1521-0111/12/8103-309–318$25.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 81:309–318, 2012

Vol. 81, No. 3
74757/3747190

Lateral Allosterism in the Glucagon Receptor Family: Glucagon-Like Peptide 1 Induces G-Protein-Coupled Receptor Heteromer Formation[S]

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Received July 14, 2011; accepted November 22, 2011

ABSTRACT

Activation of G-protein-coupled receptors (GPCRs) results in a variety of cellular responses, such as binding to the same receptor of different ligands that activate distinct downstream cascades. Additional signaling complexity is achieved when two or more receptors are integrated into one signaling unit. Lateral receptor interactions can allosterically modulate the receptor response to a ligand, which creates a mechanism for tissue-specific fine tuning, depending on the cellular receptor coexpression pattern. GPCR homomers or heteromers have been explored widely for GPCR classes A and C but to lesser extent for class B. In the present study, we used bioluminescence resonance energy transfer (BRET) techniques, calcium flux measurements, and microscopy to study receptor interactions within the glucagon receptor family. We found basal BRET interactions for some of the receptor combinations tested that decreased upon ligand binding. A BRET increase was observed exclusively for the gastric inhibitory peptide (GIP) receptor and the glucagon-like peptide 1 (GLP-1) receptor upon binding of GLP-1 that could be reversed with GIP addition. The interactions of GLP-1 receptor and GIP receptor were characterized with BRET donor saturation studies, shift experiments, and tests of glucagon-like ligands. The heteromer displayed specific pharmacological characteristics with respect to GLP-1-induced β-arrestin recruitment and calcium flux, which suggests a form of allosteric regulation between the receptors. This study provides the first example of ligand-induced heteromer formation in GPCR class B. In the body, the receptors are functionally related and coexpressed in the same cells. The physiological evidence for this heteromerization remains to be determined.

Introduction

G-protein-coupled receptors (GPCRs) are integral plasma membrane proteins that trigger cellular responses to stimuli as diverse as light, smell, taste, hormones, and neurotransmitters. GPCRs represent one of the largest protein families in the genome; in vertebrates, the main subclasses are formed by the rhodopsin-like receptors (class A), the secretin-like receptors (class B), and the glutamate-like receptors (class C). GPCRs activate heterotrimeric G-protein signaling in the cytoplasm, but receptor interactions with kinases, arrestins, molecular chaperones, receptor activity-modifying proteins, and other proteins (Bockaert et al., 2004) also contribute to specific cellular responses.

GPCRs interact laterally with each other in the plasma membrane (Bouvier, 2001). Self-association (homomerization) and association with other receptors (heteromerization) can yield dimers, trimers, or higher order oligomers. The receptors in such complexes are thought to modulate each other allosterically, creating a new receptor type with unique pharmacological properties (Smith and Milligan, 2010). Fluorescence resonance energy transfer and BRET techniques have been used extensively to monitor real-time interactions between GPCRs in living cells (Pfleger and Eidne, 2005). Data on receptor-receptor interactions have been collected in a GPCR oligomerization database (http://data.gpcr-okb.org/gpcr-okb), and some of the described interactions were confirmed to be physiologically relevant and to exist in vivo. Receptor dimers are thought to exist for many GPCRs and

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
http://dx.doi.org/10.1124/mol.111.074757.
[S] The online version of this article (available at http://molpharm.aspetjournals.org) contains supplemental material.

ABBREVIATIONS: GPCR, G-protein-coupled receptor; BRET, bioluminescence resonance energy transfer; ECD, extracellular domain; GIPR, gastric inhibitory protein receptor; GlucR, glucagon receptor; GLP-1R, glucagon-like peptide 1 receptor; GLP-2R, glucagon-like peptide 2 receptor; HBSS++, Hanks' balanced salt solution with calcium and magnesium; PBS, phosphate-buffered saline; HEK, human embryonic kidney; SecR, secretin receptor; PCR, polymerase chain reaction; GIP, gastric inhibitory protein; GLP, glucagon-like peptide.

may couple to trimeric G-proteins, in a 2:1 stoichiometry, for a minimal functional unit (Han et al., 2009).

Constitutive heteromers can exist independently of ligand binding and can remain associated throughout the life cycle of a receptor. One of many examples in class A are the dopamine receptor heteromers dopamine receptors 1/2 and 1/3, which exist in rodent brain and display distinct pharmacological features (Rashid et al., 2007; Marcellino et al., 2008). Heteromer formation upon ligand binding has been reported for dopamine receptor 2/somatostatin receptor 5 (Rocheville et al., 2000) and somatostatin receptor 1/5 (Patel et al., 2002). In other cases, ligand-induced increases in the energy transfer in BRET experiments have been interpreted as movements within an existing heteromer (e.g., melatonin receptor 1/2) (Ayoub et al., 2002).

In class C, most metabotropic glutamate receptors are known to form heterodimers (Doumazane et al., 2011), whereas the GABA B1/B2 receptors form dimers or tetramers (Pinard et al., 2010), which undergo dissociation movements upon ligand binding (Matsushita et al., 2010). Other heteromers include the sweet and umami taste receptors 1/2 and 3 (Li et al., 2002), and the discovery of a metabotropic glutamate receptor 5/dopamine receptor 2/adenosine receptor 2A heteromer provided evidence of class A/C mixed heteromers (Cabello et al., 2009).

In class B, corticotropin-releasing factor receptor 1 has been shown to interact with vasopressin V1b receptor (Young et al., 2007), whereas the secretin receptor (SecR) forms heteromers with vasoactive intestinal polypeptide receptors 1 and 2 (Harikumar et al., 2006) and other closely related receptors, of which only the SecR/parathyroid hormone receptor heteromer showed a decreased BRET signal upon ligand binding (Harikumar et al., 2008). Vasoactive intestinal polypeptide receptors 1 and 2 have also been found to form heteromers that exhibit reduced BRET signals on ligand binding. None of the aforementioned studies noted ligand-induced heteromer recruitment for class B GPCRs.

Coexpression in the same cell is mandatory for physiological relevance of receptor heteromerization. Lateral regulation of receptor function in such heteromers may occur between receptors that are clustered around the same physiological function. The members of the glucagon receptor family, including the glucagon receptor (GlucR), the glucagon-like peptide 1 and 2 receptors (GLP-1R and GLP-2R), and the gastric inhibitory protein receptor (GIPR), regulate glucose homeostasis and fulfill those criteria. Coexpression has been demonstrated, for example, in pancreatic α-cells, thalamus, and hypothalamus (GlucR and GLP-1R), pancreatic β-cells and heart (GlucR, GLP-1R, and GIPR), stomach (GLP-1R and GLP-2R), and cerebral cortex and hippocampus (GlucR and GIPR) (Brubaker and Drucker, 2002; Mayo et al., 2003; Alvarez et al., 2005).

In the present study, we assessed heteromeric interactions in the GlucR family. By fusing GlucR, GLP-1R, GLP-2R, and GIPR to the luciferase RLuc8 or the fluorescent protein YPet, we measured possible interactions in BRET experiments (Kamal et al., 2009). Ligand-induced heteromer formation was profiled pharmacologically, and effects on receptor internalization, arrestin recruitment, and calcium flux were tested.

Materials and Methods

Ligands. Human glucagon(1–29), human oxyntomodulin, and human GLP-1(7–36)NH_2 (all from Bachem, Bubendorf, Switzerland), human GLP-2(146–178) and human GIP(1–42) (both from Anaspec, Fremton, CA), human GLP-1(9–36), exendin(9–39), and exendin 4 (all from Tocris Bioscience, Bristol, UK), and (Pro^3)GIP (Phoenix Europe, Karlsruhe, Germany) were purchased from commercial sources.

Fusion Protein Constructs. Plasmids encoding RLuc8 and YPet-β-arrestin 2 were provided by Dr. Ralf Jockers (Institut Cochin, Paris, France), and human cDNAs for the glucagon family receptors were amplified from human cDNA by using standard reverse transcription-PCR. Fusion proteins consisting of full-length receptors C-terminally fused in-frame to RLuc8 or YPet were generated through PCR and DNA recombination by using Multisite Gateway Pro technology (Invitrogen, Carlsbad, CA). Through standard PCR, receptors, YPet, and RLuc8 were amplified by using primers that added specific Gateway recombination sites for two-way fusion to the products, according to the manufacturer's recommendations. The N-terminal fusion protein elements (receptors) were inserted without a stop codon into pDONR 1–5r vectors, and the C-terminal elements RLuc8 and YPet were inserted into pDONR 5–2. Receptor fusion proteins were finally generated through site-specific DNA recombination by using one receptor-containing pDONR plasmid, one RLuc8- or YPet-containing pDONR plasmid, and one expression plasmid containing an acceptor Gateway cassette.

Tissue Culture. Human embryonic kidney (HEK) cells (HEK 293T cells) were cultured in Dulbecco's modified Eagle's medium containing 100 units/ml penicillin, 100 μg/ml streptomycin (all from Invitrogen), and 10% fetal calf serum (Chemie Brunschwig, Basel, Switzerland), in a humidified incubator at 37°C and 5% CO_2. Cells were transiently transfected in 12- or 6-well plates (Nunc GmbH and Co. KG, Langenselbold, Germany), at 50% to 60% confluence, by using 10 or 20 μl of Optifect reagent (Invitrogen) and 2 or 4 μg of DNA, according to the manufacturer's protocol. The cells were used 48 h after transfection. One day before the BRET or calcium experiments, cells were detached with trypsin/EDTA (Invitrogen) and seeded in black, clear-bottomed, 384-well plates (Nunc) coated with 10 μg/ml poly-L-ornithine (Sigma-Aldrich, St. Louis, MO), at 2 to 3 $\times$ 10^4 cells per well, in growth medium.

BRET Measurements. On the day of the experiment, the medium was aspirated, cells were serum-starved for 60 min at 37°C in Hanks' balanced salt solution with calcium and magnesium (HBSS++) (Invitrogen) and washed with PBS (Invitrogen), and the bioluminescent reaction was started with the addition of coelenterazine H (Dalton Pharma Services, Toronto, Canada) at a final concentration of 6 μM, in 45 μl of HBSS++ per well. Five minutes after the addition of coelenterazine H, the dynamic BRET response was measured with a FLIPR Tetra high-throughput screening system (Molecular Devices, Sunnyvale, CA). Light emission of the donor RLuc8 (emission peak, 487 nm) was detected with a band-pass emission filter of 460 $\pm$ 20 nm, and the acceptor YPet (emission peak, 535 nm) was detected at 556 $\pm$ 30 nm. The two signals were recorded by using equal exposure times and maximal gain settings, with reading periods of 3 to 6 s, depending on signal strength. Measurements for all 384 wells were recorded simultaneously, which allowed for a dynamic result. Ligands were added online through transfer of 15 μl from a 4-fold concentrated addition plate to the reading plate with a multitip head. In two addition protocols, the second ligand was added through transfer of an additional 20 μl from a second 4-fold concentrated addition plate during the reading. The signal was read for 10 to 60 min, and the BRET ratio was defined as [(response at 556 $\pm$ 30 nm)/(response at 460 $\pm$ 20 nm) $-$ correction factor], where the correction factor corresponds to [(response at 556 $\pm$ 30 nm)/(response at 460 $\pm$ 20 nm)] of a receptor-RLuc8 BRET donor expressed in the absence of the YPet acceptor protein. Dose-response curves were graphed by plotting the average net BRET response over 5 to 10 min after a single addition of increasing concentrations of ligand.

Calcium Measurements. On the day of the experiment, cells were washed three times with PBS and incubated for 1 h, at 37°C and 5% CO_2, with 40 μl per well of calcium buffer (143 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 20 mM HEPES, 0.1% glucose, 0.125 mM

sulfinpyrazone, 1 mM $CaCl_2$) containing 3 μM levels of the green fluorescent calcium indicator fluo-4/acetoxymethyl ester (Invitrogen). Cells were washed three times with PBS and were incubated with 45 μl of calcium buffer for another 3.5 h at room temperature in the dark. The calcium response was measured with the FLIPR Tetra system by using light-emitting diode excitation (470–495 nm) in combination with a band-pass emission filter (545 $\pm$ 30 nm) to measure the calcium-induced fluorescence of fluo-4/acetoxymethyl ester. Ligands were added online through transfer of 15 μl from a 4-fold concentrated addition plate to the reading plate with a multitip head. Dose-response curves were calculated by subtracting the minimal value from the maximal value for the dynamic calcium response over 5 min. Each data point was measured in duplicate.

Fluorescence Imaging. Transfected cells were seeded onto 10 μg/ml poly-L-ornithine (Sigma-Aldrich)–coated coverslips 24 h after transfection, washed after 48 h, incubated for 30 min at room temperature with ligand in HBSS++, fixed with PBS (with 4% paraformaldehyde), and mounted with Mowiol medium (Sigma-Aldrich) on microscope slides. Images were obtained with an Axiovert 200 fluorescence microscope (Carl Zeiss AG, Oberkochen, Germany) by using yellow fluorescent protein and enhanced cyan fluorescent protein filter sets and a 63$\times$ oil-immersion objective. Images were digitally treated by using ImageJ software (http://rsbweb.nih.gov/ij).

Data Analysis. The dose-response curves were fitted to a four-parameter, nonlinear regression, sigmoidal, dose-response equation by using Prism 5.0 software (Graph Pad Software, San Diego, CA), which allowed determination of EC_{50} values. Each curve data point was measured in duplicate unless indicated otherwise. Statistical relevance in comparisons of the effects of ligands was determined by using one-way analysis of variance followed by Tukey's multiple-comparison test when more than two conditions were compared or by an unpaired two-tailed *t* test when two conditions were compared, by using Prism 5.0.

Results

Protein Structures

The relationships among the glucagon-family ligands glucagon, GLP-1, GLP-2, and GIP were compared by aligning primary protein structures (Fig. 1). Crystal structures for the complexes of GLP-1 and GIP with the extracellular domains (ECDs) of their respective receptors have been solved (Parthier et al., 2007; Underwood et al., 2010), and residues that could be modeled are indicated. The four peptides share a glucagon-like core structure of 27 amino acids, with highly conserved N-terminal (amino acids 1–9 of glucagon) and C-terminal (amino acids 20–28) domains.

Marked differences between the ligands were found in the last C-terminal residues of the peptides, which are nonconserved and variable in size, with glucagon possessing the shortest tail and GIP the longest tail. The precise interactions of those tails with the receptor are not known. Glucagon is evolutionarily more closely related to GIP than to GLP-1 or GLP-2, despite the fact that GIP is derived from a different precursor protein.

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Glucagon HSQGTFTSDYSKYLDSRRAQDFVQWLMNT------------- 29
GIP      YAEGTFISDYSIAMDKIHQQDFVNWLLAQKGKKNDWKHNITQ 42
GLP-1    HAEGTFTSDVSSYLEGQAAKEFIAWLVKGRG------------ 31
GLP-2    HADGSFSDEMNTILDNLAARDFINWLIQTKITD---------- 33
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Fig. 1. Protein sequence alignment of human glucagon, GLP-1, GLP-2, and GIP. The amino acid alignment of the ligands was constructed with ClustalW (Chenna et al., 2003). Residues of GLP-1 and GIP that were resolved through crystallization with the ECDs of GLP-1R and GIPR are underlined. Asterisks, fully conserved residues (identical residues); double dots, highly conserved residues (conserved substitutions of the same polarity or hydrophobicity); single dots, semiconserved residues (substitutions of similar shape).

Cotransfection Experiments

For assessment of possible ligand-induced interactions between the receptors of the glucagon family, HEK cells were cotransfected with equal amounts of plasmids encoding receptors fused to RLuc8 or YPet. The subcellular localization of the receptors was assessed with immunofluorescence assays (anti-RLuc8) or direct fluorescence microscopy (YPet), 48 h after transfection (data not shown). The receptors were located mainly at the cell membrane for all combinations tested except for the GIPR-RLuc8/GIPR-YPet combination, for which aggregation in the endoplasmic reticulum/Golgi was observed. The basal BRET ratio for the receptor constructs was determined, and changes in energy transfer after the addition of ligand were calculated by subtracting the BRET signal obtained after ligand addition from the basal ratio. In the following sections, GLP-1 refers to the physiological agonist GLP-1(7–36)NH_2, unless indicated differently.

Homomeric Interactions

In HEK cells coexpressing the same receptor coupled to RLuc8 or YPet, basal BRET signals were observed for GIPR/GIPR and GlucR/GlucR and, to a lesser extent, for GLP-2R/GLP-2R and GLP-1R/GLP-1R (Fig. 2, bottom). Addition of GIP or glucagon induced a small reduction in energy transfer for GIPR/GIPR and GlucR/GlucR, respectively (Fig. 2, top).

Heteromeric Interactions

Heteromer formation was assessed by double-transfecting cells with different BRET-compatible receptor combinations. Each combination was tested once as receptor A-RLuc8/receptor B-YPet and once with the same receptors and switched tags (Fig. 2).

GLP-1R/GIPR. A strong, highly significant increase in the BRET ratio was observed upon GLP-1 stimulation of cells expressing GLP-1R-RLuc8/GIPR-YPet ($p < 0.001$), which suggests recruitment of a heteromer or a conformational change in an existing one. The same effect was observed in cells expressing GLP-1R-YPet/GIPR-RLuc8 ($p < 0.01$), which confirms the independence of BRET tag orientation. GIP induced a small decrease in the basal BRET level, which suggests inverse effects of the ligands. We estimated the GLP-1R expression levels in BRET experiments through fluorescent ligand binding and compared levels with those in the insulinoma cell line INS-1E, a physiological reference (Supplementary Fig. 1), and we found an average of 17.2-fold overexpression. With decreases in BRET receptor expression levels to estimated 2.4- and 1.1-fold overexpression, compared with INS-1E cells, we observed GLP-1-induced receptor heteromerization (Supplementary Fig. 2) but no GIP effect on basal BRET values. The detection limit of the luminescence reader used in this study was reached at low receptor expression levels; therefore, such conditions could not be used for the reliable generation of data.

GLP-1R/GlucR. A small but significant decrease in the basal BRET ratio was induced by stimulation with GLP-1 ($p < 0.001$) but not glucagon in cells expressing GLP-1R-RLuc8 and GlucR-YPet.

GlucR/GLP-2R. A decrease in the basal BRET ratio for GlucR-RLuc8/GLP-2R-YPet was induced after stimulation

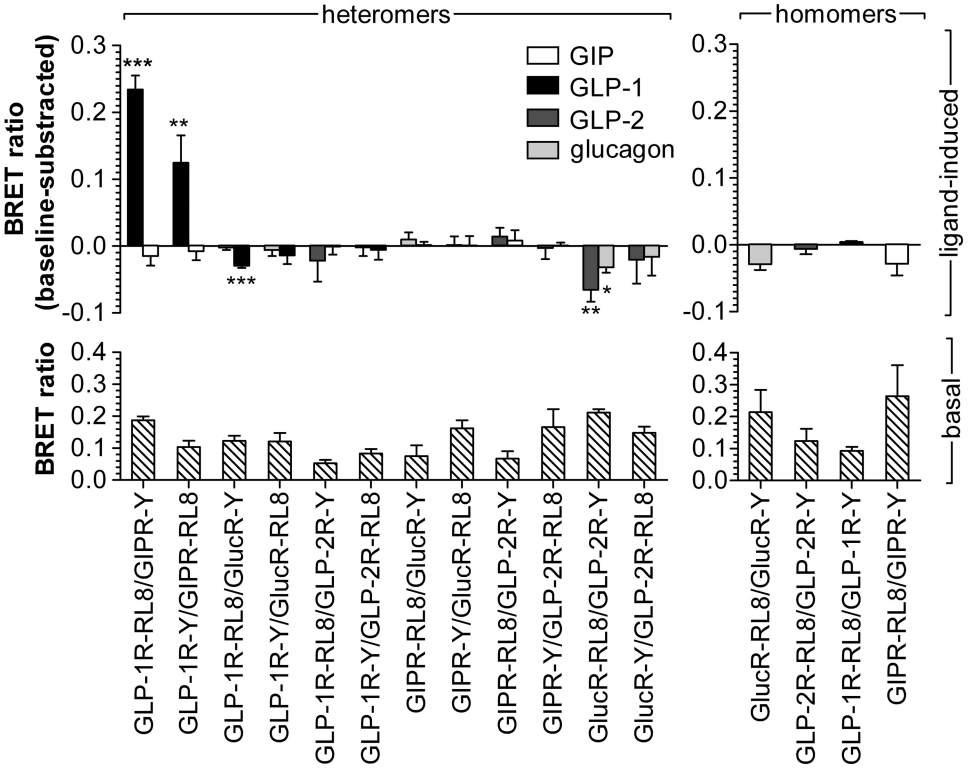

Fig. 2. Interactions between glucagon family receptors in BRET experiments. HEK cells were cotransfected with equal amounts of plasmids encoding receptors coupled to RLuc8 (RL8) or YPet (Y), and the BRET ratios were measured. Bottom, basal BRET responses. Net BRET values were determined for all receptor combinations. Each pair was tested in two BRET configurations (YPet/RLuc8 or RLuc8/YPet). Homomeric interactions were tested through transfection of cells with equal amounts of the same receptor tagged with YPet or RLuc8. Top, ligand-induced BRET ratio changes. The effects of the ligands GIP, GLP-1, GLP-2, and glucagon on the basal BRET response were assessed by subtracting the BRET value measured after ligand addition from the basal value. Each receptor combination was tested with both specific ligands for the two receptors; cells transfected with just one receptor type were tested with only one ligand. Decreases in the BRET ratio induced by ligand resulted in negative values and increases in positive values. Each condition tested was graphed as the average ± S.E.M. of three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

with glucagon ($p < 0.05$) or GLP-2 ($p < 0.01$). The same tendency could be observed when the BRET tags were inverted; however, the response was less pronounced. With all other receptor combinations, ligand addition did not significantly affect the basal BRET ratio. Because a ligand-induced BRET increase was seen only with GLP-1R/GIPR, this unique interaction was further characterized.

BRET Donor Saturation Experiments. Next, the nature of the interactions between GLP-1R and GIPR was determined in BRET donor saturation experiments (Issad and Jockers, 2006). Cells were cotransfected with a fixed amount of the BRET donor plasmid and increasing amounts of the acceptor plasmid. The energy transfer between the receptors was quantified as net BRET in the presence or absence of saturating concentrations of ligand. An increase of BRET between GIPR and GLP-1R was observed with increasing concentrations of transfected GIPR-YPet plasmid in buffer conditions (Fig. 3A), but no saturation of the curve was observed. The presence of GLP-1 increased the BRET in three independent experiments, leading to saturation of the curve that reached the maximal BRET interaction level at $BRET_{max}$ = 0.66 ± 0.05 (mean ± S.E.M.) and to a leftward shift of the curve, which allowed calculation of a half-saturation ratio ($BRET_{50}$ = 1.27 ± 0.20) at which 50% of donors were occupied by acceptors (Issad and Jockers, 2006). In a reverse BRET acceptor saturation experiment, decreasing concentrations of GLP-1R-RLuc8 produced increasing BRET signals with a fixed concentration of GIPR-YPet in the presence of GLP-1, which confirms the specificity of this interaction, also at low levels of GLP-1R (Supplementary Fig. 3). The saturation curves for GIPR alone or GLP-1R alone showed ligand-induced reductions in BRET when the donor was present in excess, which suggests that these receptors interact as homomers and that a reduction in affinity or a conformational change occurs upon ligand binding (Fig. 3, C and D). In the control experiments, a quasi-linear, nonsaturated, BRET curve was observed, which suggests nonspecific, random interactions between the nonglucagon receptor muscarinic acetylcholine receptor 4-RLuc8 (class A) and GLP-1R-YPet. The presence of GLP-1 did not alter the signal, which shows that the GLP-1 effect of heteromerization was receptor-specific (Fig. 3B).

Opposing Effects of GLP-1 and GIP on Receptor Heteromerization in BRET System

The pharmacological features of the heteromerization of GLP-1R and GIPR were characterized by transfecting HEK cells with equal amounts of GLP-1R-RLuc8 and GIPR-YPet plasmids. The dynamics of the GLP-1 response in BRET were monitored with online addition of increasing concentrations of the ligand, which resulted in rapid formation of the BRET response (Fig. 4A). Opposing effects and the dynamics of GLP-1 and GIP in receptor recruitment in BRET were tested with subsequent addition of both ligands (Fig. 4B). GLP-1 addition induced recruitment of GLP-1R to GIPR that resulted in a stable maximal BRET signal after 0.5 min. Addition of GIP 1 or 5 min after the addition of GLP-1 reversed the GLP-1-induced BRET augmentation to baseline levels, which was completed 3 to 4 min after GIP addition. GIP was still effective in decreasing the BRET signal 5 min after GLP-1 addition, which indicates that the receptor heteromer was still present at the ligand-accessible cell surface.

GLP-1, GLP-1(9–36), and GLP-2 Induction of Receptor Heteromerization

The GLP-1R and GIPR antagonists GLP-1(9–36) and (Pro^3)GIP were characterized at full doses. Both ligands are known to bind to their receptors but their receptor-activating

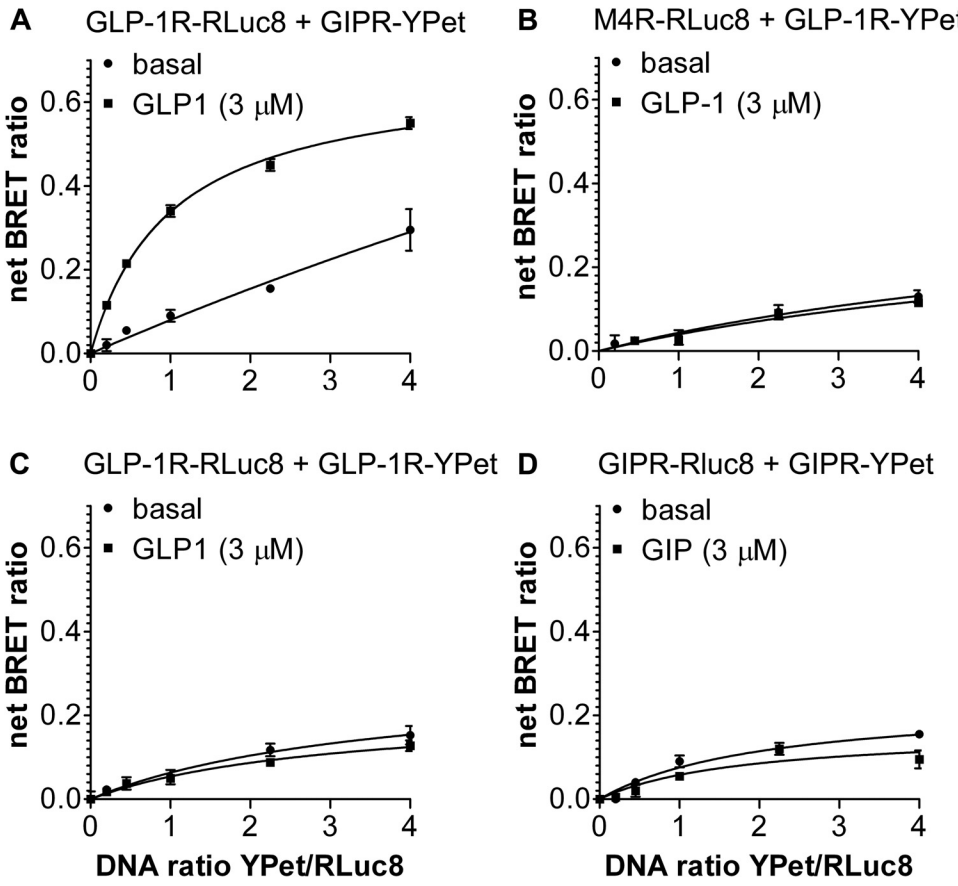

Fig. 3. BRET donor saturation experiments. The specificity of receptor homomerization and heteromerization between the GLP-1R and GIPR was tested in BRET donor saturation experiments. HEK293T cells were cotransfected with a fixed amount of BRET donor plasmid and increasing amounts of acceptor plasmid. The energy transfer was tested in the absence or with saturating concentrations of GLP-1 or GIP. A, GLP-1R/GIPR. Increasing amounts of BRET acceptor protein resulted in an increase of the BRET signal that was quasi-linear. The presence of GLP-1 increased the BRET signal at each condition tested and resulted in earlier BRET signal saturation. B, muscarinic acetylcholine receptor 4/GLP-1R. As a control condition, GLP-1R was cotransfected with muscarinic acetylcholine receptor 4 (M4R). The curve observed was quasi-linear, and no difference between the basal condition and GLP-1 treatment was observed. C and D, GLP-1R/GLP-1R (C) and GIPR/GIPR (D). Saturation experiments in cells transfected with just one receptor type showed the beginning of saturation under the conditions tested for both receptors. Both GLP-1 and GIP decreased the BRET signal when BRET acceptor protein was present in excess. All graphs show one representative experiment (duplicate data points; mean ± S.D.) of three independent experiments.

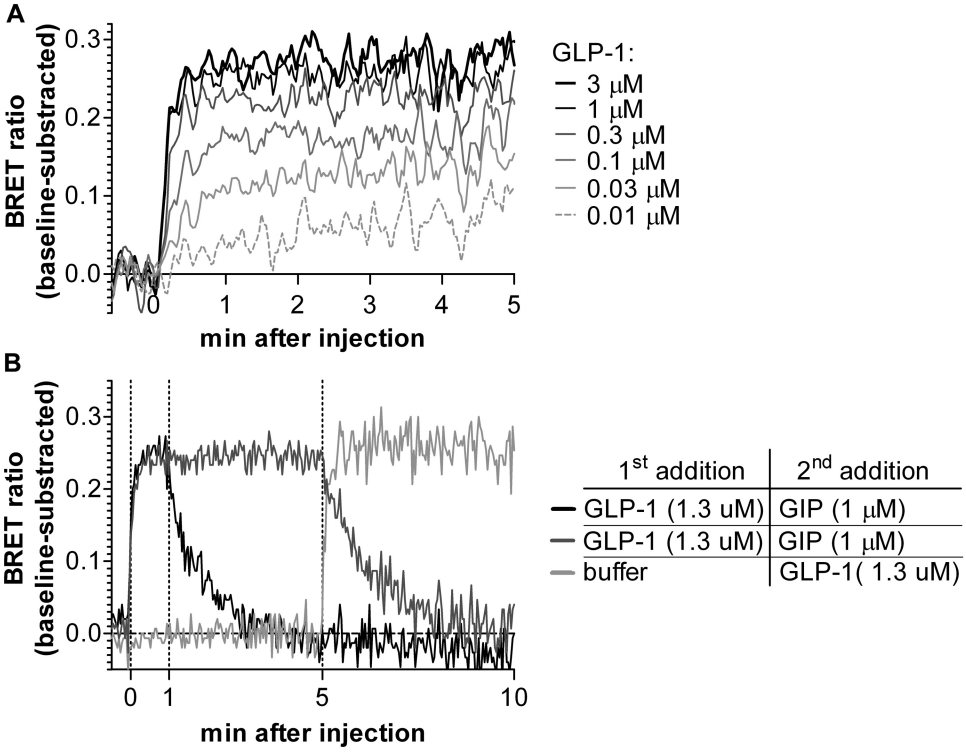

Fig. 4. Dynamics of the BRET signal of GLP1R/GIPR in response to GLP-1 and GIP. A, representation of a dynamic BRET result for 5 min, with increasing concentrations of GLP-1 added online. Traces (data point duplicates) were smoothed (average of 3 nearest neighbors; second-order smoothing polynominal), and buffer readings were subtracted. B, representation of a dynamic BRET result for 10 min. GLP-1 (black and dark gray traces) or buffer (light gray trace) was added online, followed by a second addition of GIP after 1 min (black trace) or 5 min (dark gray trace) or of GLP-1 (light gray trace) after 5 min.

N-terminal residues are truncated or mutated. Their BRET responses were compared with the GLP-1 and GIP responses (Fig. 5A). GLP-1 and GLP-1(9–36) were equally potent in recruiting the receptor heteromer (EC_{50} = 243 ± 95 nM and 266 ± 145 nM, respectively), but GLP-1(9–36) surpassed the maximal response achieved with GLP-1. GIP caused a decrease in the basal BRET ratio (IC_{50} = 33 ± 2.1 nM), which suggests some interaction between the receptors in the ab-

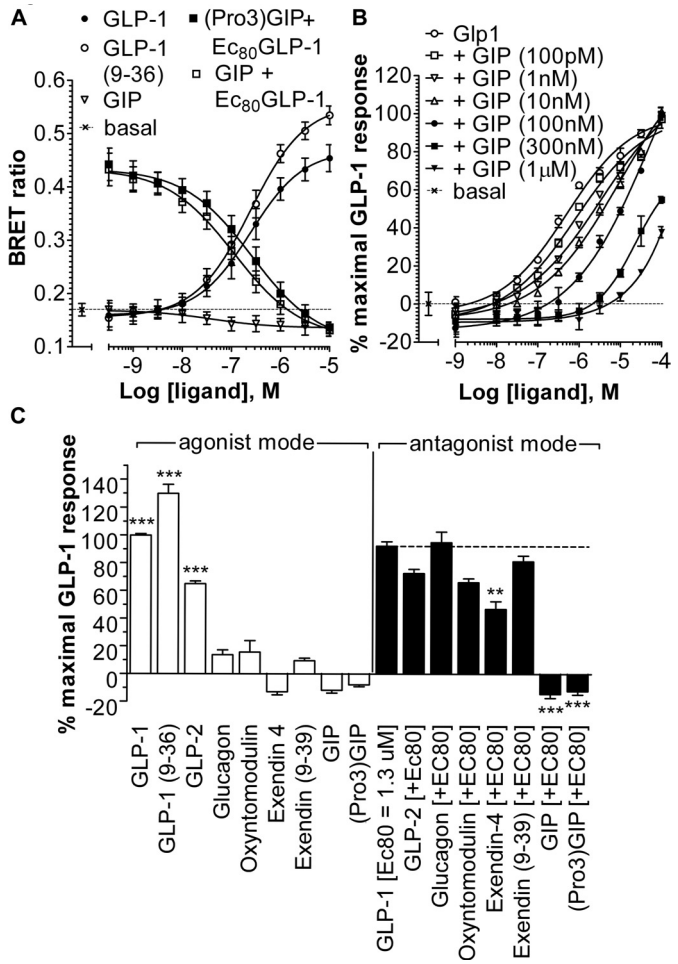

Fig. 5. Effects of GluR family agonists and antagonists on the heteromerization of GLP-1R/GIPR. A, dose-response curves were graphed by plotting the average net BRET over 10 min after a single addition of increasing concentrations of ligand. GLP-1, GLP-1(9–36), and GIP were tested for their effects alone. GIP and (Pro^3)GIP were tested at an EC_{80} concentration of GLP-1 (1.3 μM). Data represent the average ± S.E.M. of 3 independent experiments. B, dose-response curves for GLP-1 were measured in the presence of several fixed concentrations of GIP, which resulted in a shift in the GLP-1 curve. A representative experiment with quadruplicate data points (mean ± S.D.) is shown. C, additional, naturally occurring, glucagon-like ligands and their derivates were tested for their effects on the heteromerization of GLP-1R and GIPR. All ligands were tested in full dose-response conditions, and their maximal effects at 10 μM were compared with the maximal response to GLP-1 (100%). Ligands that were tested in antagonist mode were added with an EC_{80} concentration of GLP-1 (1.3 μM). EC_{50} and IC_{50} values were derived from full dose-response curves for the ligands. Data represent the average ± S.E.M. of three independent experiments. **, $p < 0.01$; ***, $p < 0.001$.

sence of ligand. The GIPR antagonist (Pro^3)GIP had an effect on the heteromer similar to that of GIP; both antagonized EC_{80} concentrations (1.3 μM) of GLP-1 (IC_{50} = 135 ± 43 nM and IC_{50} = 369 ± 188 nM, respectively) and decreased the BRET signal below the buffer baseline.

To characterize further the pharmacological characteristics of the GIP inhibition of receptor heteromerization, dose-response experiments with GLP-1 were carried out with increasing concentrations of GIP, which resulted in a decrease in the initial BRET value and a rightward shift of the GLP-1 dose-response curve (Fig. 5B). High doses of GLP-1 could overcome the GIP inhibition to yield the same maximal response as GLP-1 alone. For the heteromerization, this pharmacological profile suggests orthosteric competition of GLP-1 and GIP for the same binding site on the receptor.

Additional naturally occurring ligands and derived agonists and antagonists of GLP-1R and GIPR were tested for their potential to induce heteromerization between the receptors (Fig. 5C). GLP-1, GLP-1(9–36), and GLP-2 (EC_{50} = 407 ± 68 nM) significantly increased the BRET signal between the receptors ($p < 0.001$), whereas glucagon, oxyntomodulin, and exendin 4, known activators of GLP-1R, did not induce heteromerization. Exendin 4 decreased the basal BRET interaction in a way similar to that of GIP and (Pro^3)GIP, whereas the functional antagonist exendin(9–39) showed no activity. Probing with those ligands against an EC_{80} concentration of GLP-1 confirmed effective disruption of the BRET complex by exendin 4 ($p < 0.01$), GIP, and (Pro^3)GIP ($p < 0.001$).

Receptor Internalization

Because similar effects on receptor interactions were observed with agonist and antagonists in BRET experiments, receptor localization was studied to monitor internalization of each receptor and to detect possible GLP-1R/GIPR complexes. Cells were transfected with fluorescently tagged GLP-1R (enhanced cyan fluorescent protein) and GIPR (YPet). In the nontreated condition, both receptors were located at the membrane (Fig. 6, A and E). GLP-1 (Fig. 6, B and F), exendin 4 (Fig. 6, D and H), and glucagon (Fig. 6, E and I) caused internalization of mainly GLP-1R, whereas GIPR remained at the membrane. In contrast, GLP-1(9–36) (Fig. 6, C and G) and GLP-2 (Fig. 6, I and M) did not have effects on receptor internalization. Likewise, GIP internalized mainly GIPR (Fig. 6, K and O), whereas (Pro^3)GIP had no effect on either receptor (Fig. 6, L and P). Recruitment and disruption of the GLP-1R/GIPR heteromer by GLP-1(9–36), GLP-2, and (Pro^3)GIP (Fig. 5) seem to represent a mechanism earlier than and independent of receptor internalization.

Coexpression of GLP-1R and GIPR Altering GLP-1-Induced but Not GIP-Induced Calcium Responses in HEK Cells

GLP-1R and GIPR both induce a cellular calcium flux in response to ligand, which raises the question of whether allosteric regulation in a heteromeric complex of those receptors would result in altered calcium pharmacological characteristics. HEK cells transfected with GLP-1R alone or GIPR alone demonstrated calcium flux in response to the respective ligands, with no effect of the other ligand at concentrations of <3 μM (Fig. 7A). Coexpression of GLP-1R with GLP-2R and GlucR had no effect on the GLP-1 response (Fig. 7B). Coexpression of GLP-1R and GIPR, however, resulted in an altered, flattened, pharmacological response to GLP-1, with a significant change of the EC_{50} ($p < 0.05$) and a highly significant effect on the Hill slope ($p < 0.001$), compared with cells expressing GLP-1R alone (Table 1). Under these conditions, lower doses of GLP-1 (0.3 nM) stimulated calcium flux and the dose-response curve approached the pharmacological profile of the GIP response. The presence of GLP-1R did not affect the profile of the GIP response, compared with cells expressing GIPR alone. This observation is in line with the finding that GLP-1 but not GIP induced heteromerization of

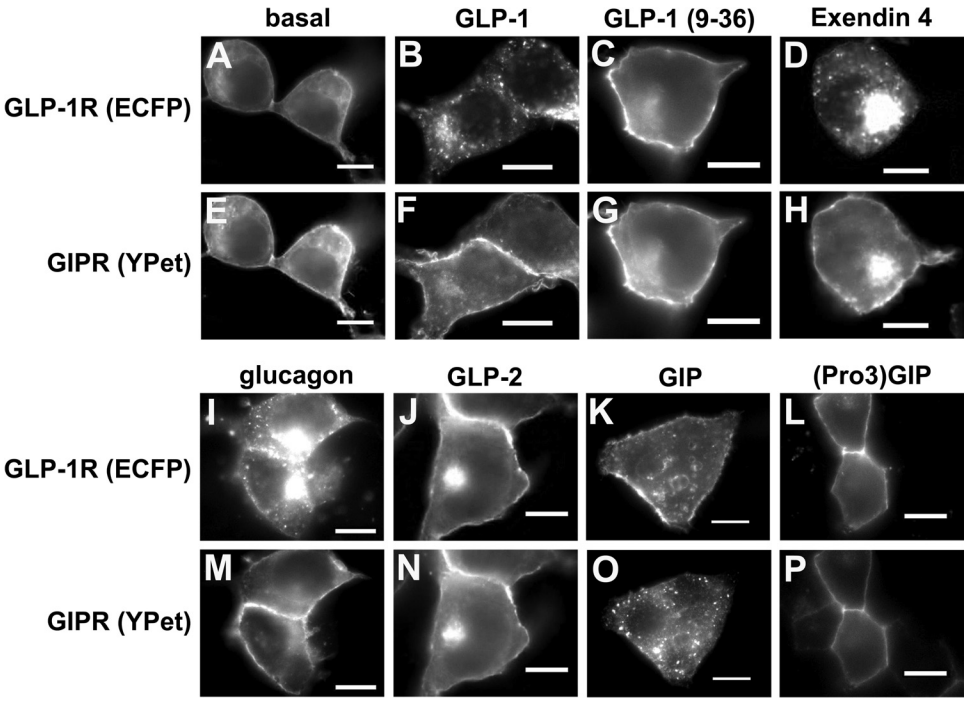

Fig. 6. Ligand-induced receptor internalization. The effects of the ligands on the internalization of GLP-1R and GIPR were studied in HEK cells cotransfected with GLP-1R-enhanced cyan fluorescent protein (ECFP) and GIPR-YPet. Cells were incubated with ligand (1 μM) for 20 min at room temperature and fixed, and the localization of each receptor was assessed with a fluorescence microscope.

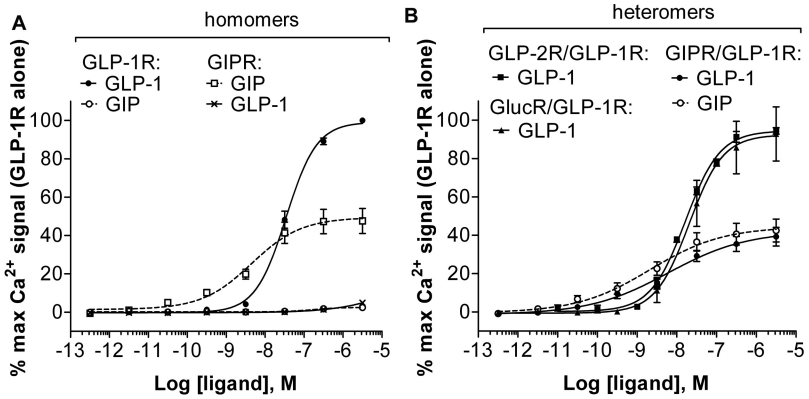

Fig. 7. Coexpression of GLP-1R and GIPR altering the GLP-1-induced but not the GIP-induced calcium response in HEK cells. Cells were transiently transfected with a single, wild-type receptor or were cotransfected with the GLP-1R and a second glucagon family receptor. Changes in intracellular calcium levels were measured by using the calcium-sensitive fluorescent dye fluo-4. A, the calcium signal in response to GIP or GLP-1 was measured in cells expressing GIPR or GLP-1R alone. The signal was normalized to the maximal response obtained with GLP-1 at the GLP-1R. B, the GLP-1 and GIP responses of the GLP-1R in the presence of a second receptor were tested and graphed, with normalization to the GLP-1 response of the GLP-1R expressed alone. Data represent the average ± S.E.M. of three to five independent experiments.

TABLE 1

Calcium release of HEK cells expressing GIPR or GLP-1R or coexpressing GLP-1R with another glucagon receptor

The EC_{50} and Hill slope values from independent experiments were averaged and compared for each condition, with GIPR (GIP, n = 4; GLP-1, n = 4), GLP-1R (GIP, n = 4; GLP-1, n = 5), GLP-1R plus GIPR (GIP, n = 4; GLP-1, n = 4), GLP-1R plus GlucR (GLP-1, n = 3), and GLP-1R plus GLP-2R (GLP-1, n = 3).

	GLP-1		GIP	
	EC_{50}	Hill Slope	EC_{50}	Hill Slope
	nM		*nM*	
GIPR			5.0 ± 1.2	0.59 ± 0.27
GLP-1R	41.2 ± 11.0	1.17 ± 0.17		
GLP-1R + GIPR	5.0 ± 2.6	0.50 ± 0.19	2.35 ± 0.5	0.63 ± 0.24
GLP-1R + GlucR	26.2 ± 10.6	1.14 ± 0.22		
GLP-1R + GLP-2R	17.0 ± 2.3	1.02 ± 0.16		

the two receptors in BRET experiments, and it suggests a pharmacological role of receptor heteromerization in calcium signaling.

Influence of GIPR on GLP-1-Induced β-Arrestin Recruitment to GLP-1R

In pancreatic cells, β-arrestin recruitment contributes importantly to GLP-1-induced insulin release (Dalle et al., 2011). On the basis of BRET results that showed recruitment of β-arrestin 2 to GLP-1R (Jorgensen et al., 2007), we used a similar approach to evaluate the effects of heteromerization on arrestin recruitment.

Cells transfected with luciferase-tagged GLP-1R and YPet-β-arrestin 2 responded to GLP-1 with a dose-dependent increase in the BRET signal (Fig. 8A). Coexpression of GIPR markedly decreased the BRET response of GLP-1 at concentrations of >30 nM (Fig. 8B), reducing the maximal plateau of arrestin recruitment to GLP-1R by ~25% at potencies of GLP-1 that remained comparable (EC_{50} for GLP-1R = 32.1 nM; EC_{50} for GLP-1R/GIPR = 17.7 nM). Because the GLP-1

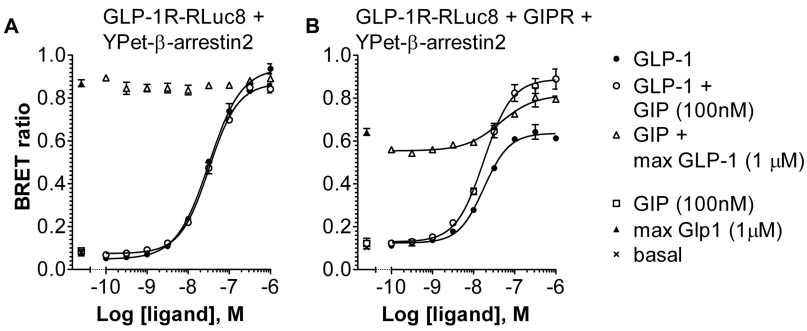

Fig. 8. Coexpression of GLP-1R and GIPR altering the GLP-1-induced recruitment of β-arrestin to the receptor. HEK cells were transiently cotransfected with GLP-1R-RLuc8 plus YPet-β-arrestin 2 or GLP-1R-RLuc8 plus GIPR (wild-type) plus YPet-β-arrestin 2. The GLP1-induced recruitment of YPet-β-arrestin 2 to the luciferase-tagged GLP-1R resulted in an increase in the BRET ratio. The dose-response effects of GLP-1, of GLP-1 at a fixed concentration of GIP (100 nM), of GIP at saturating levels of GLP-1 (1 μM), and of a fixed dose of GIP (100 nM) alone were compared in the absence (A) or presence (B) of coexpressed GIPR. A representative experiment, with duplicate data points (mean ± S.D.), is shown.

effect on receptor heteromerization was observed at similar concentrations (Fig. 5A), we assessed the possibility of reversing this effect on arrestin recruitment with the addition of GIP. In the presence of a fixed concentration of GIP (100 nM), the maximal response of GLP-1 with the coexpressed receptors was similar to the level of the GLP-1 response with GLP-1R alone (Fig. 8). In addition, with saturating concentrations of GLP-1 (1 μM), GIP was able to increase the BRET signal in a dose-dependent manner. The addition of 100 nM GIP alone did not induce a BRET signal in either transfection condition, with or without GIPR present. In the absence of GIPR, addition of a fixed concentration of GIP (100 nM) did not have an effect on the GLP-1 response and increasing concentrations of GIP did not alter the maximal GLP-1 response, which suggests no activity of GIP at GLP-1R. The arrestin recruitment results create a link to the receptor heteromerization phenomenon observed, suggesting a physiologically relevant mechanism of allosteric regulation through this interaction and allowing for the development of a heteromerization model.

Discussion

In the present study, we grouped functionally related class B receptors that are known to be coexpressed in the same cells that respond to the closely related ligands glucagon, GLP-1, GLP-2, and GIP, which are involved in glucose homeostasis (Baggio and Drucker, 2007). We observed, for the first time, dose-dependent GLP-1-induced formation of a heteromer between GLP-1R and GIPR. This effect was still detectable when receptor expression levels were decreased to match those measured in a pancreatic cell line endogenously expressing GLP-1R (Supplementary Fig. 2), which excludes the possibility of an artifact resulting from receptor overexpression. The functional GLP-1R antagonist GLP-1(9–36) and GLP-2 mimicked the GLP-1 effect. No such behavior was observed for other members of the glucagon receptor family. Both GIP and (Pro^3)GIP dose-dependently inhibited GLP-1-induced GLP-1R/GIPR heteromerization. BRET donor saturation experiments indicated that specific receptor recruitment, rather than a conformational change of an already existing heteromer, was induced by GLP-1 (Issad and Jockers, 2006). This is, to our knowledge, the first example of ligand-induced receptor heteromerization in class B.

In a study that tested the potential of class B prototypic SecR to form intrafamily heteromers with nine other class B GPCRs (including GLP-1R and GLP-2R) in response to ligand, the authors observed constitutive interactions of SecR with most of the receptors tested and ligand-induced decreases in heteromerization (Harikumar et al., 2008). No complex formation upon ligand binding was reported for the receptors tested. We also observed reduction of the basal BRET between GLP-1R and GIPR after GIP addition (Fig. 5) and a small but significant ligand-induced BRET decrease between GlucR/GLP-1R and GlucR/GLP-2R. Confirmation of such small changes in the BRET ratio at lower expression levels is technically challenging with our experimental configuration. Therefore, we cannot exclude the possibility that the basal receptor interaction observed results from random collisions of overexpressed receptors. Consequently, ligand-induced BRET decreases might be the result of receptor internalization.

Class B ligands are considered "two-domain" peptides. Their C-terminal domain conveys receptor specificity by binding to the ECD, whereas the N-terminal domain activates the receptor via the transmembrane domain (Hoare, 2005). GLP-1 and its antagonists, GLP-1(9–36) and GLP-2, induced receptor heteromerization in the BRET assay, but GLP-1(9–36) and GLP-2 did not alter the surface localization of the receptors (Fig. 6, C, G, J, and N), which suggests that this receptor heteromerization is independent of GLP-1R activation and makes involvement of the C-terminal receptor-binding domain of the ligand more likely. In contrast, GLP-1 induced GLP-1R internalization, whereas most of the GIPR population remained at the membrane (Fig. 6, B and F). This indicates either that most of the GLP-1R population is activated and internalized independent of the GIPR or that the heteromeric complex is dissolved before the GLP-1R is internalized. The finding that GLP-1(9–36) induced a stronger BRET response than did GLP-1 supports this possibility (Fig. 5C). The BRET heteromerization of GLP-1R/GIPR reached its maximum 10 to 30 s after ligand addition (Fig. 4A), whereas GLP-1R internalization could be observed 1 to 2 min after addition, which indicates two independent sequential events. The possibility of disrupting the BRET complex induced by GLP-1 with subsequent addition of GIP even after 5 min (Fig. 4B) shows that the heteromers stay at the cell surface or are internalized and cycled back to the membrane where GIP can bind.

Recruitment of the GLP-1R/GIP heteromer by BRET was detectable at ligand concentrations of >10 nM, higher than the 5 to 30 pM concentrations of GLP-1 found in human blood (Vilsbøll et al., 2001). In the lamina propria of the gastrointestinal mucosa, where GLP-1 is released from L-cells, locally high concentrations of GLP-1 could act in a paracrine way (e.g., on afferent nerve terminals that coexpress GLP-1R and, to much lesser extent, GIPR) (Nakagawa et al., 2004). GLP-1 contributes to insulin release and glucose increases through afferent sensory neurons (Ahrén, 2004). Neuronal GIPR ex-

pression has been reported for various cell types of the central and peripheral nervous systems and was linked to endocrine or paracrine signaling (Buhren et al., 2009). Half of the GLP-1 produced in the intestine is metabolized to GLP-1(9–36) as it enters the circulation (Hansen et al., 1999), which makes this antagonist the predominant bioavailable form of GLP-1. Our observation that GLP-1(9–36) can induce receptor heteromerization adds to the debate regarding the physiological role of this entity.

Unlike GLP-1, the lizard-derived bioactive analog exendin 4 did not induce heteromerization. Exendin 4 (39 amino acids) possesses an extended C terminus similar to that of GIP (42 amino acids). These residues have not been resolved in ligand/receptor crystal structures (Parthier et al., 2007; Runge et al., 2008; Underwood et al., 2010), and exendin 4 may interact with regions of the receptor ECD important for receptor heteromerization differently, compared with the relatively short, heteromer-inducing, ligands GLP-1, GLP-1(9–36), and GLP-2 (Fig. 1). A short consensus repeat region situated near the C terminus of the bound ligand, which is known to promote protein-protein interactions, is present in the ECD of corticotropin-releasing factor receptor 2 and is conserved in GLP-1R (Grace et al., 2004), which raises the issue of possible involvement in GLP-1R/GIPR heteromerization.

GIP and (Pro^3)GIP both inhibited GLP-1-induced receptor heteromerization, which raises the question of whether those ligands act by binding to a separate binding site on the heteromer or by orthosterically competing with GLP-1 for the same binding site. GIP did not activate GLP-1R expressed alone in the calcium response or in arrestin recruitment. High concentrations of GIP did not affect the pharmacological response of GLP-1R to GLP-1, which suggests that GIP does not compete with GLP-1 for the ligand binding site on GLP-1R (Fig. 8A). The BRET heteromerization shift experiment (Fig. 5B), however, suggests a competition mechanism, because the BRET-decreasing GIP effect could be overcome by high concentrations of GLP-1, which implies competition of GLP-1 and GIP for the same binding site on the GIPR.

In the arrestin-recruitment and calcium-response experiments, altered GLP-1 pharmacological characteristics of the GLP-1R, with reduced amplitude, were observed in the presence of the GIPR at concentrations of GLP-1 of >30 nM. This corresponds to the concentrations at which receptor heteromerization became apparent (Fig. 5A), whereas GLP-1R calcium responses and arrestin recruitment through GLP-1 were detectable at lower concentrations. This raises the possibility that the activation of GLP-1R is induced through high-affinity binding of GLP-1 to its receptor, whereas GLP-1R/GIPR heteromerization is mediated by low-affinity binding of GLP-1 to the GIPR.

When GLP-1R and GIPR were coexpressed in the arrestin experiment, coincubation with GIP rescued the normal GLP-1 pharmacological features and restored the GLP-1R response to that observed when GLP-1R was expressed alone (Fig. 8B). This suggests that the changed pharmacological profile resulted from allosteric regulation of GLP-1R through the recruitment of GIPR at high GLP-1 concentrations.

On the basis of these results, a model for GLP-1R/GIPR heteromerization can be derived (Fig. 9). In this model, GLP-1 serves as a high-affinity ligand for GLP-1R, inducing functional responses at low concentrations (Fig. 9A). At higher concentrations and in the absence of GIP, GLP-1 becomes also a low-affinity ligand for GIPR, and its binding induces a receptor heteromer in which both GLP-1R and GIPR bind GLP-1 (Fig. 9B). Because the maximal response in our calcium-response and β-arrestin-recruitment experiments resulted in a decrease of the maximal response, the interaction with the GIPR seems to represent a form of allosteric regulation of the GLP-1R. Finally, addition of the high-affinity ligand GIP can displace GLP-1 from the GIPR, dissolving the receptor heteromer and restoring the normal GLP-1R pharmacological characteristics (Fig. 9C).

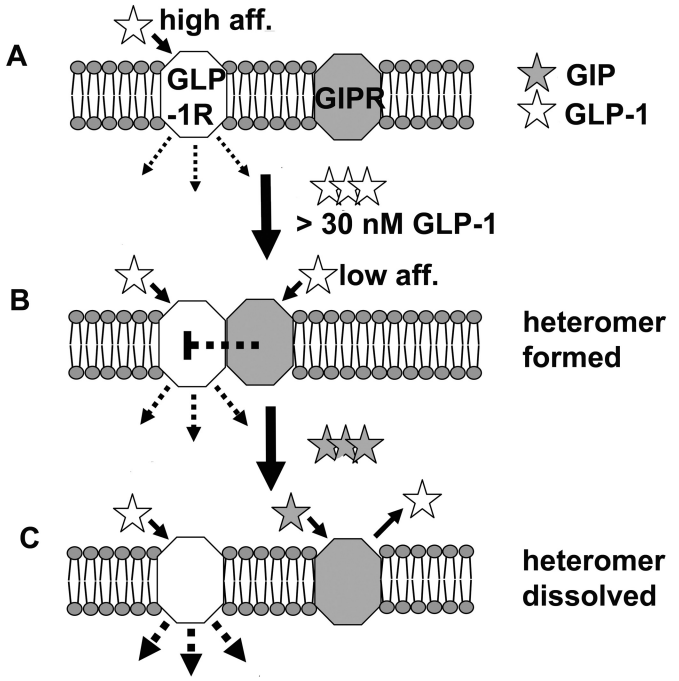

Fig. 9. Heteromerization model for GLP-1R/GIPR. A, in the basal state, the main GLP-1R and GIPR populations exist mainly as monomers or homomers. GLP-1 is a high-affinity ligand for GLP-1R that activates a signaling response starting at concentrations of <30 nM. B, GLP-1 is also a low-affinity ligand for the GIPR and binds at concentrations of >30 nM. GLP-1R and GIPR form heteromers when GLP-1 is bound to both receptors. Heteromerization with the GIPR regulates the maximal GLP-1 responses of arrestin recruitment and calcium mobilization. C, in the presence of GIP, a high-affinity ligand for GIPR, GLP-1 orthosterically competes with GIP for the same binding sites. With its higher affinity, GIP can displace GLP-1 from the GIPR, which results in dissociation of the receptor heteromer and restoration of the normal arrestin recruitment profile of GLP-1R.

Oligomerization of receptors can have multiple effects on the behavior of each receptor in the complex. Ligand binding can increase the affinity of the receptor, modulate the signaling response, affect the potencies of agonists, or induce switching of G-protein coupling (Smith and Milligan, 2010). GLP-1R can couple to different G-proteins (Montrose-Rafizadeh et al., 1999), and we showed that coexpression of GLP-1R and GIPR changed the EC_{50} and the Hill slope of the GLP-1 response, compared with GLP-1R expressed alone (Fig. 7). Increased calcium flux at low concentrations and a reduced maximal response may reflect altered G-protein (e.g., Gq) coupling to the heteromeric GLP-1R/GIPR complex, giving the GLP-1 response a more–GIP-like character. Changes in the Hill slope have been described as characteristic pharmacological "fingerprints" for GPCR dimers and their allosteric behavior (Franco et al., 2008). We also observed a decreased maximal BRET response in arrestin recruitment for GLP-1R

coexpressed with GIPR (Fig. 8). If this BRET decrease reflects a reduction in the total number of GLP-1R/β-arrestin complexes formed when GIPR is present, then heteromerization with GIPR may represent a form of protection from receptor desensitization.

In conclusion, the present study shows, for the first time to our knowledge, that class B GPCRs can form ligand-induced heteromers in a heterologous overexpression system. GIPR and GLP-1R are coexpressed in pancreatic and nerve cells, and their roles in type 2 diabetes mellitus make them interesting targets for drug development. Additional evidence is needed to prove the in vivo existence and pharmacological relevance of such heteromers, and understanding their role may open the door to possible therapies targeting the GLP-1R/GIPR heteromer.

Acknowledgments

We thank Dr. Ralf Jockers (Institut Cochin, Paris, France), who kindly provided the plasmids encoding RLuc8 and YPet-β-arrestin2 for research purposes and who assisted with the interpretation of BRET results.

Authorship Contributions

Participated in research design: Schelshorn, Hampe, Breton, V. Mutel, and Lütjens.

Conducted experiments: Schelshorn, Joly, and S. Mutel.

Contributed new reagents or analytic tools: Schelshorn, S. Mutel, and Hampe.

Performed data analysis: Schelshorn, Joly, and S. Mutel.

Wrote or contributed to the writing of the manuscript: Schelshorn, Joly, Mutel, Hampe, Breton, Mutel, and Lütjens.

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