Novel Screening Assay for the Selective Detection of G-Protein-Coupled Receptor Heteromer Signaling

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ABSTRACT

Drugs targeting G-protein-coupled receptors (GPCRs) make up more than 25% of all prescribed medicines. The ability of GPCRs to form heteromers with unique signaling properties suggests an entirely new and unexplored pool of drug targets. However, current *in vitro* assays are ill equipped to detect heteromer-selective compounds. We have successfully adapted an approach, using fusion proteins of GPCRs and chimeric G proteins, to create an *in vitro* screening assay (in human embryonic kidney cells) in which only activated heteromers are detectable. Here we show that this assay can demonstrate

heteromer-selective G-protein bias as well as measure transinhibition. Using this assay, we reveal that the δ -opioid receptor agonist ADL5859, which is currently in clinical trials, has a 10-fold higher potency against δ -opioid receptor homomers than δ/μ -opioid receptor heteromers ($pEC_{50} = 6.7 \pm 0.1$ versus 5.8 ± 0.2). The assay enables the screening of large compound libraries to identify heteromer-selective compounds that could then be used *in vivo* to determine the functional role of heteromers and develop potential therapeutic agents.

Introduction

G-protein-coupled receptors (GPCRs) form the largest protein family within the human genome. GPCRs are involved in many physiologic processes, including regulation of blood pressure and sensing of light and taste (Perez, 2003). Moreover, these receptors play roles in many disease states, including cancer, hypertension, and drug addiction (Abdalla et al., 2001; Koob and Volkow, 2010; Lappano and Maggiolini, 2011). GPCRs are located on the cell membrane and are thus easily accessible to both endogenous and exogenous drug compounds. These attributes of GPCRs have contributed to the success of many of these drugs (Ma and Zimmel, 2002; Overington et al., 2006). Yet most GPCR drugs approved by the Food and Drug Administration target receptors that have been targeted previously (Ma and Zimmel, 2002; Overington

et al., 2006; Swinney and Anthony, 2011). Still, increased knowledge of GPCR pharmacology, including deorphanization, biased agonism, allosteric modulation, and heteromerization (Levoye et al., 2006; Conn et al., 2009; Whalen et al., 2011), should provide new avenues for developing drugs against novel binding domains or targets.

Much attention has been given to the finding that GPCRs can interact with themselves (homomers) and with other GPCRs (heteromers) (Maurice et al., 2011). Although monomeric GPCRs are the minimal signal unit (Whorton et al., 2007), it has been reported that heteromers can have pharmacological properties distinct from those of the individual GPCRs of which they are made (Dalrymple et al., 2008; Ferre et al., 2010). GPCR heteromers could thus represent a broad new range of accessible pharmaceutical targets (Dalrymple et al., 2008; Ferre et al., 2010). However, the physiologic role of GPCR heteromers has been difficult to determine, in part because no pharmacological tools currently exist that selectively target heteromers, thus distinguishing their functional role from those of their homomers. Furthermore, coexpression of two GPCRs *in vitro* leads to the formation of both homomers and heteromers, which has hindered the identification of heteromer-selective binding and activity.

Han et al. (2009) recently developed an approach to study homomer pharmacology. In this assay, a carboxy-terminally

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ABBREVIATIONS: ADL5859, *N,N*-diethyl-4-(5-hydroxy-2,2',4'-piperidine-4-yl)benzamide; ARM1000390, *N,N*-diethyl-4-(phenyl-4-piperidinylidenemethyl)-benzamide hydrochloride; D₁R, dopamine D₁ receptor; D₂R, dopamine D₂ receptor; DAMGO, [D-Ala², N-MePhe⁴, Gly-ol]-enkephalin; DOR, δ -opioid receptor; ELISA, enzyme-linked immunosorbent assay; GPCR, G-protein-coupled receptor; H₂R, histamine H₂ receptor; H₄R, histamine H₄ receptor; H8, helix 8; HEK, human embryonic kidney; KOR, κ -opioid receptor; MOR, μ -opioid receptor; PCR, polymerase chain reaction; SKF81297, 6-chloro-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine-7,8-diol; SNC80, 4-[(R)-[(2*S*,5*R*)-4-allyl-2,5-dimethylpiperazin-1-yl](3-methoxyphenyl)methyl]-*N,N*-diethylbenzamide; TBS, Tris-buffered saline; TM, transmembrane domain; U69,593, *N*-methyl-2-phenyl-*N*-[(5*R*,7*S*,8*S*)-7-(pyrrolidin-1-yl)-1-oxaspiro[4.5]dec-8-yl]acetamide; WT, wild-type.

truncated dopamine D₂ receptor (D₂R) was fused to a chimeric G protein (Conklin et al., 1993) to create a signaling-mutated receptor. Activity was shown to be rescued, via functional complementation, through coexpression of a full-length wild-type (WT) (i.e., not fused) receptor, which itself is unable to signal without interacting with the chimeric G protein. The goal of the current study was to determine the feasibility of applying this complementation strategy to other family A GPCR homomers and, more importantly, to examine whether this method could be applied to selectively isolate heteromer-mediated G-protein signal transduction. Here we demonstrate that we have developed a robust, albeit artificial, screening assay that shows selective signaling from family A GPCR heteromers of diverse types. By extension, this assay should allow for the identification of heteromer-selective ligands for many family A GPCRs. Such heteromer-selective compounds could then be used *in vivo* to determine the roles of GPCR heteromers in both naïve and disease states.

Materials and Methods

Molecular Cloning of Receptor-Chimeric G-Protein Fusions.

The chimeric G_{q14} and G_{q5} were kindly provided by Dr. E. Kostenis (University of Bonn, Bonn, Germany). The start codon of the G_{q14} was removed by polymerase chain reaction (PCR) using the respective forward and reverse primers 5'-TAATAGGATCCATAGGGTGCTGCCTGAGCGAG-3' and 5'-TACACGGGCCCTTAGAAGAGGCCACACTCCTTC-3' using G_{q14} as a template. The PCR product was cloned into pcDNA3.1 after digestion with BamHI and ApaI, resulting in a "G_{q14}-no start." DOR₃₇₃-G_{q14} and DOR₃₄₄-G_{q14} were then cloned using a standard T7 forward primer (5'-TACGACTCACTATAGGGAGAC-3') and the reverse primers 5'-TTATAGGATCCGGCGGCAGCGCCACC-3' and 5'-ACATAGGATCCACTCCCGGGTCTTGCGC-3' using FLAG-DOR as a template, followed by restriction of the PCR fragment and G_{q14}-no start with NheI and BamHI. DOR₃₃₇-G_{q14}, DOR₃₃₃-G_{q14}, and DOR₃₂₃-G_{q14} were cloned using the T7 forward primer and the reverse primers 5'-ATGGATCCGGGCGTGCGACAGAGCTGGCGG-3', 5'-ATAAGGATCCGAGCTGGCCGGAAGCAGCGC-3', and 5'-ATCGGATCCAGGAAGCGTAGAGAACCG-3' using FLAG-DOR as a template, followed by restriction of the PCR fragment and DOR_{q1}-G_{q14} with HindIII and BamHI. DOR₃₃₃-G_{q5} was cloned by restriction of DOR₃₃₃-G_{q14} and G_{q5} with AvrII and EcoRI. MOR₃₅₄-G_{q14} was cloned using the T7 forward primer and 5'-CGAGATCTTGGGATGCAGAACTCTCTAAAAC-3' as a reverse primer using FLAG-MOR as a template, followed by restriction of the PCR fragment with HindIII and BglII and DOR_{q1}-G_{q14} with HindIII and BamHI. D₁R₃₅₀-G_{q14} was cloned using the respective forward and reverse primers 5'-AGATGGATCCAAGTCTGTAGCATCCTAAGAGG-3' and 5'-GAACTGCTAGCCATGAAGACGATCATCGCC-3', followed by restriction of the PCR fragment and DOR₃₃₃-G_{q14} with NheI and BamHI. D₁R₃₅₀-G_{q5} was cloned by restriction of D₁R₃₅₀-G_{q14} and DOR-G_{q5} with AvrII and EcoRI.

All constructs contained an N-terminal cleavable hemagglutinin signal peptide followed by a FLAG (DYKDDDDK) or HA (YPYDVP-DYA) peptide for enhanced cell surface expression and biochemical detection, respectively.

Calcium Mobilization. Human embryonic kidney (HEK-293) cells were maintained at 37°C humidified in 7% CO₂/93% air atmosphere in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum. To measure calcium mobilization following activation of a G_i- or G_s-coupled receptor, we used chimeric G_q proteins that contained either the last four or five N-terminal amino acids of the G_i protein (G_{q14}) or G_s protein (G_{q5}) (Kostenis, 2001). Cells were transiently transfected using Lipofectamine 2000 (Invitrogen, Life Technologies, Grand Island, NY) with a single GPCR, a GPCR with a chimeric G protein, a GPCR-chimeric G-protein fusion, or a GPCR with a GPCR-G-protein chimera fusion

(200 ng total DNA for every 40,000 cells) 24 hours before measuring calcium release. One day after transfection, cells were loaded for 60 minutes with a Ca²⁺ fluorophore (Molecular Devices, Sunnyvale, CA) and stimulated with increasing amounts of ligand. Intracellular Ca²⁺ release was measured immediately after agonist application in a Flex apparatus (Molecular Devices) for 2 minutes. For measurements in 384-well format, HEK-293 cells (15,000 cells/well) were transfected with 100 ng/well of DNA using JetPEI (Polyplus Transfection, Illkirch, France). Two days after transfection, cells were loaded for 60 minutes with a Ca²⁺ fluorophore and stimulated with increasing amounts of ligand. Intracellular Ca²⁺ release was measured immediately after agonist application in a Flex apparatus for 2 minutes. For the calcium mobilization experiments, each data point was obtained in triplicate and the experiment was performed at least three times.

Enzyme-Linked Immunosorbent Assay. The ELISA assay was performed as previously described (van Rijn et al., 2008). In short, HEK-293 cells transiently expressing N-terminal FLAG-DOR receptors or truncated receptors in a 96-well plate (40,000 cells/well) were fixed for 30 minutes with 4% paraformaldehyde. Permeabilization, when applicable, was carried out using 0.5% NP40 in Tris-buffered saline (TBS) for 30 minutes. Cells were blocked for 4 hours using 0.1 M NaHCO₃ 1% milk powder in TBS. Cells were incubated with primary antibodies (mouse Anti-FLAG-M2; Sigma, St. Louis, MO) overnight and secondary antibodies (goat anti-mouse HRP; Jackson ImmunoResearch Laboratories, West Grove, PA) for 2 hours. Cells were incubated with 3,3',5,5'-tetramethylbenzidine (Sigma), and reactions were stopped with 0.5M H₂SO₄. The reaction mixture was measured at 450 nm. Between every step cells were washed at least three times with TBS. For the ELISA experiments, each data point was obtained at least in sextuplicate and the experiment was performed three times.

Fluorescent Imaging. HEK-293 cells transiently expressing N-terminal FLAG-DOR receptors or truncated receptors were incubated with monoclonal M1 anti-FLAG antibody (Sigma-Aldrich, St. Louis, MO) for 30 minutes to label surface receptors. Subsequently, cells were fixed with 3.7% formaldehyde in phosphate-buffered saline for 20 minutes at room temperature and permeabilized with 0.1% Triton X-100. Cells were then incubated with a subtype-selective fluorescent anti-mouse antibody directed against M1 (Alexa488 IgG2b; Invitrogen) for 30 minutes. After staining, cells were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and analyzed using a Zeiss LSM 510 META Axioplan 2 confocal microscope (Carl Zeiss Inc., Thornwood, NY).

Data Analysis. Dose-response curves for the calcium release assay were generated using Graphpad Prism (La Jolla, CA). The software automatically calculates 50% effective concentration values (pEC₅₀).

For one experiment, relative efficacy values (α) were calculated by setting the maximal effective response (E_{max}) at 100% for the response obtained for the agonist (morphine) in the absence of the antagonist (naltrindole).

Drugs and Reagents. Leu-enkephalin, 4-[(R)-[(2S,5R)-4-allyl-2,5-dimethylpiperazin-1-yl](3-methoxyphenyl)methyl]-N,N-diethylbenzamide (SNC80), N,N-diethyl-4-(phenyl-4-piperidinylidenemethyl)-benzamide hydrochloride (ARM1000390), quinpirole, deltorphin II, 6-chloro-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine-7,8-diol (SKF81297), histamine, and dimaprit were purchased from Tocris Bioscience (Minneapolis, MN). N-Methyl-2-phenyl-N-[(5R,7S,8S)-7-(pyrrolidin-1-yl)-1-oxaspiro[4.5]dec-8-yl]acetamide (U69,593) and [D-Ala², N-MePhe⁴, Gly-ol]-enkephalin (DAMGO) were purchased from Sigma-Aldrich. N,N-Diethyl-4-(5-hydroxyspiro[chromene-2,4'-piperidine]-4-yl)benzamide (ADL5859) was purchased from Axon MedChem (Groningen, The Netherlands). N-[2-(Dimethylamino)-2-(4-methoxyphenyl)ethyl]-2,3-dihydro-1H-indene-5-carboxamide (methoxyphenyl indene) was purchased through Molport (Riga, Latvia). PfuUltra Hotstart PCR Master Mix was from Agilent (Santa Clara, CA). Custom-designed primers were purchased from Eurofins MWG

Operon (Huntsville, AL). Restriction enzymes and ligase were obtained from New England BioLabs (Ipswich, MA). Competent TOP10 cells and cell culture medium were purchased from Invitrogen, Life Technologies.

Results

Creation of Truncated Receptor–Chimeric G_{q14} -Protein Fusion Protein Attenuates Signal Transduction

Previous reports by Han et al. (2009) have shown that connecting a chimeric G_{q15} protein to a carboxy-terminal-tail-truncated D_2R produces a receptor that is unable to signal. We created fusion proteins with a chimeric G_{q14} protein fused to the carboxy-terminal tail of either a full-length δ -opioid receptor (DOR) or to DORs with increasingly truncated C-terminal tails (Fig. 1A; also see *Methods*). Shortening of the C-terminal tail attenuated signal transduction induced by the DOR-selective agonist SNC80 and the peptide agonist leu-enkephalin (Fig. 1B; Table 1). Signal transduction was significantly attenuated when the receptor was truncated beyond the palmitoylation site (DOR₃₃₃- G_{q14}) and was entirely abolished if the putative helix 8 (H8) was truncated as well (DOR₃₂₃- G_{q14}) (Fig. 1B; Table 1). Fusion of the G protein to truncated DOR affected the distribution of the fusion protein compared with WT DOR; whereas nearly 100% of the WT DORs and full-length DOR₃₇₂- G_{q14} were expressed at the cell surface (Fig. 1, C–E), a significant fraction of fused truncated DORs were located intracellularly (Fig. 1, C and F–I). However, the majority of truncated DOR- G_{q14} fusion proteins were expressed on the cell surface (Fig. 1, F–I).

Coexpression of WT Receptor Restores Signal Transduction of Silenced, Fused Receptor–Chimeric G-Protein Complex and Reveals Heteromer-Selective Signaling

Han and coworkers (2009) restored signal transduction of the “muted” D_2R_{trunc} - G_{q15} by coexpression of a WT D_2R . Similarly, coexpression of DOR₃₃₃- G_{q14} receptors with WT DORs restored receptor signal transduction (Fig. 2A; Table 2). To exclude that coexpressing DOR₃₃₃- G_{q14} with WT DOR causes increased surface expression of DOR₃₃₃- G_{q14} , which could potentially explain the functional complementation we observe, we performed an ELISA on the same pool of transfected cells. No increase in expression of the FLAG-DOR₃₃₃- G_{q14} was observed when it was coexpressed together with HA-DOR. In contrast, there was a decrease in the surface expression of FLAG-DOR₃₃₃- G_{q14} when coexpressed with HA-DOR, as compared with transfection with an empty expression vector (Fig. 2B). The DOR₃₃₃- G_{q14} fusion protein was the shortest truncation of the DOR that could be rescued by complementation, as no G-protein signaling was induced by coexpression if the receptor was truncated directly after transmembrane domain 7 (TM7; DOR₃₂₃- G_{q14}), eliminating the putative H8 (Fig. 2C). We observed similar functional complementation of homomeric receptors of the μ -opioid receptor (MOR), truncated after H8 and fused to the G_{q14} -protein (MOR₃₅₄- G_{q14}), when it was coexpressed with WT full-length MOR (Fig. 2D). These results demonstrate that this method of functional complementation can be used to study homomeric opioid receptors.

Next we examined whether this approach would allow the selective detection of G-protein signaling from heteromers. We found that the κ -opioid receptor (KOR)-selective ligand U69,593 potently induced calcium release in cells in which the DOR₃₃₃- G_{q14} and KOR were expressed together (Fig. 2E; Table 2). Similar functional complementation was observed for the selective MOR agonist DAMGO in cells coexpressing DOR₃₃₃- G_{q14} and the MOR (Fig. 2F; Table 2). Importantly, none of the WT opioid receptors induced calcium release when they were expressed in HEK-293 cells in the absence of either DOR₃₃₃- G_{q14} or “nonfused” chimeric G_{q14} proteins (Fig. 2G). We also demonstrated that the functional complementation was bidirectional. Specifically, in cells coexpressing WT DOR with the MOR₃₅₄- G_{q14} fusion protein, the DOR agonist SNC80 induced calcium release (Fig. 2H; Table 2). Thus, we have demonstrated the ability to detect a signal exclusively from heteromers but not homomers in cells expressing a truncated, G-protein-fused opioid receptor and a full-length WT opioid receptor (Fig. 2I).

Functional Complementation of G_i -Coupled and G_s -Coupled Receptors

G_i -Coupled Receptors. To examine whether functional complementation would occur between G_i -coupled receptors belonging to different subclasses of family A GPCRs, we coexpressed the DOR₃₃₃- G_{q14} with either the D_2R or the histamine H_4 receptor (H_4R). Neither quinpirole nor histamine stimulation results in calcium release in HEK-293 cells expressing only DOR₃₃₃- G_{q14} or cells expressing D_2R or H_4R in the absence of the chimeric G_{q14} protein (Fig. 3, A and B). However, we were able to induce calcium release with the D_2R -selective agonist quinpirole or the H_4R -selective agonist histamine when DOR₃₃₃- G_{q14} was coexpressed with D_2R or H_4R , respectively (Fig. 3, A and B), suggesting that functional complementation occurs between different G_i -coupled receptors.

G_s -Coupled Receptors. Next, we truncated the dopamine D_1 receptor (D_1R) after H8 and fused it to the chimeric G_{q85} protein (D₁R₃₅₀- G_{q85}) to determine if we could observe functional complementation for receptors that preferentially signal through G_s proteins. We observed that the D_1R -selective agonist SKF81297 was unable to induce calcium release in cells expressing only D₁R₃₅₀- G_{q85} (Fig. 4A), but we were able to restore signal transduction coexpressing WT D_1R (Fig. 4A), suggesting that this technique of functional complementation can be employed to study both G_i - and G_s -coupled receptors.

Functional Complementation between G_s - and G_i -Coupled Receptors. We next investigated whether we could observe functional complementation between D_1R and DOR using cells coexpressing D₁R₃₅₀- G_{q85} and DOR. However, we were unable to observe any calcium release by SKF81297 or SNC80 in these cells (Fig. 4B; Table 3). Interestingly, SKF81297 and SNC80 did induce calcium release in cells expressing D₁R₃₅₀- G_{q14} and DOR (Fig. 4C; Table 3). We also determined that SKF81297 did not induce calcium release in cells expressing D₁R₃₅₀- G_{q14} alone (Fig. 4C). To further study this observation, we fused truncated DOR₃₃₃ to the G_{q85} protein. DORs preferentially signal through G_i proteins; hence we did not expect DOR₃₃₃- G_{q85} to signal. Indeed, no calcium release was observed when SNC80 was

Fig. 1. Increasing truncation of the DOR carboxy-terminal tail attenuates signal transduction of a DOR-G_{qi4} fusion protein without preventing cell surface expression. (A) The murine DOR was truncated after TM7 at position 323, after putative H8 (position 333), after the palmitoylation site (position 337), or before several phosphorylation sites (position 344) and subsequently fused with a chimeric G_{qi4} protein. (B) Calcium release induced by the DOR-selective agonist SNC80 in HEK-293 cells expressing full-length DOR with the chimeric G_{qi4} protein or the DOR-G_{qi4} fusion proteins (C) Surface and intracellular distribution of WT FLAG-DOR, FLAG-DOR₃₇₂-G_{qi4}, and FLAG-DOR₃₃₃-G_{qi4} as assessed by ELISA. (D-I) Confocal microscopic analysis of the localization of WT DOR (D) and DOR-G_{qi4} fusion proteins (F-I) in HEK-293 cells.

TABLE 1

Potency of DOR-selective agonists SNC80 and leu-enkephalin in inducing calcium release

Data are from HEK-293 cells expressing wild-type δ -opioid receptor (DOR) with the chimeric G_{qi4} protein or DOR- G_{qi4} fusion proteins

Ligand	pEC50 (Mean $\pm$ S.E.M.) in Cells Expressing:					
	DOR + G_{qi4}	DOR ₃₇₂ - G_{qi4}	DOR ₃₄₄ - G_{qi4}	DOR ₃₃₇ - G_{qi4}	DOR ₃₃₃ - G_{qi4}	DOR ₃₂₃ - G_{qi4}
SNC80	8.4 $\pm$ 0.2	8.3 $\pm$ 0.3	6.4 $\pm$ 0.2***	6.8 $\pm$ 0.3***	7.0 $\pm$ 0.1***	<5
Leu-enkephalin	7.9 $\pm$ 0.2	8.1 $\pm$ 0.1	7.4 $\pm$ 0.2	7.3 $\pm$ 0.2	7.6 $\pm$ 0.2	<5

*** $P < 0.001$ vs. DOR + G_{qi4} . pEC50 = -Log(50% effective concentration).

applied to cells expressing DOR₃₃₃- G_{qs5} (Fig. 4D; Table 3). Yet similar to the calcium release induced in cells coexpressing D₁R₃₅₀- G_{qi4} and DOR, SKF81297 induced calcium release in cells coexpressing DOR₃₃₃- G_{qi4} and D₁R (Fig. 4E; Table 3), suggesting that heteromers of DOR and D₁R preferentially couple only to G_i proteins. We also observed functional complementation when coexpressing DOR₃₃₃- G_{qs5} and the

histamine H₂ receptor (H₂R), which preferentially couples to G_s proteins (Hill et al., 1997), using the H₂R-selective agonist dimaprit (Fig. 4F; Table 3), suggesting that the lack of G_{qs} -mediated signal transduction observed when coexpressing DOR and D₁R is unique to this combination. However, calcium release occurred only when the H₂R-DOR₃₃₃- G_{qs5} complex was bound by dimaprit, but not when bound by the

Fig. 2. Heterologous expression of WT opioid receptors enables functional complementation of signal transduction through the interaction of the DOR- G_{qi4} fusion protein with a WT receptor. (A) Calcium release induced by the DOR-selective agonist SNC80 in cells expressing DOR₃₃₃- G_{qi4} alone or together with WT DOR. (B) Surface expression of FLAG-DOR₃₃₃- G_{qi4} from the same pool of cells used in (A), as assessed by ELISA. (C) SNC80 does not induce calcium release when the truncated DOR₃₂₃- G_{qi4} is coexpressed with WT DOR. (D) Coexpression of WT MOR in cells expressing MOR₃₅₄- G_{qi4} enables calcium release by the MOR-selective agonist DAMGO. (E, F) Calcium release induced by the KOR-selective agonist U69,593 (E) and by DAMGO (F) in HEK-293 cells expressing DOR₃₃₃- G_{qi4} with either WT KOR (E) or WT MOR (F). (G) In the absence of chimeric G_{qi4} , DOR-, MOR-, and KOR-selective agonists are unable to induce calcium release in cells expressing WT DOR, MOR, or KOR. (H) Calcium release induced by SNC80 in cells expressing the MOR₃₅₄- G_{qi4} fusion protein with WT DOR. (I) Schematic representation of the functional composition of homomers and heteromers expressed in this experiment.

TABLE 2

Potency of opioid receptor agonists in inducing calcium release

Data are for the δ -opioid receptor (DOR)-selective agonist SNC80, the μ -opioid receptor (MOR)-selective agonist DAMGO, and the κ -opioid receptor (KOR)-selective agonist U69,593 in HEK-293 cells expressing wild-type (WT) DOR, MOR, or KOR with G_{q14} ; DOR₃₃₃- G_{q14} in the absence or presence of WT DOR, MOR, or KOR; or MOR₃₅₄- G_{q14} in the absence or presence of WT DOR or MOR.

Receptor	pEC ₅₀ (Mean $\pm$ S.E.M.) of:		
	SNC80	DAMGO	U69,593
DOR + G_{q14}	8.4 $\pm$ 0.2	5.3 $\pm$ 0.1	<5
MOR + G_{q14}	N.A.	8.1 $\pm$ 0.1	N.A.
KOR + G_{q14}	N.A.	N.A.	9.3 $\pm$ 0.2
DOR ₃₃₃ - G_{q14}	7.0 $\pm$ 0.1	<5	<5
DOR ₃₃₃ - G_{q14} + DOR	7.6 $\pm$ 0.2	N.A.	N.A.
DOR ₃₃₃ - G_{q14} + MOR	N.A.	6.4 $\pm$ 0.3	N.A.
DOR ₃₃₃ - G_{q14} + KOR	N.A.	N.A.	7.7 $\pm$ 0.3
MOR ₃₅₄ - G_{q14}	<5	6.6 $\pm$ 0.2	N.A.
MOR ₃₅₄ - G_{q14} + DOR	7.2 $\pm$ 0.3	N.A.	N.A.
MOR ₃₅₄ - G_{q14} + MOR	N.A.	7.0 $\pm$ 0.1	N.A.

N.A., not applicable.

DOR-selective agonist SNC80 (Fig. 4F; Table 3). We also observed calcium release with dimaprit when DOR₃₃₃- G_{q14} was coexpressed with H₂R (Fig. 4G; Table 3). SNC80 also induced a calcium response in cells expressing DOR₃₃₃- G_{q14} with H₂R, thus suggesting that the DOR-H₂R heteromer may be able to signal through either G_s or G_i proteins, and suggesting that this was dependent on the ligand. We also observed functional complementation between two G_s -coupled receptors using cells coexpressing D₁R₃₅₀- G_{q5} and H₂R (Fig. 4H). We next determined whether we could detect a heteromer-selective signal not only in a 96-well but also in a 384-well format. Using cells expressing the D₁R₃₅₀- G_{q5} -H₂R heteromer, we demonstrated that the assay is functional in a 384-well format as well (Fig. 4H).

Transinhibition. Knockout, knockdown, or pharmacological inhibition of DORs has been reported to attenuate morphine tolerance, place preference, and dependence (Miyamoto et al., 1993; Zhu et al., 1999; Shippenberg et al., 2009). Some of these effects have been attributed to DOR-MOR heteromers (Rozenfeld et al., 2007). Devi and coworkers have shown that both DOR agonists and antagonists act as positive modulators for morphine activity in cells and tissues coexpressing DOR and MOR (Gomes et al., 2004; Gupta

et al., 2010). We next examined whether the positive modulation was due to allosteric enhancement of morphine's activity at the MOR-DOR heteromer upon DOR ligand binding. At 10 nM, naltrindole does not compete with morphine in cells expressing MOR alone (pEC₅₀, 8.1 $\pm$ 0.1 versus 8.0 $\pm$ 0.1; α , 100 $\pm$ 0.1 versus 106 $\pm$ 3; n = 3) (Fig. 5A). Nevertheless, in cells coexpressing DOR₃₃₃- G_{q14} with MOR, we found that naltrindole significantly (P = 0.004) attenuated morphine signaling (pEC₅₀, 7.0 $\pm$ 0.2 versus 7.0 $\pm$ 0.1; α , 100 $\pm$ 0.1 versus 60 $\pm$ 4; n = 3) (Fig. 5B). We determined that morphine was unable to induce calcium release in cells expressing only DOR₃₃₃- G_{q14} (Fig. 5B). These assays were performed with the same transfected cells to control for expression levels. These data suggest that the increased activity of morphine produced by naltrindole in cells or tissues expressing both MOR and DOR is not due to increased activity of morphine at the MOR-DOR heteromer via a positive allosterism produced by naltrindole. On the contrary, it appears that naltrindole acts as a negative allosteric modulator for the effects of morphine on the MOR-DOR heteromer.

Screening for Homomer- and Heteromer-Selective Compounds

We envision that the key use of this heteromer assay will be to identify compounds that are selective for a GPCR heteromer. To validate the heteromer assay for this purpose, we tested four DOR-selective compounds for their activity on DOR homomers (DOR₃₃₃- G_{q14} + DOR), MOR homomers (MOR₃₅₄- G_{q14} + MOR), and DOR-MOR heteromers (DOR₃₃₃- G_{q14} + MOR; MOR₃₅₄- G_{q14} + DOR). The set included deltorphin II, an amphibian-derived peptide (Kreil et al., 1989); a DOR agonist currently in phase 2 clinical trials, ADL5859 (Le Bourdonnec et al., 2008); as well as two agonists, SNC80 and ARM1000390, that differ in their ability to internalize the DOR (Pradhan et al., 2009). Deltorphin II displayed similar activity on the DOR homomer and the DOR-MOR heteromers (Fig. 6A; Table 4). However, the other three compounds, all of which had been designed to exhibit a high degree of DOR selectivity (Calderon et al., 1994), have a higher potency against DOR homomers than DOR-MOR heteromers or MOR homomers (Table 4). In particular, ADL5859 is

Fig. 3. Functional complementation between G_i -coupled receptors belonging to different family A GPCR subclasses. (A) The D₂R-selective agonist quinpirole induces calcium release only in cells transiently expressing D₂R and DOR₃₃₃- G_{q14} , but not in cells expressing DOR₃₃₃- G_{q14} alone or D₂R in the absence of G_{q14} . (B) The H₄R-selective agonist histamine induces calcium release only in cells transiently expressing H₄R and DOR₃₃₃- G_{q14} , but not in cells expressing DOR₃₃₃- G_{q14} alone or H₄R in the absence of G_{q14} .

Fig. 4. Use of G_{q14} - and G_{q5} -GPCR fusion proteins reveals preferential interaction of a GPCR heteromer with specific G proteins. (A) Coexpression of WT D_1R in cells expressing D_1R_{350} - G_{q5} enables calcium release by the D_1R -selective agonist SKF81297. (B, C) Calcium release induced by SKF81297 and the DOR-selective agonist SNC80 in HEK-293 cells expressing WT DOR and D_1R_{350} - G_{q5} (B) or D_1R_{350} - G_{q14} (C) or D_1R_{350} - G_{q14} alone (C). (D, E) Calcium release induced by SNC80 or SKF81297 in cells expressing WT D_1R and DOR_{333} - G_{q5} (D) or DOR_{333} - G_{q14} (E). (F, G) Calcium release induced by the H_2R -selective agonist dimaprit and SNC80 in HEK-293 cells expressing WT H_2R and DOR_{333} - G_{q5} (F) or DOR_{333} - G_{q14} (G). (H) Calcium release induced by dimaprit-treated cells expressing WT H_2R and D_1R_{350} - G_{q5} using a 96-well and a 384-well format.

significantly more potent at DOR homomers than at DOR-MOR heteromers (Fig. 6B; Table 4).

Discussion

Here we show that the ability of GPCRs truncated after the putative H8 and fused to chimeric G_q proteins to induce calcium release is severely attenuated (Fig. 2A) or abolished (Figs. 2D and 4A). More importantly, we demonstrate functional complementation in several diverse heteromeric

TABLE 3

Potency of receptor agonists in inducing calcium release

Data are for the δ -opioid receptor (DOR)-selective agonist SNC80, the dopamine D_1 receptor (D_1R)-selective agonist SKF81297, and the histamine H_2 receptor (H_2R)-selective agonist dimaprit in HEK-293 cells expressing DOR_{333} - G_{q14} in the absence or presence of wild-type (WT) H_2R or D_1R , DOR_{333} - G_{q5} in the absence or presence of WT H_2R or D_1R , D_1R_{350} - G_{q5} in the absence or presence of WT H_2R or DOR, or D_1R_{350} - G_{q14} in the absence or presence of WT DOR.

Receptor	pEC ₅₀ (Mean $\pm$ S.E.M.) of:		
	SNC80	SKF81297	Dimaprit
DOR_{333} - G_{q14}	7.0 $\pm$ 0.1	<5	<5
DOR_{333} - G_{q14} + H_2R	6.7 $\pm$ 0.2	N.A.	5.8 $\pm$ 0.2
DOR_{333} - G_{q14} + D_1R	6.6 $\pm$ 0.2	8.8 $\pm$ 0.4	N.A.
DOR_{333} - G_{q5}	<5	<5	<5
DOR_{333} - G_{q5} + H_2R	<5	N.A.	5.7 $\pm$ 0.1
DOR_{333} - G_{q5} + D_1R	<5	<5	N.A.
D_1R_{350} - G_{q14}	<5	<5	<5
D_1R_{350} - G_{q14} + DOR	7.7 $\pm$ 0.1	8.8 $\pm$ 0.3	N.A.
D_1R_{350} - G_{q5}	<5	<5	<5
D_1R_{350} - G_{q5} + DOR	<5	<5	N.A.

N.A., not applicable.

complexes when these fusion proteins are coexpressed with a WT receptor. When used with the proper controls, this assay can be used to identify molecules with selective activity at heteromeric GPCRs. Specifically, for any heteromeric target of interest, for example, DOR-MOR, one would screen three receptor combinations: DOR_{333} - G_{q14} + DOR, MOR_{354} - G_{q14} + MOR, and DOR_{333} - G_{q14} + MOR. A fourth combination, i.e., MOR_{354} - G_{q14} + DOR, could be tested as well (Fig. 6). Ligands that show increased activity in the heteromeric cell line compared with the homomer-only-expressing cells would be heteromer-selective. Conversely, ligands that are more active in the homomer- than the heteromer-expressing cells, such as ADL5859 (Fig. 6B), would be homomer-selective. Furthermore, here we show that this strategy allows for the detection of heteromer-specific pharmacology, including different G-protein preference (Fig. 4, B-E) and negative allosteric modulation (Fig. 5, A and B). Our finding that heteromers between DOR and D_1R show a strong preference for engaging G_i over G_s proteins is also interesting, as a similar switch in G-protein coupling preference by heteromerization has recently been described for the serotonin 5-HT_{2A} and metabotropic glutamate 2 receptor (Fribourg et al., 2011), which was suggested to be caused by crosstalk between the two receptors through a heteromer interface (Bruno et al., 2009).

Because of the artificial nature of the heteromer assay, which allows for high expression levels, it could be proposed that functional complementation is achieved by the WT receptors being in close proximity to the chimeric G-protein-fused receptors and directly interacting with only the

Fig. 5. Transinhibition of heteromer function. Calcium release induced by the MOR-selective agonist morphine in the absence or presence of 10 nM DOR-selective antagonist naltrindole (NTI) in HEK-293 cells expressing WT MOR and G_{qi4} (A) or WT MOR and DOR₃₃₃-G_{qi4} (B) or DOR₃₃₃-G_{qi4} alone (B).

chimeric G protein without engaging in heterodimer formation. Yet our finding that the D₁R does not interact with G_{qs} proteins when fused to the DOR suggests that this is not the case.

It is important to note that the robustness of the assay is derived from expressing the chimeric G-protein-fused receptors and WT receptors at levels that are not necessarily natural. Similar to other *in vitro* assays that study GPCR heteromers, such as resonance energy transfer techniques and coimmunoprecipitation, a heteromer-derived signal in this assay is no guarantee of the heteromer's existence *in vivo*. However, in contrast to the other methods, the heteromer assay described here will ultimately provide us with a tool that allows the study of heteromer function *in vivo*.

Although several assays exist to probe for GPCR heteromer formation, few high-throughput screening assays are available that allow for the identification of heteromer-selective compounds. One approach has involved fusing a G protein to a receptor that is unable to bind ligands due to a point mutation in the binding domain, and coexpressing this fusion protein with a separate receptor that is unable to be activated due to a point mutation in the signaling domain of the receptor, thus gaining functional complementation through mandatory transactivation (Milligan et al., 2007). Such an approach was recently used in an elegant study to show that the luteinizing hormone receptor forms dimers *in vivo* (Rivero-Muller et al., 2010). In this case, neither GPCR is

a WT receptor, as both carry different point mutations and must be expressed as fusion proteins, whereas the assay we describe requires the creation of only one fusion protein. Dimerix Bioscience (Bundoora, Australia), with GPCR-HIT, and DiscoveRx (Fremont, CA), with PathHunter, also have proprietary dimer assays, which use a tagged GPCR and β -arrestin fusion proteins that can only be activated by the tagged GPCR. The tagged GPCR thus allows for the detection of the formation of dimers that signal through β -arrestin by functional transcomplementation. Importantly, whereas our G-protein-based heteromer assay eliminates both homomer signals (Fig. 2I), the aforementioned β -arrestin-based assays only remove one of the two homomer signals. Still, when used in parallel, these assays could identify ligand bias for G protein versus β -arrestin for any heteromeric-specific ligand.

It is worth mentioning that not every GPCR interacts well with the chimeric G proteins (Coward et al., 1999). Also, the current setup does not allow for the detection of heteromers containing G_q-coupled receptor, as their homomers would already induce a signal on their own. Nevertheless, several options exist for expanding this assay to include the study of G_q-coupled GPCRs. Similar to G_{qs} and G_{qi} chimeric proteins, G_{iq} (Grisshammer and Hermans, 2001) and G_{sq} (Hsu and Luo, 2007) chimeras have been produced. Fusing a G_q-coupled receptor to such a chimera would allow the selective activation of heteromers consisting of two G_q-coupled GPCRs (Fig. 7A). Selective activation of a heteromer consisting of

Fig. 6. Screening for heteromer- and homomer-selective agonists. Calcium release induced by the DOR-selective agonists deltorphin II (A) and ADL5859 (B) in HEK-293 cells expressing DOR₃₃₃-G_{qi4} and WT MOR, MOR₃₅₄-G_{qi4} and WT DOR, MOR₃₅₄-G_{qi4} and WT MOR, or DOR₃₃₃-G_{qi4} and WT DOR.

TABLE 4

Potency of DOR-selective agonists deltorphin II, SNC80, ARM1000390, and ADL5859 in inducing calcium release

Data are from HEK-293 cells expressing DOR₃₃₃-G_{q14} together with wild-type (WT) δ -opioid receptor (DOR) or μ -opioid receptor (MOR), or MOR₃₅₄-G_{q14} together with WT DOR or MOR.

Ligand	pEC ₅₀ (Mean $\pm$ S.E.M.) in Cells Expressing:			
	DOR ₃₃₃ -G _{q14} + DOR	MOR ₃₅₄ -G _{q14} + DOR	DOR ₃₃₃ -G _{q14} + MOR	MOR ₃₅₄ -G _{q14} + MOR
Deltorphin II	7.8 $\pm$ 0.1	7.3 $\pm$ 0.3	7.9 $\pm$ 0.1	<5
SNC80	7.7 $\pm$ 0.2	7.2 $\pm$ 0.2	7.0 $\pm$ 0.3	<5
ARM1000390	6.9 $\pm$ 0.2	6.5 $\pm$ 0.1	6.4 $\pm$ 0.1	<5
ADL5859	6.7 $\pm$ 0.1	5.9 $\pm$ 0.3	5.8 $\pm$ 0.2*	<5

* $P < 0.05$ vs. DOR₃₃₃-G_{q14} + WT DOR.

a G_q-coupled GPCR and a G_i-coupled GPCR could be achieved by fusing one of the receptors to the pertussis-toxin-insensitive G_{iq} protein and measuring cAMP inhibition in the presence of pertussis toxin (Fig. 7B).

As the physiologic role of specific heteromers becomes more apparent, it may become evident that heteromer activation can have both desirable and undesirable effects. Therefore, either prevention or disruption of heteromer formation could also be a therapeutic strategy. To this end, an increasing effort has been made to develop protein-protein interaction inhibitors (Mullard, 2012). Since this assay detects an exclusively heteromer-derived signal, it allows for the study of the receptor interface via point mutations at the predicted heteromer interface. Moreover, small peptide (TM-like) fragments could be developed with the potential of disrupting the interface (He et al., 2011). However, it may also be possible to disrupt heteromers with ligands that change the conformation of the GPCR in a way that is unfavorable to sustaining or inducing heteromer formation (or that favors homomer formation).

Recently, the crystal structures of the opioid receptors have been resolved. Although the DOR was not crystallized in a dimer lattice (Granier et al., 2012), several GPCRs have been crystallized as dimers (Fotiadis et al., 2003; Wu et al.,

2010), including the MOR (Manglik et al., 2012) and KOR (Wu et al., 2012). Interestingly the crystal structure of the MOR revealed the potential of a tight interaction between TM5 and TM6 of the MOR, a region highly conserved between MOR and DOR. In several instances, H8, a region that has been known to be important for receptor signal transduction (Tetsuka et al., 2004; Feierler et al., 2011), is involved in the dimer interface (Salom et al., 2006; Manglik et al., 2012; Wu et al., 2012). In our experiments, it appears that H8 needs to remain intact for functional complementation to occur, which would be in line with those previous findings. The crystal structures should allow for improved computer models for predicting how the binding of ligands to one pharmacophore might affect the homomer/heteromer interface and, allosterically, the binding pocket of the second receptor (Maurice et al., 2011). Indeed, one possible explanation for the observed inhibition of morphine-induced calcium release from the DOR-MOR heteromer with the DOR antagonist naltrindole could be decreased DOR-MOR heteromer formation.

In conclusion, here we describe an in vitro assay that provides a robust method enabling the study of GPCR heteromer pharmacology at diverse targets without the confounding effects of homomeric signaling. When used appropriately, this assay could lead to the discovery of new heteromer-selective compounds. This in turn will facilitate research on the role of heteromers in human (patho-) physiology.

Authorship Contributions

Participated in research design: van Rijn, Harvey, Whistler.

Conducted experiments: van Rijn, Harvey, Brissett, DeFriel.

Performed data analysis: van Rijn, Harvey, Whistler.

Wrote or contributed to the writing of the manuscript: van Rijn, Harvey, Whistler.

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Fig. 7. Assay diagram for the detection of a heteromer-selective assay to detect heteromers of G_q-coupled receptors. (A) Selective activity of heteromers consisting of two (R1 and R2) G_q-coupled receptors can be achieved by fusing one of the G_q-coupled receptors to a G_{sq} or G_{iq} protein and measuring cAMP responses. (B) Selective activity of heteromers consisting of one G_q-coupled receptor and a G_i-coupled receptor can be achieved by fusing one of the receptors to a pertussis-toxin-insensitive G_{iq} protein and measuring cAMP responses in the presence of pertussis toxin (PTX).

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