1R-Nluc (Table 1; Figure 3C,D). GIP was unable to recruit Venus-mG$_s$ to GLP-1R-Nluc in the presence of unlabeled GLP-1R, however a GIP stimulated BRET signal between Venus-mG$_s$ to GLP-1R-Nluc was detectable in the presence of unlabeled GIPR. A similar observation was seen when GIPR was labeled with Nluc except that the presence of unlabeled GLP-1R significantly increased GIP-mediated Venus-mG$_s$ recruitment to GIPR (Figure 3E,F).

Compared to GLP-1R, GIPR was a relatively poor recruiter of Venus-mG$_q$ and as expected neither GLP-1 nor GIP displayed any detectable activity at the other's receptor (Table 1; Figure 4A,B). The presence of unlabeled GLP-1R had no significant effect on GLP-1 stimulated recruitment of Venus-mG$_q$ to GLP-1R-Nluc, however the presence of unlabeled GIPR significantly inhibited Venus-mG$_q$ recruitment to GLP-1R-Nluc (Figure 4C,D). No detectable GIP stimulated recruitment of Venus-mG$_q$ to GLP-1R-Nluc was observed in the presence of either unlabeled GLP-1R or GIPR. In the reciprocal experiments using GIPR-Nluc in the presence of unlabeled receptors, GIP again stimulated Venus-mG$_q$ to GIPR relatively poorly. As with the Venus-mG$_s$ assays, the presence of unlabeled GLP-1R improved the GIP stimulated BRET signal between Venus-mG$_q$ and GIPR-Nluc (Figure 4E,F).

To explain why the presence of unlabeled GLP-1R should improve the GIP stimulated BRET signal between GIPR-Nluc and Venus-mG$_s$ and Venus-mG$_q$ we investigated whether GLP-1R could improve



(B)

GLP-1	HAEGTFTSDVSSYLEGQAAKEFIAWLVKGR
GIP	YAEGTFISDYSIAMDKIHQQDFVNWLLAQKGKKNDWKHNITQ
Ex-4 (9-39)	DLSKQMEEEVARLFIEWLKNGGPSSGAPPPS
(Pro3) GIP	YAPGTFISDYSIAMDKIHQQDFVNWLLAQKGKKNDWKHNITQ
GLP-1 (9-36)	EGTFTSDVSSYLEGQAAKEFIAWLVKGR
Oxyntomodulin	HSQGTFTSDYSKYLDSRRAQDFVQWLMNTKRNRNNIA
P18	YXEGTFTSDYSIYLDKQAAXEFVNWLLAGGPSSGAPPPSK

FIGURE 2 Agonist induced changes in BRET between GLP-1R and GIPR. (A) HEK-293 cells expressing a fixed ratio of GIPR-SYFP2 and GLP-1R-Nluc were stimulated with 1 μM of one of the panel ligands shown. Stimulation GLP-1 or GLP-1 (9–36) resulted in a significant ($p < .001$) increase in BRET ratio, whereas stimulation with GIP resulted in a significant ($p < .05$) decrease in BRET ratio. The mean ± SEM is shown from at least three independent experiments. (B) Peptide ligands used in this study. X = aminoisobutyric acid. GLP-1, Ex-4(9 = 39), and GLP-1 (9–36) are C-terminally amidated.

TABLE 1 Recruitment of Venus-labeled mG_s or mG_q subunits or YFP-labeled arrestin3 to Nluc-labeled receptors in the presence or absence of unlabeled receptor

		Venus-mG_s	Venus-mG_q	Arr3-YFP
Receptor	Agonist	pEC_{50}	pEC_{50}	pEC_{50}
GLP-1R-Nluc	GLP-1	$7.8 \pm 0.06_{(7)}$	$7.8 \pm 0.11_{(7)}$	$7.1 \pm 0.14_{(4)}$
GLP-1R-Nluc	GIP	ND	ND	ND
GIPR-Nluc	GIP	$8.5 \pm 0.06_{(6)}$ **	$8.0 \pm 0.23_{(6)}$	ND
GIPR-Nluc	GLP-1	ND	ND	ND
GLP-1R-Nluc + GLP-1R	GLP-1	$7.5 \pm 0.12_{(3)}$	$7.7 \pm 0.10_{(4)}$	$7.0 \pm 0.11_{(4)}$
GLP-1R-Nluc + GLP-1R	GIP	ND	ND	ND
GLP-1R-Nluc + GIPR	GLP-1	$7.7 \pm 0.10_{(5)}$	$7.9 \pm 0.19_{(4)}$	$7.3 \pm 0.24_{(4)}$
GLP-1R-Nluc + GIPR	GIP	$8.6 \pm 0.45_{(5)}$	ND	ND
GIPR-Nluc + GIPR	GIP	$7.9 \pm 0.16_{(4)}$	$7.6 \pm 0.18_{(3)}$	ND
GIPR-Nluc + GIPR	GLP-1	ND	ND	ND
GIPR-Nluc + GLP-1R	GIP	$8.2 \pm 0.12_{(4)}$	$7.8 \pm 0.15_{(3)}$	ND
GIPR-Nluc + GLP-1R	GLP-1	$8.1 \pm 0.25_{(4)}$	$7.3 \pm 0.16_{(3)}$	ND

Note: The mean ± SEM is shown from at least three independent experiments (the number of experiments is shown in parentheses). ND refers to assays that exhibited no detectable activity at 1 μM of agonist. pEC_{50} refers to −log EC_{50}/M.

**$p < .001$ significantly different from GLP-1 at GLP-1R.

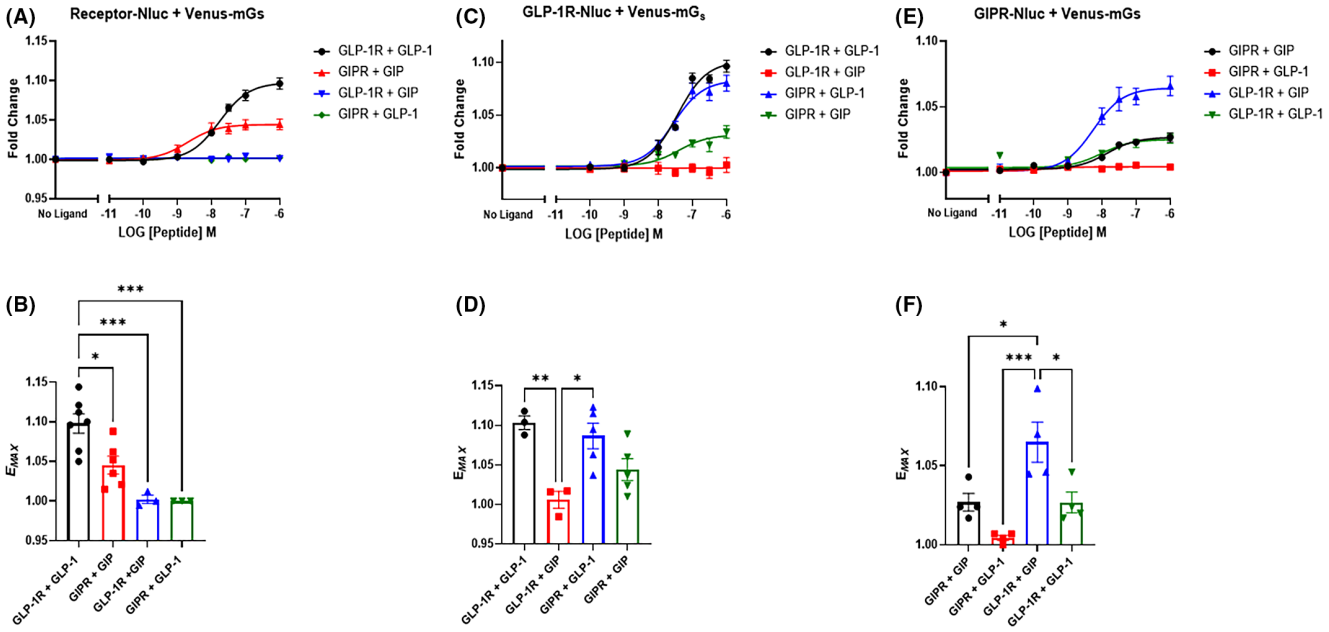


FIGURE 3 Venus-mG_s recruitment to GLP-1R and GIPR. Venus-mG_s recruitment to Nluc-labeled receptor assessed by BRET. (A) Venus-mG_s recruitment to either GLP-1R-Nluc or GIPR-Nluc, stimulated by either GLP-1 or GIP. (B) Maximum Venus-mG_s recruitment to GLP-1R-Nluc or GIPR-Nluc. (C) Venus-mG_s recruitment to GLP-1R-Nluc in the presence of unlabeled GLP-1R or GIPR stimulated by either GLP-1 or GIP (D). Maximum Venus-mG_s recruitment to GLP-1R-Nluc in the presence or absence of unlabeled GLP-1R or GIPR. (E) Venus-mG_s recruitment to GIPR-Nluc in the presence of unlabeled GLP-1R or GIPR stimulated by either GLP-1 or GIP (F). Maximum Venus-mG_s recruitment to GIPR-Nluc in the presence or absence of unlabeled GLP-1R or GIPR. BRET ratios were expressed as fold-change over non-stimulated. The mean ± SEM displayed as error bars, from at least three independent experiments.

GIPR cell surface expression using confocal microscopy. HEK-293 cells were transiently transfected with plasma membrane-targeted mCherry-CAAX and SYFP2 and mTurquoise labeled GLP-1R and GIPR. The localization of GLP-1R-SYFP2 was highly correlated with mCherry-CAAX at the plasma membrane, whereas in contrast GIPR-SYFP2 was poorly expressed at the plasma membrane. The presence of GIPR-mTurquoise did not affect the colocalization of GLP-1R-SYFP2 with mCherry-CAAX and the presence of GLP-1R-mTurquoise did not improve the cell surface expression of GIPR-SYFP2 (Figure 5A,B).

A similar pattern of recruitment to Venus-mG_q was observed for Arr3-YFP. GLP-1 stimulated dose-dependent recruitment of

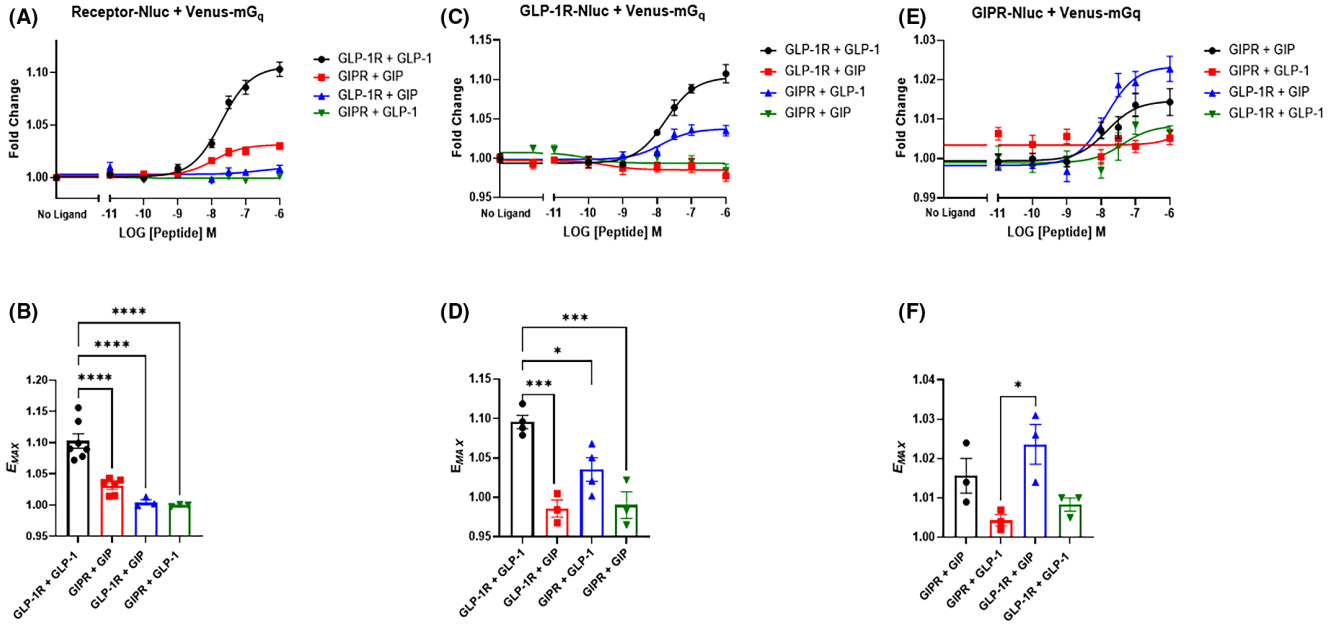


FIGURE 4 Venus-mG$_q$ recruitment to GLP-1R and GIPR. Venus-mG$_q$ recruitment to Nluc-labeled receptor assessed by BRET. (A) Venus-mG$_q$ recruitment to either GLP-1R-Nluc or GIPR-Nluc stimulated by either GLP-1 or GIP. (B) Maximum Venus-mG$_q$ recruitment to GLP-1R-Nluc or GIPR-Nluc. (C) Venus-mG$_q$ recruitment to GLP-1R-Nluc in the presence of unlabeled GLP-1R or GIPR stimulated by either GLP-1 or GIP (D). Maximum Venus-mG$_q$ recruitment to GLP-1R-Nluc in the presence or absence of unlabeled GLP-1R or GIPR. (E) Venus-mG$_q$ recruitment to GIPR-Nluc in the presence of unlabeled GLP-1R or GIPR stimulated by either GLP-1 or GIP (F). Maximum Venus-mG$_q$ recruitment to GIPR-Nluc in the presence or absence of unlabeled GLP-1R or GIPR. BRET ratios were expressed as fold-change over non-stimulated. The mean ± SEM displayed as error bars, from at least three independent experiments.

Arr-3-YFP to GLP-1R-Nluc, whereas no GIP stimulated recruitment Arr-3-YFP to GIP-Nluc could be detected (Table 1; Figure 6A,B). The presence of unlabeled GLP-1R had no significant effect on GLP-1 stimulated recruitment of Arr-YFP to GLP-1R-Nluc, whereas the presence of unlabeled GIPR significantly inhibited Arr-YFP recruitment to GLP-1R-Nluc (Figure 6C,D). GIP could not elicit any Arr-YFP recruitment to GLP-1R-Nluc in the presence of either unlabeled GLP-1R or GIPR. In this assay, however, the presence of unlabeled GLP-1R did not result in any detectable GIP stimulated recruitment of Arr-3-YFP to GIPR-Nluc (data not shown).

4 | DISCUSSION

G-protein-coupled receptors (GPCRs) are the largest family of membrane-spanning receptors in the human genome and are involved in a wide range of physiological and pathological process. Approximately one-third of approved drugs act on this family[41] and yet, with a large number of GPCRs whose endogenous ligands are still to be identified (the orphan receptors), there is huge potential in targeting these receptors to develop novel therapeutics. GPCRs have been shown to form both homo- and heteromers, with potential functional consequences (e.g., effector efficacy cross-talk and trans-inhibition and activation).[42] This may not only be physiologically relevant but might also provide further opportunities for developing novel pharmacological agents. Since GLP-1R and GIPR are both expressed on pancreatic β cells, the possibility exists that they may form functionally relevant heteromers in vivo and that targeting this process may be therapeutically useful.[43]

Using real-time FRET and BRET saturation experiments we confirm that GLP-1R and GIPR form heteromers when expressed in HEK-293 cells (Figure 1), which is in agreement with previously published work.[34,44–46] Treatment with 1 μM GLP-1 resulted in an increase in both the FRET signal and BRET$_{MAX}$, whereas treatment with 1 μM GIP caused a decrease in FRET signal and BRET$_{MAX}$, which is also in agreement with previous work.[44,45] While this may be interpreted as an increase or decrease in the number of heteromers formed, the changes in RET may instead be reporting conformational changes within the heteromer. A more sophisticated experimental approach, such as single-molecule FRET, will be required to determine which is the case. Using a fixed ratio of GLP-1R-Nluc and GIPR-SYFP2, we tested a panel of known GLP-1R and GIPR ligands (shown in Figure 2B). As predicted by the real-time FRET and BRET saturation experiments, treatment with 1 μM GLP-1 caused a significant increase in BRET ratio, whereas 1 μM GIP caused a significant decrease (Figure 2A). The only other ligand tested that caused a significant increase in BRET ratio was the metabolic breakdown product of GLP-1, GLP-1 (9–36). This is in agreement with Schelshorn et al., and suggests that the observed increase in BRET ratio does not require GLP-1R activation.[44] In contrast, the GLP-1R antagonist exendin-4 (9–39) had no significant effect on BRET ratio. (Pro3) GIP, previously reported to be a GIPR antagonist but subsequently shown to be a low potency GIPR agonist, also had no effect on BRET ratio.[47,48] Oxyntomodulin, like GLP-1 a posttranslational product of

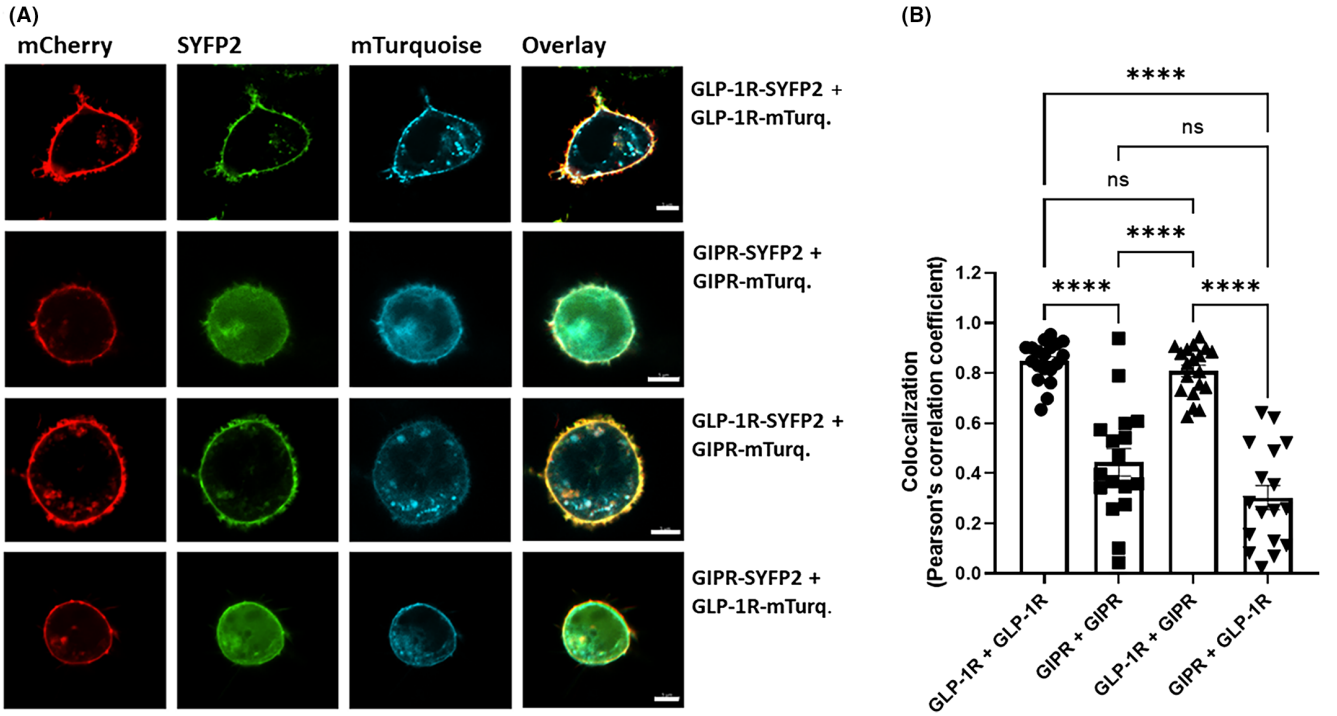


FIGURE 5 Visualization of the cellular location of SYFP2- and mTurquoise-labeled receptors transiently expressed in HEK-293 cells by confocal microscopy. (A) Representative live cell images of HEK-293 cells transiently co-transfected with plasma membrane-targeted mCherry-CAAX (red) and SYFP2-labeled receptor (green) and mTurquoise-labeled receptor (turquoise). GLP-1R-SYFP2 appears to be expressed primarily at the plasma membrane, whereas GIPR-SYFP2 appears to be located not only at the plasma membrane but also in intracellular compartments. Co-expression of GIPR-mTurquoise had no effect on cell surface expression of GLP-1R-SYFP2 and GLP-1R-mTurquoise did not improve the expression of GIPR-SYFP2 and the plasma membrane. The images are representative of at least three independent experiments. Scale bar = 5 μm. (B). Colocalization of the SYFP2-labeled receptors with plasma membrane-targeted mCherry. GLP-1R-SYFP2 colocalizes with membrane-targeted mCherry to a significantly (****$p < .001$) greater extent than GIPR-SYFP2. Co-expression of GIPR-mTurquoise had no significant effect on the colocalization of GLP-1R-SYFP2 with mCherry-CAAX. The presence of GLP-1R-mTurquoise did not improve the colocalization of GIPR-SYFP2 with mCherry-CAAX. Data are the mean ± SEM from values measured in $n = 19$

proglucagon and an endogenous dual GLP-1R/glucagon receptor agonist, similarly had no effect on BRET ratio. In the case of both (Pro3) GIP and oxyntomodulin, the lack of observable change in BRET ratio may be due to their low potency at GIPR and GLP-1R, respectively. P18 is a dual GLP-1R/GIPR agonist that has been shown to be a G-protein-biased agonist at GLP-1R.[8,29] In contrast to other dual GLP-1R/GIPR agonists that have recently been shown to cause a decrease in BRET ratio between GLP-1R and GIPR,[46] we observed no effect on BRET ratio with P18. Simultaneous activation with GLP-1 and GIP also had no effect on BRET ratio, most likely because the conformational change resulting from simultaneous activation of the two receptors results in no net change in BRET ratio.

BRET-based mini G protein and arrestin recruitment assays were used to investigate the impact of GIPR on GLP-1R signaling and vice versa. Both GLP-1 and GIPR dose dependently recruited Venus-mG$_s$ to their respective Nluc-labeled receptors, although GIP was significantly more potent than GLP-1 in this regard and GLP-1 recruited Venus-mG$_s$ to GLP-1R with a significantly higher E_{MAX}. Neither peptide displayed any detectable activity at the other's receptor in this assay (Figure 3A,B; Table 1). Co-expression of unlabeled GLP-1R had no significant effect on GLP-1 stimulated Venus-mG$_s$ recruitment to GLP-1R-Nluc. As expected, GIP had no effect in this assay as even in more distal assays, such as a cAMP reporter gene assay, GLP-1 and GIP are 100 000-fold more selective for their own receptors.[47] No significant difference in GLP-1 stimulated Venus-mG$_s$ recruitment to GLP-1R-Nluc was observed when co-expressed with unlabeled GIPR (Figure 3C,D; Table 1). This appears to be in contradiction to Roed et al., who demonstrated that the presence of GIPR inhibits GLP-1R-mediated cAMP production.[45] There are two possible explanations for this discrepancy. First, we utilized a mG$_s$ recruitment assay, which is directly downstream of receptor activation, whereas Roed et al., utilized a cAMP accumulation assay. It is possible that any inhibition by GIPR on GLP-1R-mediated signaling only becomes apparent using a more amplified assay. An alternative explanation is that GIPR does not inhibit Gαs recruitment to GLP-1R but, as previously reported, inhibits GLP-1R internalization. GLP-1R is one of the growing number of GPCRs that continues to signal once internalized and inhibiting internalization significantly reduces GLP-1R meditated cAMP accumulation and insulin secretion.[18,49] Interestingly, a BRET signal could be detected when unlabeled GIPR was stimulated with GIP in the presence of GLP-1R-Nluc (Figure 3C,D). This suggests that Venus-mG$_s$ recruited to GIPR was in close enough proximity to

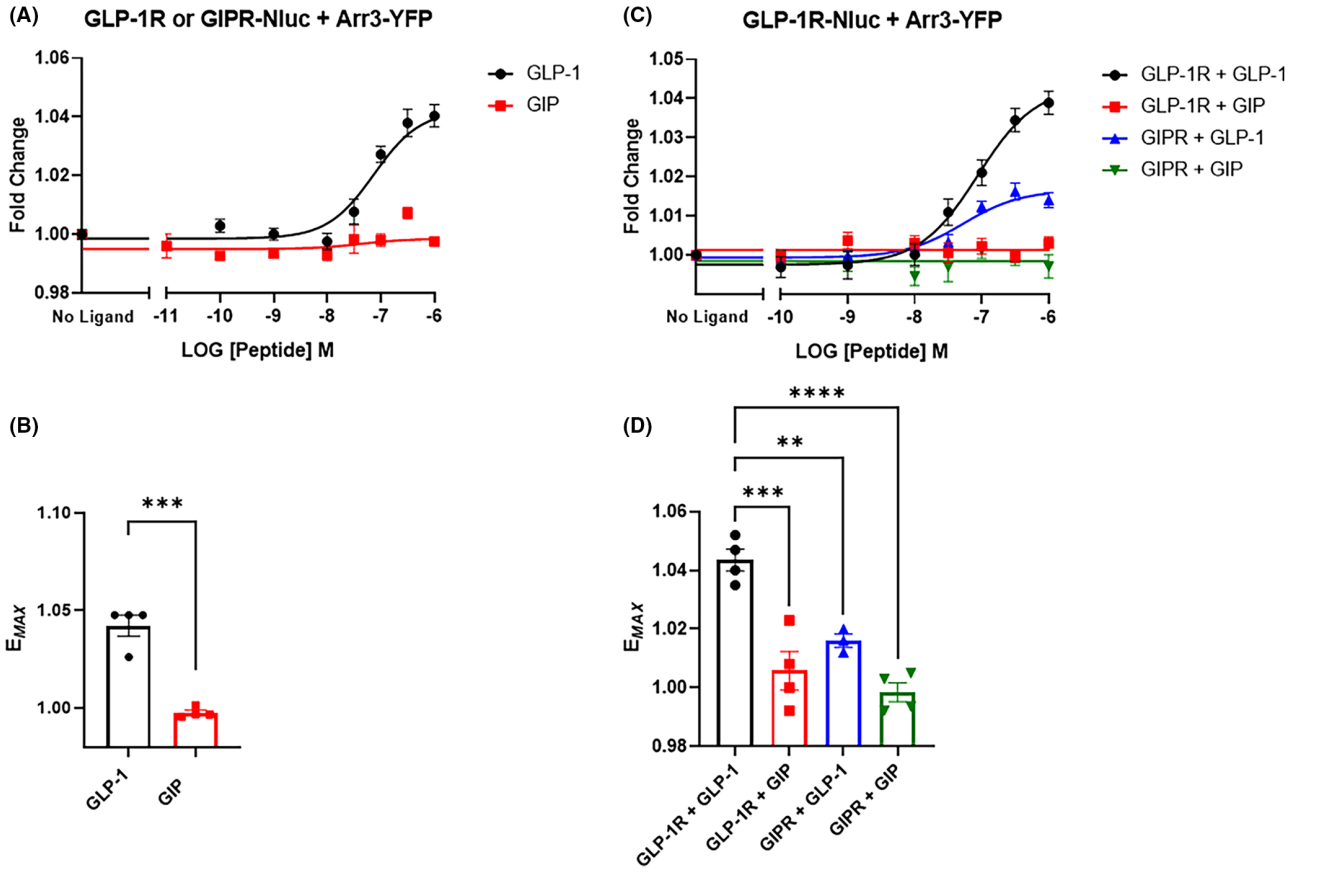


FIGURE 6 Arrestin3 recruitment to GLP-1R and GIPR. Arrestin3-YFP recruitment to Nluc-labeled receptor assessed by BRET. (A) Arrestin3-YFP recruitment to either GLP-1R-Nluc or GIPR-Nluc stimulated by either GLP-1 or GIP. (B) Maximum Arrestin3-YFP recruitment to GLP-1R-Nluc or GIPR-Nluc. (C) Arrestin3-YFP recruitment to GLP-1R-Nluc in the presence of unlabeled GLP-1R or GIPR stimulated by either GLP-1 or GIP (D). Maximum Arrestin3-YFP recruitment to GLP-1R-Nluc in the presence or absence of unlabeled GLP-1R or GIPR. BRET ratios were expressed as fold-change over non-stimulated. The mean ± SEM displayed as error bars, from at least three independent experiments.

GLP-1R-Nluc to generate a BRET signal, further supporting the observation that GIPR and GLP-1R form heteromers. When the reciprocal experiments were performed with Nluc-labeled GIPR a BRET signal could also be observed when unlabeled GLP-1R was stimulated with GLP-1 (Figure 3E,F). Surprisingly, the presence of unlabeled GLP-1R significantly enhanced GIP stimulated recruitment of Venus-mG$_s$ to GIPR-Nluc (Figure 3E,F).

Compared to GLP-1R, GIPR was a relatively poor recruiter of Venus-mG$_q$ (Figure 4A,B), which is in agreement with previous observations.[50–52] This difference in signaling characteristics has been proposed to be one of the reasons why pharmacological doses of GLP-1 remain insulinotropic under hyperglycemic conditions, whereas comparable doses of GIP are not.[21] In contrast to the Venus-mG$_s$ recruitment assay, the presence of unlabeled GIPR significantly inhibited GLP-1-mediated recruitment of Venus-mG$_q$ to GLP-1R-Nluc (Figure 4C,D). This supports the growing body of work that suggests that, at least in vitro, GIPR acts as a negative regulator of several aspects of GLP-1R-mediated signaling such as calcium and pERK accumulation and receptor internalization.[44,45] Interestingly, the Gα_q pathway has been shown to be involved in agonist-induced internalization of GLP-1R.[53] Further work will be required to determine whether GIPR's ability to inhibit GLP-1R internalization is mediated through its attenuation of Gα_q recruitment to GLP-1R. As with Venus-mG$_s$ recruitment to GIPR-Nluc, the presence of unlabeled GLP-1R improved GIP stimulated Venus-mG$_q$ recruitment to GIPR-Nluc (Figure 4E,F).

We speculated that the enhancement of GIP stimulated recruitment of Venus-mG$_s$ and Venus-mG$_q$ to GIPR-Nluc in the presence of unlabeled GLP-1R may be due to improved cell surface expression of GIPR. To test this hypothesis, HEK-293 cells were transiently transfected with membrane-targeted mCherry-CAAX and SYFP2- and mTurquoise-labeled GLP-1R or GIPR and observed using confocal microscopy. SYFP2-labeled GLP-1R was expressed primarily at the plasma membrane, whereas GIPR-SYFP2 was also expressed intracellularly (Figure 5A,B) which is in agreement with previous studies.[13,28] Co-expression of GIPR-mTurquoise did not have any significant effect on surface expression of GLP-1R-SYFP2 and the presence of GLP-1R-mTurquoise did not improve surface expression of GIPR-SYFP2 (Figure 5A,B). The observed improvement of G protein recruitment to GIPR in the presence of GLP-1R is therefore unlikely to be due to enhanced cell surface expression of GIPR. An alternative explanation could be that GLP-1R is somehow acting as a positive allosteric modulator at GIPR.

While arrestin recruitment to GLP-1R is well documented, arrestin recruitment to GIPR remains controversial.[22,25–27] The reasons for this discrepancy are likely to be due to modifications made to the receptor and the configuration of the arrestin recruitment assay.[28] Nevertheless, we were unable to detect any agonist-stimulated arrestin3 (β-arrestin 2) recruitment to GIPR with the assay utilized in this study (Figure 6A,B). In agreement with both Schelshorn et al. and Roed et al., the presence of unlabeled GIPR significantly inhibited arrestin3 recruitment to GLP-1R (Figure 6C,D).[44,45] Knockdown of arrestin2 (β-arrestin 1) in cultured INS-1 cells has been shown to reduce GLP-1 stimulated cAMP production and insulin secretion, suggesting that arrestin recruitment is an integral component of GLP-1R signaling.[54] As mentioned previously, endocytosis is also a key component of GLP-1R signaling, however this process appears to be independent of arrestin recruitment.[18,23,45,51] Nonetheless, GLP-1 receptor agonists that preferentially signal through G proteins over arrestin have been shown to cause less receptor internalization. Such agonists produce greater long-term insulin release and less nausea than more balanced agonists.[55] Not only has tirzepatide, a dual GLP-1/GIP receptor agonist, been shown to be a G protein biased at GLP-1R, it has also been shown to cause a decrease in BRET between GLP-1R and GIPR.[13,46] Whether this contributes to its efficacy and favorable side effect profile in the treatment of T2DM and obesity remains to be determined.

In summary, in this study, we demonstrate that GLP-1R and GIPR form heteromers when expressed in HEK-293 cells with functional consequences for GLP-1R and GIPR-mediated signaling. While these data are mostly in agreement with previously published work the discrepancies can be explained by differences in the assays used. We also observed an enhancement of G protein recruitment to GIPR in the presence of GLP-1R, suggesting that GLP-1R may act as a positive allosteric modulator at GIPR. With dual GLP-1R/GIPR agonists currently in clinical trials, a greater understanding of the interaction between these two receptors is warranted.

AUTHORS' CONTRIBUTIONS

Suleiman Al-Sabah and Cornelius Krasel designed the research study. Bashaier Al-Zaid, Siby Chacko, Cornelius Krasel, Tina Karimian, Peter Lanzerstorfer, and Suleiman Al-Sabah performed the research. Bashaier Al-Zaid, Cornelius Krasel, Peter Lanzerstorfer, and Suleiman Al-Sabah analyzed the data. Suleiman Al-Sabah wrote the original draft. Charles Ifeamalume Ezeamuzie, Moritz Bünemann, Cornelius Krasel, Peter Lanzerstorfer, and Suleiman Al-Sabah reviewed and edited the manuscript, provided supervision, project administration and acquired funding.

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DISCLOSURE

The authors have no conflict to declare with the contents of this article.

DATA AVAILABILITY STATEMENT

Further information and requests for data and reagents should be directed to and will be fulfilled by the corresponding author, Suleiman Al-Sabah. Please contact (sulaiman.alsabah@ku.edu.kw).

ETHICS STATEMENT

No animals, human tissue, human volunteers or patients were used in this study.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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