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ARTICLE

Signaling profiles in HEK 293T cells co-expressing GLP-1 and GIP receptors

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Glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) are regarded as 'incretins' working closely to regulate glucose homeostasis. Unimolecular dual and triple agonists of GLP-1R and GIPR have shown remarkable clinical benefits in treating type 2 diabetes. However, their pharmacological characterization is usually carried out in a single receptor-expressing system. In the present study we constructed a co-expression system of both GLP-1R and GIPR to study the signaling profiles elicited by mono, dual and triple agonists. We show that when the two receptors were co-expressed in HEK 293T cells with comparable receptor ratio to pancreatic cancer cells, GIP predominately induced cAMP accumulation while GLP-1 was biased towards β-arrestin 2 recruitment. The presence of GIPR negatively impacted GLP-1R-mediated cAMP and β-arrestin 2 responses. While sharing some common modulating features, dual agonists (peptide 19 and LY3298176) and a triple agonist displayed differentiated signaling profiles as well as negative impact on the heteromerization that may help interpret their superior clinical efficacies.

Keywords: glucagon-like peptide-1 receptor; glucose-dependent insulinotropic peptide receptor; peptide 19; LY3298176; heteromerization; type 2 diabetes

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INTRODUCTION

Glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP) are incretin hormones responsible for glucose homeostasis. After a meal, GLP-1, secreted by intestinal L cells, directly acts on the pancreatic β-cell to stimulate insulin secretion in a glucose-dependent manner [1, 2]. GIP, also known as gastric inhibitory peptide, is secreted by intestinal K cells and displays insulinotropic function following nutrient intake. Unlike GLP-1 that suppresses glucagon secretion, GIP enhances the release of glucagon [3]. Both hormones are implicated in the treatment of type 2 diabetes and obesity [3–6], and their cognate receptors, GLP-1R and GIPR, are present in islets of Langerhans to mediate insulin biosynthesis and β-cell proliferation [7–9].

While GLP-1R is a validated therapeutic target, the potential of using GIP or GIPR agonists to treat metabolic diseases is hampered by reduced incretin effects in diabetic patients [10]. Thus, a strategy involving co-agonism of GLP-1R and GIPR was developed and successfully tested in the clinic [11–14]. Compared with dulaglutide (a GLP-1R mono-agonist), the dual agonist LY3298176 exhibited superb benefits such as glucose tolerance, appetite suppression, and weight loss [15].

The insulinotropic action of GLP-1 and GIP are materialized by a cascade of signaling events controlled by their respective receptors [16, 17]. $G\alpha_s$ coupling induces cAMP accumulation and upregulates pro-insulin genes. Receptor activation causes inhibition of K^+ channels, progressively opens voltage-dependent Ca^{2+} channels and accelerates Ca^{2+} influx, resulting in the exocytosis of insulin from β-cells.

Pharmacological characterization conducted previously almost exclusively focused on a single receptor without consideration of the synergistic action of GIP and GLP-1 [18]. This hinders our understanding of signal crosstalk and cumulative effects of GLP-1R and GIPR in the same cellular environment. Furthermore, oligomerization of G protein-coupled receptors (GPCRs) diversifies the classic unary stoichiometry of their signaling [19, 20]. Heteromerization of GLP-1R and GIPR has been reported and proven as an allosteric regulatory mechanism of downstream signaling [21, 22]. It is valuable to define its role on the cell surface where GLP-1R and GIPR are co-expressed.

Here, we report the development of a quantitative method to study multiple signaling pathways in HEK 293T cells expressing GLP-1R or GIPR jointly or separately. The relative expression levels of both receptors resembled that found in two pancreatic cancer cell lines. Signal transduction pathways (cAMP accumulation and β-arrestin 2 recruitment) as well as ligand-induced dimerization were profiled with different peptides including GLP-1, GIP, dual agonists (peptide-19, LY3298176) and a triple agonist.

MATERIALS AND METHODS

Peptides and reagents

Human GLP-$1_{(7\text{-}36)}NH_2$ was purchased from Sigma-Aldrich (St. Louis, MO, USA). $Exendin_{(9\text{-}39)}NH_2$ was bought from Aladdin (Shanghai, China). $GIP_{(1\text{-}42)}$ was procured from GenScript (Nanjing, China). Dual agonists, triple agonist and $GIP_{(3\text{-}30)}NH_2$ were synthesized by GL Biochem (Shanghai, China). All peptides had

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Table 1. Peptide sequences.

Peptide	Sequence
$GLP\text{-}1_{(7\text{-}36)}$	HAEGTFTSDVSSYLEGQAAKEFIAWLVKGR-NH_2
$GIP_{(1\text{-}42)}$	YAEGTFISDYSIAMDKIHQQDFVNWLLAQKGKKNDWKHNITQ
$Exendin_{(9\text{-}39)}$	LSKQMEEEAVRLFIEWLKNGGPSSGAPPPS-NH_2
$GIP_{(3\text{-}30)}$	EGTFISDYSIAMDKIHQQDFVNWLLAQK-NH_2
Peptide 19	Y-{Aib}-EGTFTSDYSIYLDKQAA-{Aib}-EFVNWLLAGGPSSGAPPPS-{C16 acyl-Lys}-NH_2
LY3298176	Y-{Aib}-EGTFTSDYSI-{Aib}-LDKIAQ-{C20 diacid-γGlu-$(AEEA)_2$-Lys}-AFVQWLIAGGPSSGAPPPS-NH_2
Triple agonist	H-{Aib}-QGTFTSD-{C16 acyl-γGlu-Lys}-SKYLDERAAQDFVQWLLDGGPSSGAPPPS-NH_2

Uncommon amino acids are denoted by curly bracket. Aib, aminoisobutyric acid, which is impervious to DPP-4 degradation; {C16 acyl-γE-Lys}, C16 acyl binds to amino of glutamate, and side-chain carboxyl of glutamate binds to the side-chain amino of lysine; {C16 acyl-Lys}, C16 acyl binds to the side-chain amino of lysine; {C20 diacid-γGlu-$(AEEA)_2$-Lys}, C20 diacid binds to amino of glutamate and followed by two AEEAs that bind to the side-chain amino of lysine. The structures of uncommon amino acids are shown in Supplementary Figure S1.

a purity of more than 95% by HPLC analysis and had the correct mass spectrometry controlled molecular weight. Amino acid sequences are listed in Table 1.

Monoclonal myc tag antibody (9E10) was purchased from Sigma-Aldrich. Rabbit anti-mouse IgG secondary antibody Alexa Fluor 488 conjugate was obtained from Life Technologies (Carlsbad, CA, USA).

Cell culture and transfection

HEK 293T, Mia-PaCa-2 and PANC-1 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) supplemented with 10% fetal bovine serum (FBS, Life Technologies) and maintained in a humidified chamber with 5% CO_2 at 37 °C. KYSE-150 was purchased from The European National Collection of Authenticated Cell Cultures (ECACC, Porton Down, Salisbury, UK) and cultured in Roswell Park Memorial Institute 1640 medium (RPMI-1640, Life Technologies) supplemented with 10% FBS.

The pcDNA3.1 GIPR-Ypet and pcDNA3.1 GLP-1R-Rluc were obtained by inserting Ypet or Rluc into pcDNA3.1 embracing wild-type receptors using ClonExpress II system (Vazyme, Nanjing, China). All the constructs were verified by sequencing. Transfections were performed in 60%–80% confluent cells by Lipofectamine 2000 (Life Technologies) following the manufacturer's instructions.

cAMP accumulation assay

Ligand-simulated cAMP accumulation was quantified by LANCE Ultra cAMP kit (PerkinElmer, Boston, MA, USA) following the manufacturer's instructions. Briefly, twenty-four hours after transfection with pcDNA3.1 plasmid, HEK 293T cells were digested by 0.02% EDTA and seeded onto 384-well white plates (PerkinElmer) with a density of 3000 cells per well with 5 μL simulation buffer (HBSS supplemented with 5 mM HEPES, 0.5 mM IBMX and 0.1% BSA, pH 7.4). Then, peptides were gradient diluted and allocated for stimulation at room-temperature (RT) for 45 min before adding 5 μL Eu-cAMP tracer and 5 μL ULight-anti-cAMP antibody successively. After 60 min incubation at RT, TR-FRET signals (excitation wavelength at 320 nm and emission wavelength at 615 nm and 665 nm, the top mirror is Lance/Delfia Dual) were recorded by an EnVision multimode plate reader (PerkinElmer). The cAMP concentrations were interpolated by a standard curve derived at the same time.

β-Arrestin 2 recruitment

β-Arrestin 2 recruitment was assessed by a bioluminescence resonance energy transfer (BRET) assay as previously described [23]. Briefly, HEK 293T cells in a 10 cm plate were transfected with 1.5 μg Rluc8 tagged receptor and 8 μg mVenus tagged β-arrestin 2. Twenty-four hours post transfection, transiently transfected cells were seeded into poly-*D*-lysine (Sigma-Aldrich) coated 96-well plates (50,000 cells/well) and grown overnight. Before detection, the medium was replaced by 80 μL BRET assay buffer (HBSS supplemented with 10 mM HEPES and 0.1% BSA, pH 7.4) for 30 min incubation at 37 °C. Coelentrazine-h (50 μM, 10 μL, Yeasen Biotech, Shanghai, China) was then added and incubated at RT for 5 min. Individual agonists (10 μL) with gradient concentrations were added to continuously examine ligand-induced specific BRET signals (ratio of 535 nm over 470 nm emission) using an EnVision plate reader (PerkinElmer) following a baseline measurement.

Ligand-induced receptor dimerization

Receptor dimerization was studied by a BRET assay. HEK 293T cells were seeded into poly-*D*-lysine coated 96-well plates and transfected with GLP-1R-Rluc8 and GIPR-Ypet together at 1:2 ratio, which was optimized in advance. Forty-eight hours post-transfection, the culture medium was replaced by 80 μL BRET assay buffer (HBSS supplemented with 10 mM HEPES and 0.1% BSA, pH 7.4), and the BRET signals were measured continuously as above before and after ligand treatment.

Quantitative PCR analysis

Pancreatic cancer cell lines (Mia-PaCa-2 and PANC-1) and esophageal cancer cell line (KYSE-150) were cultured in 6-well plates and total RNA was extracted by TRIzol (Invitrogen). The RNA was reverse transcribed immediately by HiScript II SuperMix (Vazyme) with parallel cleaning of the genome. Real-time quantitative PCR analysis was then performed on an ABI ViiA7 System (Life Technologies) using SYBR™ Green Master Mix (Life Technologies) in 10 μL solution containing 0.1 μg/μL cDNA and 0.5 μM primers. GAPDH was the internal control.

The primers used were designed by the primer blast (National Center for Biotechnology Information, Bethesda, MD, USA) and verified by mono-peak melting curve. The sequences are as followed: GLP-1R-forward: 5′-CTGCTGCTCGGGATGGTG-3′, GLP-1R-reverse: 5′-GAGCTTCTTTCCCCTCGCTT-3′, GIPR-forward: 5′-GGGACTATGCTGCACCCAAT-3′, GIPR-reverse: 5′-GCCGCCTGAACAAACTCAAG-3′, GAPDH-forward: 5′-GAAGGTGAAGGTCGGAGT-3′, GAPDH-reverse: 5′-GAAGATGGTGATGGGATTTC-3′.

Membrane expression

Cell surface expression analysis was performed by flow cytometry as previously described [24]. Human GLP-1R and GIPR plasmids are constructed with 2-myc epitope tag at the N terminus. Briefly,

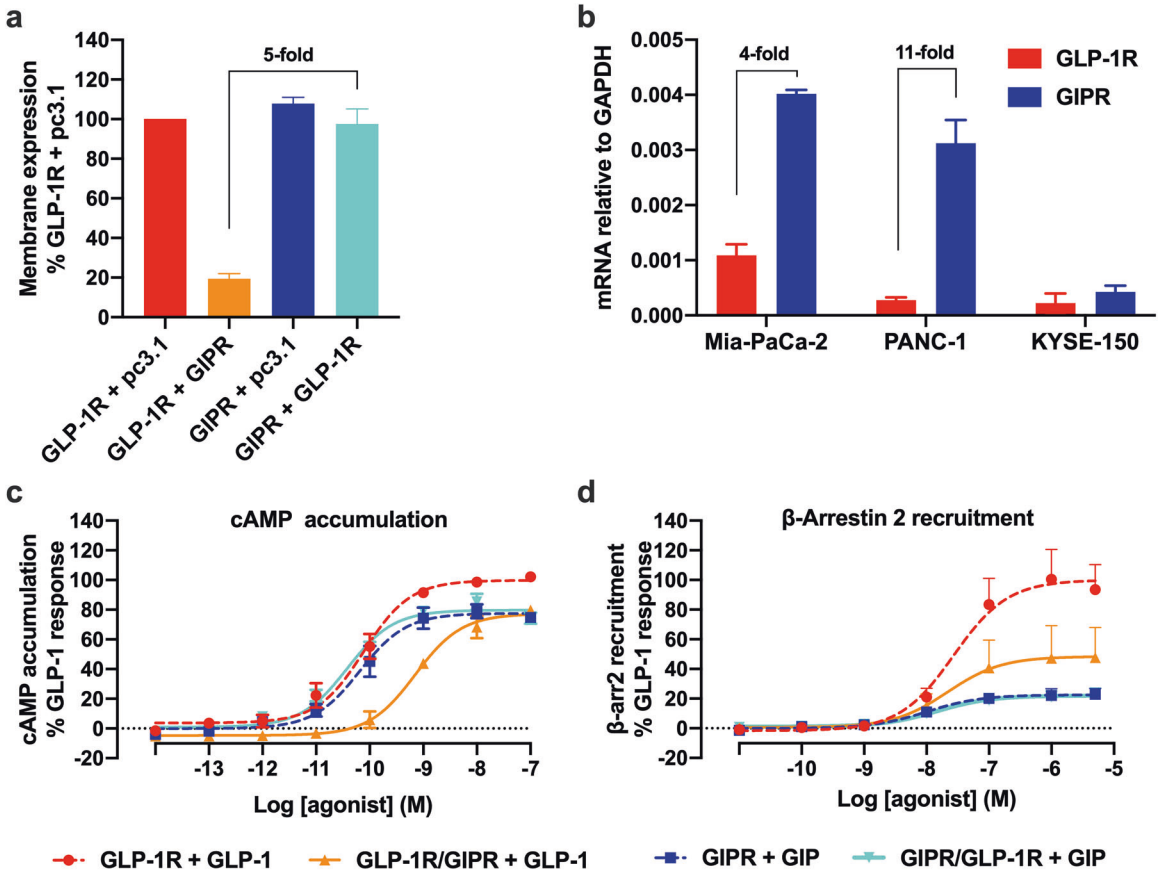

Fig. 1 cAMP accumulation and β-arrestin 2 recruitment profiles in cells co-expressing GLP-1R and GIPR. Engineered human cells (**a**) resembled the relative expression levels of GLP-1R and GIPR in two pancreatic cancer cell lines (Mia-PaCa-2 and PANC-1); **b** KYSE-150 is an esophageal cancer cell line. cAMP accumulation (**c**) and β-arrestin 2 recruitment (**d**) induced by endogenous agonists in HEK 293T cells expressing GLP-1R and GIPR jointly or separately. Data shown are means ± SEM of at least three independent experiments performed in duplicate or triplicate. The concentration–response curves were obtained from non-linear regression based on three-parameter logistic equation. pc3.1, pcDNA3.1; β-arr2, β-arrestin 2.

myc-tag-labelled GLP-1R plasmids were transfected with unlabeled GIPR plasmids or blank vectors in 1:1 ratio. Twenty-four hours post transfection, the cells were harvested using 0.02% EDTA and blocked by 5% BSA. They were then incubated with anti-myc antibody at RT for 1 h followed by Alexa Fluor 488 conjugated secondary antibody on ice for 1 h. After washing with 1% BSA, the cells were loaded to the flow cytometry (Acea Biosciences, Hangzhou, China) and the results were analyzed by NovoExpress (Acea). On the scatterplot (x = FITC, y = SSC) quadrant gates were drawn to make the blank cells in the third quadrant and receptor-expressing cells in the fourth quadrant. Expression level was calculated as below:

$$\text{Expression} = (\text{Median}_{\text{Quadrant 4 FITC}} - \text{Median}_{\text{Quadrant 3 FITC}}) \times \text{Percentage}_{\text{Quadrant 4}}$$

Statistical analysis

Data were analyzed using Prism 6 (GraphPad, San Diego, CA, USA). Assuming consistency to the law of mass action, the concentration-response curves were obtained from the least squares regression of three-parameter dose–response curve. EC_{50} and E_{max} values were calculated from the concentration-response curves. For the time-course data, we first performed the baseline correction based on the first 15 readings to offset well-to-well variances and then extracted the value of vehicle group. The area-under-the-curve data after ligand administration relative to the vehicle group were enumerated by Prism. The concentration–response values were based on the net areas. Statistical significance is evaluated using one-way ANOVA followed by Dunnett's multiple comparisons test.

RESULTS

Membrane expression

According to the data in the human protein atlas (https://www.proteinatlas.org/), both GLP-1R and GIPR are expressed in the pancreas. Compared to three other members in the secretin receptor family (GLP-2R, glucagon receptor and parathyroid hormone receptor 1), co-expression of GIPR led to significant changes in membrane expression and cAMP responses of GLP-1R (Fig. 1a, c and Supplementary Fig. S3).

Co-expression of the two receptors was realized by transient transfection of HEK 293T cells with pcDNA3.1 GLP-1R and pcDNA3.1 GIPR at 1:1 ratio. For cells that only express GLP-1R or GIPR, an equal amount of blank vector was transfected as well. The membrane expression of GLP-1R decreased dramatically to 19.5% ± 2.6%, while that of GIPR remained unchanged (Fig. 1a). Due to reduction in GLP-1R expression, the membrane expression of GIPR was higher than GLP-1R in the co-expressing HEK 293T cells (Fig. 1a). Coincidentally, higher GIPR expression was also found in two pancreatic cancer cell lines, Mia-PaCa-2 and PANC-1 (4.0- and 10.6-fold higher, respectively), but not in unrelated esophageal cancer cell line KYSE-150 (Fig. 1b).

Table 2. cAMP accumulation and β-arrestin 2 recruitment elicited by GLP-1 and GIP.

		cAMP accumulation		β-Arrestin 2 recruitment	
Receptor	Agonist	E_{max}%	pEC_{50}	E_{max}%	pEC_{50}
GLP-1R	GLP-1	99.7 ± 0.2	10.15 ± 0.18	100 ± 0	7.52 ± 0.04
GLP-1R/GIPR	GLP-1	77.1 ± 5.2**	9.17 ± 0.08**	60 ± 5.4**	7.61 ± 0.1
GIPR	GIP	78.1 ± 3.1	10.15 ± 0.22	27.8 ± 2.9	7.98 ± 0.13
GIPR/GLP-1R	GIP	80.5 ± 1.7	10.35 ± 0.13	25.1 ± 2.7	7.91 ± 0.09

EC_{50} is the estimated concentration producing half the maximal response which is displayed as pEC_{50} (the negative logarithm of the EC_{50}). E_{max} is the maximal response (shown as percentage of E_{max} of GLP-1 induced GLP-1R response). Data shown are means ± SEM of at least three independent experiments performed in duplicate or triplicate. Statistical significance is evaluated in comparison with GLP-1R or GIPR (co-transfected with a blank vector) using one-way ANOVA followed by Dunnett's test.
**$P < 0.01$.

cAMP accumulation elicited by cognate peptides
The incretin (GLP-1 or GIP)-stimulated cAMP accumulation was first measured in HEK 293T cells expressing GLP-1R and GIPR jointly or separately. Table 2 shows that GLP-1R expressing cells had a greater E_{max} value compared to GIPR expressing cells. In the co-expression cells, the potency of GLP-1R-mediated cAMP accumulation decreased by tenfold (Fig. 1c) with a modestly reduced E_{max} value (Table 2). In the presence of GLP-1R, the GIPR-mediated cAMP accumulation was not affected in the co-expressing cells (Fig. 1c).

β-Arrestin 2 recruitment elicited by cognate peptides
Since previous efforts suggested that we were unable to observe agonism of GIP, dual and triple agonists on β-arrestin 1 recruitment in our experimental conditions [25], we only assessed β-arrestin 2 recruitment in the present study (Supplementary Fig. S5). Using a BRET assay, we found that co-expression of GIPR significantly decreased GLP-1R mediated β-arrestin 2 recruitment without affecting that induced by GIPR in the presence of GLP-1R (Fig. 1d, Table 2), consistent with the membrane expression and cAMP accumulation results. However, the impaired GLP-1R mediated β-arrestin 2 recruitment was still vigorous compared to that mediated by GIPR (Fig. 1d, Table 2).

cAMP accumulation elicited by dual and triple agonists
Although downregulated by GIPR, supplement of GIP resulted in partial reversal of GLP-1R mediated cAMP response and the E_{max} value (Supplementary Fig. S4, Table S2). This probably indicates an intracellular cumulative effect of dual and triple agonists that comes from the agonism of GLP-1R and GIPR. In the cells co-expressing the two receptors, unimolecular dual agonists exhibited better potencies compared with that only expressing one receptor (Fig. 2a, c, e). Low E_{max} values observed in GIPR-expressing cells upon peptide 19, LY3298176 or triple agonist stimulation were partially recovered by co-expression of GLP-1R (Table 3). Peptide 19 and LY3298176 showed a similar response pattern that differs from triple agonist (Fig. 2a, c, e). However, comparable E_{max} values among them were observed in co-expressing cells (Table 3). The concentration-response curve of triple agonist in co-expression cells was almost overlaid with that of HEK 293T cells only expressing GLP-1R (Fig. 2e).

To evaluate dual agonist-induced cAMP responses in co-expression cells, specific receptor antagonists were used to block the orthosteric binding sites. When the GLP-1R antagonist $exendin_{(9\text{-}39)}NH_2$ was introduced, concentration-response curves of peptide 19 and LY3298176 remained unchanged, whereas that of triple agonist shifted to the right; when the GIPR antagonist $GIP_{(3\text{-}30)}NH_2$ was employed, all three curves moved to the right (Fig. 2b, d, f).

β-Arrestin 2 recruitment elicited by dual and triple agonists
The negative impact of GIPR co-expression on GLP-1R mediated β-arrestin 2 recruitment was also observed in triple agonist-stimulated cells (Fig. 3c, d, Table 4) whereas the effect of GIPR on peptide 19 and LY3298176 treated cells was not statistically significant (Fig. 3a, b, d, Table 4).

Heteromerization of GLP-1R and GIPR
Hydrophobic cores in transmembrane domains are critical to GPCR insertion into the lipid bilayer, which also make receptors amenable to oligomerization [26]. Heteromerization of GLP-1R and GIPR was investigated by transfecting HEK 293T cells with GLP-1R-RLuc8 and GIPR-YPet plasmids at a ratio of 1:2. BRET signals were monitored continuously, and an increase in BRET ratio indicates recruitment of a heterodimer in real-time. Opposing effects of GLP-1 and GIP (Fig. 4b, c) are in agreement with previous observations [21]. Peptide 19, LY3298176 and triple agonist showed similar concentration-response curves and kinetic features as GIP (Fig. 4a, d, e, f), suggesting a reduced heteromerization between GLP-1R and GIPR.

DISCUSSION

GLP-1 and GIP are "incretins" working in close coordination to maintain glucose homeostasis. Meanwhile, GLP-1R and GIPR are co-expressed in the pancreas to promote insulin secretion and β-cell preservation. In order to better understand their pharmacological properties in the same cellular environment, we developed a method to study receptor-specific signaling profiles by co-expressing GLP-1R and GIPR in HEK 293T cells. The negative impact of GIPR on the GLP-1R signaling has been documented in the literature [27]. We went a step further by measuring GLP-1R *vs.* GIPR mediated signaling quantitatively. It was found that when the two receptors were co-expressed in HEK 293T cells, GIP predominately induced cAMP responses while GLP-1 was biased towards β-arrestin 2 recruitment.

At individual level, the interplay as well as synergy between GLP-1 and GIP are essential in glucose homeostasis. As shown in GLP-1R or GIPR knockout mice, GIP plays an important role in the compensatory enhancement of insulin secretion produced by either GLP-1R deficiency or in high insulin demand [28, 29]. Exogenous GLP-1 was able to render maximal insulin secretion by diabetics to the level equal to that of normal subjects, while GIP supplement could not effectively restore insulin secretion in patients ill with diabetes [30]. The current work implies that the intracellular interplay and synergy of GLP-1R and GIPR should be taken into consideration. It appears that GIPR acts as a negative regulator to tune cellular responses when co-expressed with GLP-1R as demonstrated in this study.

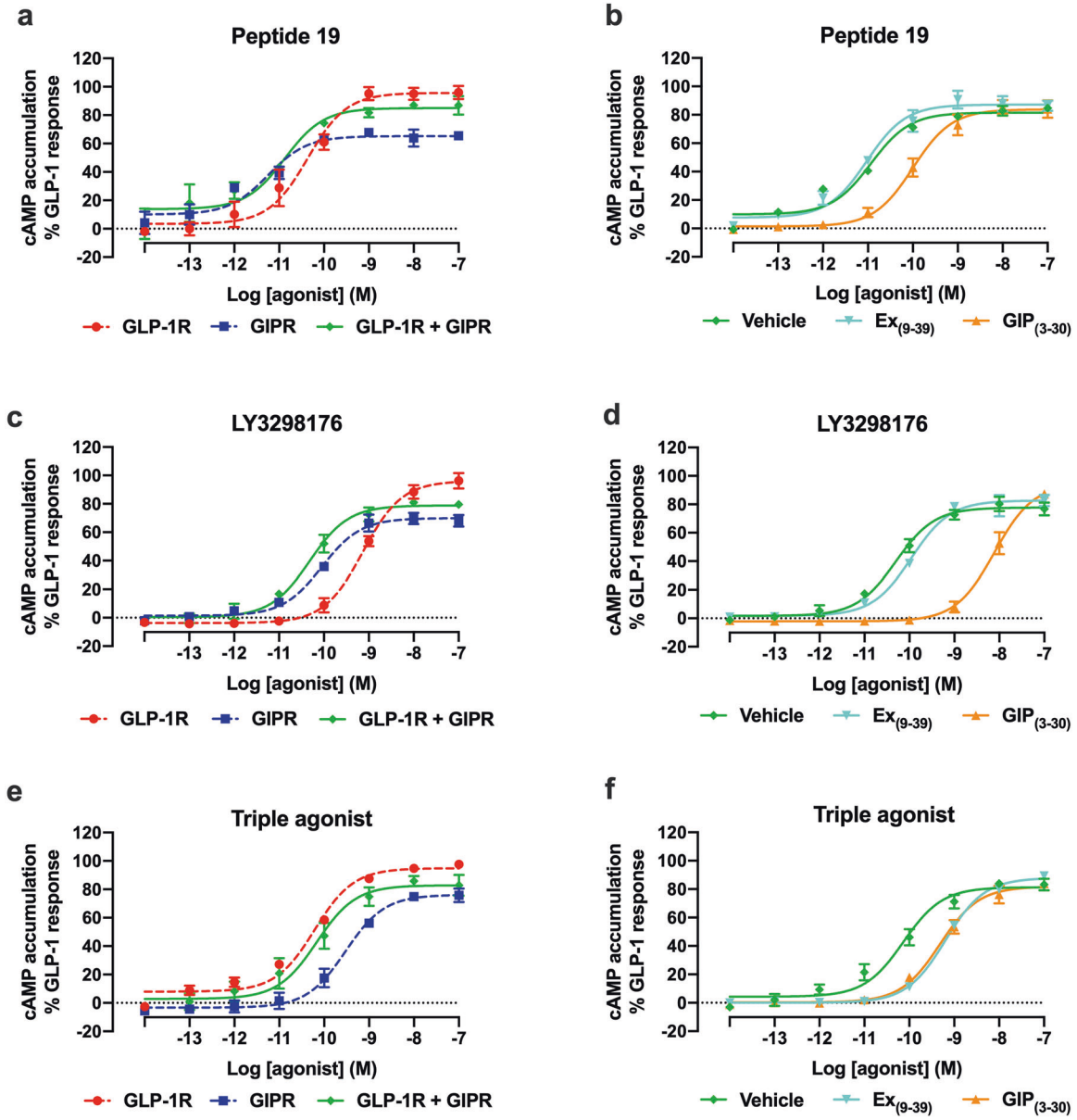

Fig. 2 cAMP accumulation elicited by dual and triple agonists. cAMP accumulation induced by unimolecular dual agonists peptide 19 (**a**) and LY3298176 (**c**), as well as triple agonist (**e**) in HEK 293T cells expressing GLP-1R and GIPR jointly or separately. Using the same co-expression system, effects of exendin$_{(9-39)}$ (Ex$_{(9-39)}$) and GIP$_{(3-30)}$ introduced before agonist stimulation at 5 μM, on peptide 19 (**b**), LY3298176 (**d**) and triple agonist (**f**) elicited cAMP responses were analyzed. cAMP concentrations were interpolated by a standard curve and normalized to the maximal response of GLP-1 in cells expressing GLP-1R alone. Data shown are means ± SEM of at least three independent experiments performed in triplicate. The concentration–response curves were obtained from non-linear regression based on three-parameter logistic equation.

Unimolecular dual agonists of GLP-1R and GIPR show significant clinical benefits including weight loss, appetite suppression, glucose tolerance as well as reduced adverse gastrointestinal effects [13, 15, 31]. While sharing some common signaling profiles of the native peptides GLP-1 and GIP (Supplementary Fig. S6), three unimolecular dual or triple agonists also modulated ligand-induced cAMP and β-arrestin 2 responses in a differentiated manner. They all elicited strong downstream cAMP signals in cells co-expressing GLP-1R and GIPR, but the β-arrestin 2 recruitment mediated by GLP-1R upon peptide 19 stimulation was extra-ordinarily robust (Fig. 3 and Table 4), implying that the impact of GIPR appears to be ligand-specific. Therefore, the superiority of dual agonism is not only displayed by a synergy across different target tissues, but also exhibited as an intracellular interplay to achieve cumulative effects when both receptors are co-expressed.

Although both GLP-1R and GIPR transduce cellular signals mainly through the G_s pathway, their link with β-arrestin 1/2 should be noted. Arrestins not only mediate receptor desensitization and internalization through β-arrestin-AP2-clathrin pathway [32], they also act as molecular scaffolds modulating G protein-independent signaling, for example, ERK signaling [33]. Reduction of GLP-1R mediated β-arrestin 2 recruitment in GIPR co-expressing cells is indicative of such a signaling modulation.

It is long-established that GPCR functions as monomer and couples to G protein on a 1:1 stochiometric basis [34].

Table 3. cAMP accumulation elicited by unimolecular dual and triple agonists.

	Peptide 19		LY3298176		Triple agonist	
	E_{max}%	pEC_{50}	E_{max}%	pEC_{50}	E_{max}%	pEC_{50}
GLP-1R/GIPR	85.1 ± 1.5	10.93 ± 0.03	77.2 ± 7.0	10.25 ± 0.09	84.9 ± 3.2	10.18 ± 0.14
GLP-1R	95.4 ± 2.6*	10.41 ± 0.09*	96.1 ± 2.3**	9.14 ± 0.05**	93.2 ± 1	10.46 ± 0.21
GIPR	66.7 ± 0.2**	11.13 ± 0.06	71.4 ± 2.9	10.19 ± 0.11	76.9 ± 1.7	9.54 ± 0.05**
GLP-1R/GIPR + $Ex_{(9-39)}$	86.9 ± 6.2	11.1 ± 0.14	85.7 ± 6.8	9.91 ± 0	88.3 ± 1.1	9.22 ± 0.05**
GLP-1R/GIPR + $GIP_{(3-30)}$	83.9 ± 5.6	9.98 ± 0.08**	95 ± 1.0	8.1 ± 0.11**	81.9 ± 2.9	9.32 ± 0.08**

EC_{50} is the estimated concentration producing half the maximal response which is displayed as pEC_{50} (the negative logarithm of the EC_{50}). E_{max} is the maximal response (shown as percentage of E_{max} of GLP-1 induced and GLP-1R mediated cAMP accumulation). Data shown are means ± SEM of at least three independent experiments performed in triplicate. GLP-1R/GIPR, GLP-1R and GIPR co-expression. $Exendin_{(9-39)}NH_2$ ($Ex_{(9-39)}$) or $GIP_{(3-30)}NH_2$ ($GIP_{(3-30)}$) was added before agonist stimulation at the 5 μM. Statistical significance is evaluated in comparison with GLP-1R and GIPR co-expressing engineered cells using one-way ANOVA followed by Dunnett's test.
*$P < 0.05$.
**$P < 0.01$.

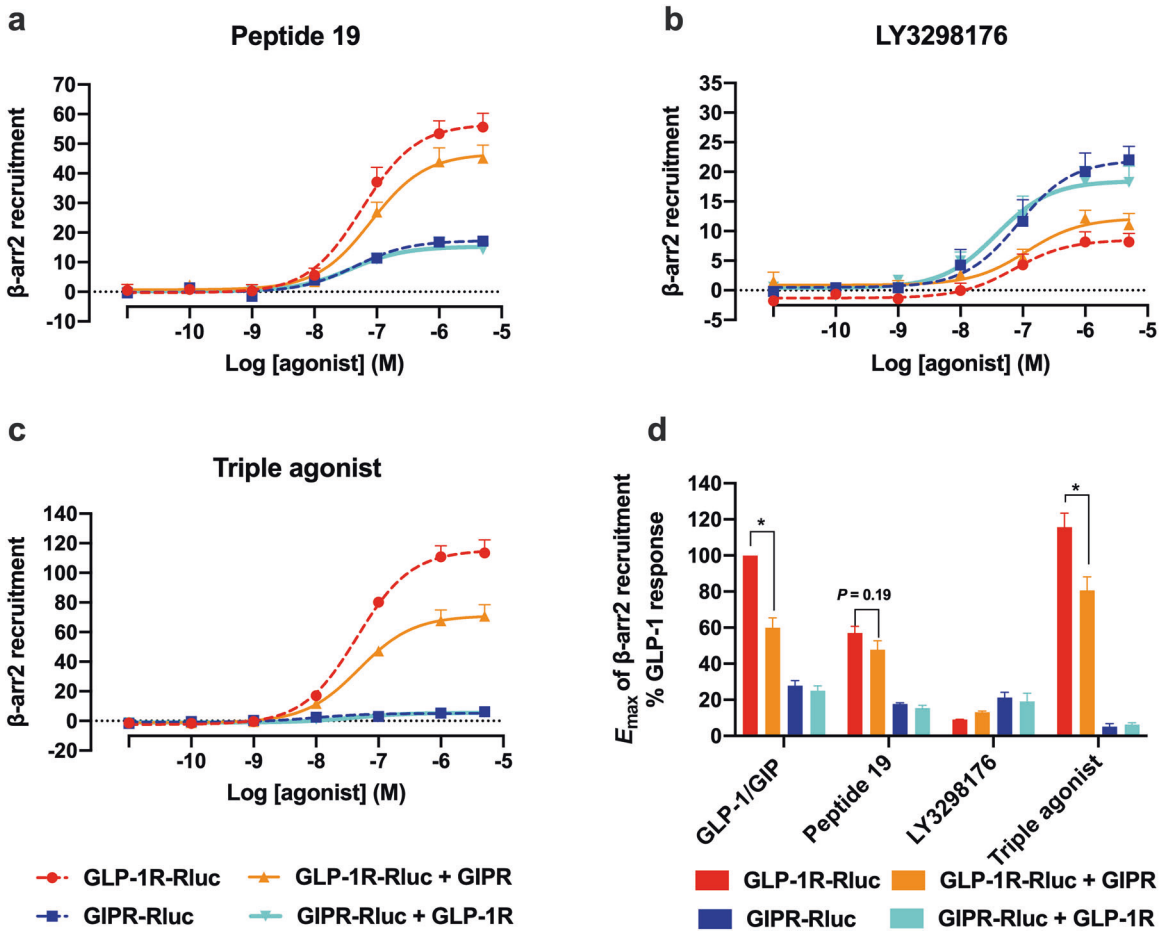

Fig. 3 β-Arrestin 2 recruitment elicited by dual and triple agonists. β-Arrestin 2 recruitment stimulated by unimolecular dual agonists peptide 19 (**a**) or LY3298176 (**b**), and triple agonist (**c**) in HEK 293T cells expressing GLP-1R and GIPR jointly or separately. E_{max} values are displayed as a percentage response caused by GLP-1 (**d**). Data shown are means ± SEM of at least three independent experiments performed in duplicate. The concentration–response curves are obtained from non-linear regression based on three-parameter logistic equation. β-arr2, β-arrestin 2. Statistical significance is evaluated in comparison with GLP-1R or GIPR (co-transfected with a blank vector) using one-way ANOVA followed by Dunnett's test. *$P < 0.01$.

Nevertheless, incremental evidence suggests it also possesses the oligomerization property [35]. Obviously, dimerization, whether homodimerization or heteromerization, would broaden receptor conformational dynamics and signaling profiles. As shown in this study, peptide 19, LY3298176 and triple agonist all exhibited negative impact on the heteromerization between GLP-1R and GIPR that is important for GLP-1R internalization and desensitization [22]. The inhibition of heteromerization by the dual/

Table 4. β-Arrestin 2 recruitment induced by mono, dual and triple agonists.

		GLP-1R-Rluc	GLP-1R-Rluc/GIPR	GIPR-Rluc	GIPR-Rluc/GLP-1R
GLP-1/GIP	E_{max}%	100 ± 0	60 ± 5.4**	27.8 ± 2.9	25.1 ± 2.7
	pEC_{50}	7.52 ± 0.04	7.61 ± 0.1	7.98 ± 0.13	7.91 ± 0.09
Peptide 19	E_{max}%	57.1 ± 3.6	47.8 ± 5	17.7 ± 0.6	15.4 ± 1.5
	pEC_{50}	7.23 ± 0.07	7.1 ± 0.11	7.31 ± 0.15	7.36 ± 0.11
LY3298176	E_{max}%	9.1 ± 0.1	13.2 ± 0.6	21.3 ± 3	19 ± 4.6
	pEC_{50}	7.23 ± 0.16	6.99 ± 0.21	7.51 ± 0.27	7.62 ± 0.03
Tri-agonist	E_{max}%	115.7 ± 7.7	80.7 ± 7.4**	5.2 ± 1.7	6.3 ± 1.1
	pEC_{50}	7.35 ± 0.05	7.35 ± 0.08	7.49 ± 0.48	7.22 ± 0.14

EC_{50} is the estimated concentration producing half the maximal response which is displayed as pEC_{50} (the negative logarithm of the EC_{50}). E_{max} is the maximal response (shown as percentage of E_{max} of GLP-1 induced and GLP-1R mediated β-arrestin 2 recruitment). Data shown are means ± SEM of at least three independent experiments performed in duplicate. Statistical significance is evaluated in comparison with GLP-1R or GIPR (co-transfected with a blank vector) using one-way ANOVA followed by Dunnett's test.
**$P < 0.01$.

triple agonists may be a beneficial feature in strengthening the overall downstream signaling.

In summary, we quantitatively profiled cAMP and β-arrestin 2 signaling in the human embryonic kidney 293T cells with GLP-1R and GIPR expressed separately and jointly. In the co-expression system possessing comparable receptor ratio with pancreatic cancer cells, GIPR mainly transduces G_s-mediated signals such as cAMP accumulation, while GLP-1R is biased towards β-arrestin 2 recruitment. Unimolecular dual and triple agonists are also in favor of cAMP responses. Inhibition of GLP-1R and GIPR heteromerization by dual agonists could strengthen the signaling intensity.

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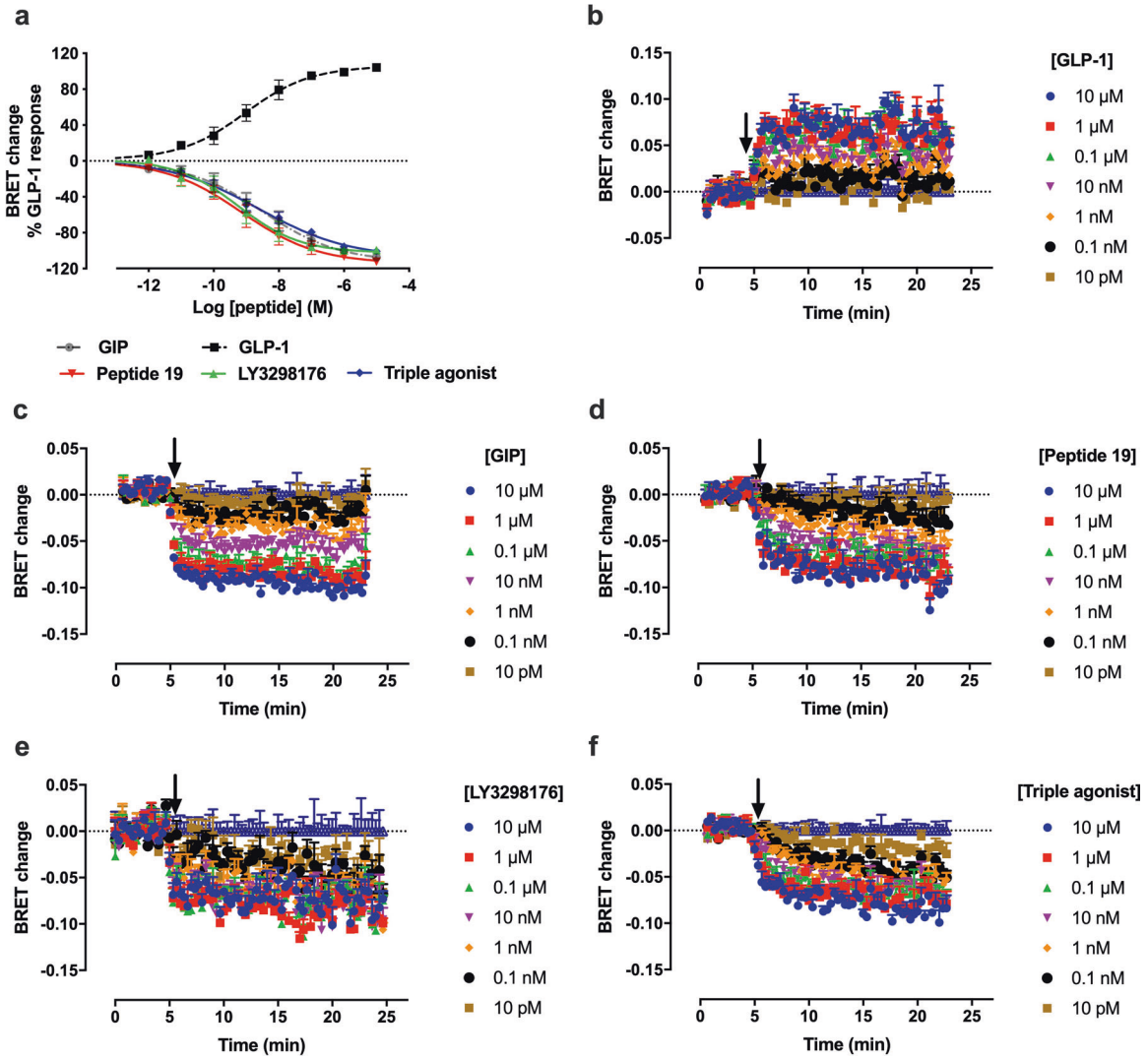

Fig. 4 Ligand-induced GLP-1R and GIPR heteromerization. Concentration-response of heteromerization elicited by GLP-1, GIP, peptide 19, LY3298176 and triple agonist (**a**). Kinetic traces of heteromerization elicited by GLP-1(**b**), GIP (**c**), peptide 19 (**d**), LY3298176 (**e**), and triple agonist (**f**). Arrow indicates peptide addition after 10 cycles of basal reading. Data shown are means ± SEM of duplicate in a single measurement which is representative of three independent experiments.

AUTHOR CONTRIBUTIONS

YZW designed expression constructs, conducted cell-based assays, analyzed the data, and drafted the manuscript. DHY supervised the experiments, analyzed the data, and edited the manuscript. MWW conceived the idea, designed the studies, and edited the manuscript.

ADDITIONAL INFORMATION

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