Detergent- and phospholipid-based reconstitution systems have differential effects on constitutive activity of G-protein–coupled receptors

Received for publication, July 8, 2019, and in revised form, July 23, 2019; Published, Papers in Press, July 30, 2019, DOI 10.1074/jbc.AC119.009848

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Edited by Henrik G. Dohlman

A hallmark of G-protein–coupled receptors (GPCRs) is the conversion of external stimuli into specific cellular responses. In this tightly-regulated process, extracellular ligand binding by GPCRs promotes specific conformational changes within the seven transmembrane helices, leading to the coupling and activation of intracellular “transducer” proteins, such as heterotrimeric G proteins. Much of our understanding of the molecular mechanisms that govern GPCR activation is derived from experiments with purified receptors reconstituted in detergent micelles. To elucidate the influence of the phospholipid bilayer on GPCR activation, here we interrogated the functional, pharmacological, and biophysical properties of a GPCR, the β2-adrenergic receptor (β2AR), in high-density lipoprotein (HDL) particles. Compared with detergent-reconstituted β2AR, the β2AR in HDL particles had greatly enhanced levels of basal (constitutive) activity and displayed increased sensitivity to agonist activation, as assessed by activation of heterotrimeric G protein and allosteric coupling between the ligand-binding and transducer-binding pockets. Using [19F] NMR spectroscopy, we directly linked these functional differences in detergent- and HDL-reconstituted β2AR to a change in the equilibrium between inactive and active receptor states. The contrast between the low levels of β2AR constitutive activity in cells and the high constitutive activity observed in an isolated phospholipid bilayer indicates that β2AR basal activity depends on the reconstitution system and further suggests that various cellular mechanisms suppress β2AR basal activity physiologically. Our findings provide critical additional insights into GPCR activation and reveal how dramatically reconstitution systems can impact membrane protein function.

G-protein–coupled receptors (GPCRs),4 the largest family of membrane proteins, sense changes in the extracellular environment and convert them into specific intracellular responses. In the simplest model of receptor activation, GPCRs reside in a quiescent inactive state in the absence of ligand. Binding of an agonist to the extracellular “orthosteric” binding pocket initiates conformational changes within the seven transmembrane helices, resulting in the coupling of specific heterotrimeric G proteins and activation of downstream signaling cascades (1). Tight regulation of G-protein activation at the receptor level is enforced by GPCR kinases and β-arrestins, which phosphorylate and desensitize GPCRs, respectively (2). The allosteric connection between orthosteric ligand binding and transducer coupling maintains low levels of receptor constitutive activity in the absence of ligand, thus maximizing the agonist-induced response. In recent years, biophysical studies on GPCRs such as the β2-adrenergic receptor (β2AR), a prototypical receptor, have begun to delineate the conformational equilibria associated with agonist activation. For example, [19F] NMR with purified β2AR reconstituted in lauryl maltose neopentyl glycol (MNG) detergent showed an equilibrium between two inactive conformations in the absence of ligand, and addition of the agonist isoproterenol stabilized ~50% of the receptor population in an active conformation (3). In a similar study, [19F] NMR studies of MNG-bound adenosine A2 receptor revealed two analogous inactive states in addition to two distinct activation intermediates, whose populations were enhanced by partial and full agonists, respectively (4).

In addition to regulation by orthosteric ligands, the activity of many GPCRs is known to be modulated by diverse components in the cellular milieu, including other membrane proteins, phospholipids, and ions (5). Most biophys-
cal studies to date have utilized purified GPCRs in detergent micelles, but technical advancements in lipid bilayer reconstitutions have led to increasing evidence for a role of the membrane in modulating the conformational landscape of GPCRs (6–9). Herein, we systemically compared purified β2AR in a lipid bilayer and in detergent with regard to its ability to activate G proteins, its ability to bind agonists, and the conformational equilibria underlying these functions.

Results

To study receptor function in a lipid bilayer or detergent micelle model system, purified β2AR was reconstituted into high-density lipoprotein (HDL) particles (HDL–β2AR) containing a 3:2 lipid ratio of POPC/POPG or in MNG detergent (MNG–β2AR) (Fig. 1A). The very-low critical micelle concentration of MNG relative to other commonly-used detergents (i.e. dodecyl-d-maltopyranoside (DDM)) minimizes the possibility that detergent disassociation could alter conformational exchange between states (10) or possibly interfere with nucleotide exchange in the G protein. The principal cellular response of ligand-dependent activation of GPCRs is intracellular coupling to and activation of heterotrimeric G proteins, where the receptor acts as a guanine nucleotide-exchange factor promoting exchange of GDP for GTP. To directly test the effect of lipid and detergent on β2AR function, we measured the ability of HDL–β2AR and MNG–β2AR to activate purified heterotrimeric Gαi protein in vitro as measured by an increase in GTP hydrolysis. Both β2AR preparations increase G-protein activation in a concentration-dependent manner upon isoproterenol (ISO) stimulation, but HDL–β2AR activates GTP hydrolysis at concentrations ~25-fold lower than MNG–β2AR (Fig. 1B, white bars). Interestingly, at receptor concentrations where HDL–β2AR and MNG–β2AR have equivalent levels of isoproterenol-stimulated G-protein activity (i.e. 5 nM HDL–β2AR and 125 nM MNG–β2AR), only HDL–β2AR enhances GTP hydrolysis in the absence of ligand (Fig. 1B). These findings indicate that a phospholipid bilayer and detergent micelle have different effects on the constitutive activity of the β2AR.

The high level of HDL–β2AR constitutive activity implies that, compared with MNG–β2AR, a greater fraction of the receptor is in an active state in the absence of ligand. Because of the reciprocal allosteric communication between the extracellular agonist-binding site and the intracellular transducer-binding site of GPCRs, stabilization of the intracellular regions in an active conformation—by mutation or by an interacting protein—increases the affinity of agonists for the receptor (11). To pharmacologically measure the relative levels of active receptor in HDL- and MNG–β2AR, we compared the affinity of isoproterenol for each receptor in the absence and presence of two single-domain antibodies (nanobodies) that stabilize an active (Nb80) and inactive (Nb60) receptor state using radioligand competition binding (12, 13).

As we demonstrated previously, there is an ~7,200-fold difference in isoproterenol affinity when the entire receptor population is locked in the inactive state by Nb60 versus locked in an active conformation by Nb80 (13). The affinity of isoproterenol for HDL–β2AR is reduced by ~40-fold by Nb60 (control logIC50 = 7.03 ± 0.02 and Nb60 logIC50 = 5.44 ± 0.04) and increased ~180-fold by Nb80 (Nb80 logIC50 = 9.29 ± 0.05), suggesting that the unliganded receptor consists of an ensemble of inactive and active states whose proportions are acutely sensitive to nanobody (Fig. 2). These findings are not specific to the type of lipid used; HDL–β2AR reconstituted with a more physiologically relevant lipid mixture, a HEK293 cell lipid extract, exhibits a similar profile (Fig. S1). In contrast to HDL–β2AR, isoproterenol affinity for MNG–β2AR did not change to the same extent with Nb60 (3-fold decrease; control logIC50 = 6.52 ± 0.04 and Nb60 logIC50 = 6.07 ± 0.03) and Nb80 (60-fold increase, logIC50 = 8.32 ± 0.04) (Fig. 2). The similar affinity of isoproterenol for MNG–β2AR in the presence and absence of Nb60 implies that the activation equilibrium lies strongly toward inactive states under these reconstitution conditions, consistent with decreased efficacy of MNG–β2AR in G-protein activation compared with HDL–β2AR. Moreover, the difference in agonist affinity between Nb60- and Nb80-bound receptor is 40-fold higher for HDL–β2AR compared with MNG–β2AR, which is driven by HDL–β2AR having both a higher absolute affinity with Nb80 and a lower absolute affinity with Nb60. This indicates that the membrane environment is critically important for allosterically facilitating the full dynamic range of activation for the β2AR. Importantly, the differential effects of Nb60 and Nb80 on agonist affinity for MNG- and HDL–β2AR were not specific to the radioligand tracer used (Fig. S2).

Finally, we directly measured the conformational distribution of MNG- and HDL–β2AR by 19F NMR spectroscopy. The outward movement of transmembrane (TM) 6 in the intracellular regions is a hallmark of GPCR activation (1). Labeling the
endogenous residue Cys-265, located on the intracellular end of TM6, with an environmentally-sensitive trifluoroacetanilide probe allows conformational changes in TM6 to be monitored by $^{19}$F NMR (3). Cys-341, which is incompletely palmitoylated in cells, is also partially labeled but does not undergo any chemical shift changes after receptor activation (Fig. 3A). Consistent with previous studies (3), the NMR spectra showed that unliganded MNG–$\beta_2$AR exists in two inactive states (S1 and S2), which appear as a single broad peak due to millisecond timescale exchange between these states (Fig. 3A and Fig. S3). In contrast to S1, S2 lacks an interaction between R1313.50 (superscripts indicate Ballesteros-Weinstein numbering for GPCRs (14)) in TM3 and E2683.50 in TM6, referred to as the ionic lock (3). The addition of the agonist isoproterenol only converts 50% of the MNG–$\beta_2$AR into an active state, consistent with our pharmacological findings (Figs. 2 and 3A and Fig. S3). Previous studies have shown that complete conversion of MNG–$\beta_2$AR into conformations with TM6 in an activated position requires both agonist binding and the intracellular coupling of the G protein or other active state–stabilizing agents, such as Nb80 (3, 12).

In contrast to MNG–$\beta_2$AR, we found that ∼40% of unliganded HDL–$\beta_2$AR already adopts an active state (Fig. 3B and Fig. S3). Isoproterenol stabilizes the entire population in active states, even in the absence of transducer. The addition of Nb80 does not alter the NMR spectra compared with isoproterenol-bound HDL–$\beta_2$AR, although it should be noted that nanobody binding could stabilize conformational changes in other intracellular regions more fully than isoproterenol alone, as indicated by sharpening of the NMR peak. The higher population of active conformations of HDL–$\beta_2$AR in the absence and presence of isoproterenol (Fig. 3C) is consistent with its enhanced ability to activate G protein relative to MNG–$\beta_2$AR. There are also notable variations in peak widths between MNG- and HDL–$\beta_2$AR, which most likely arise from different exchange rates between active and inactive conformations. In the case of MNG–$\beta_2$AR, the active state peak line width of 560 Hz is reduced to ∼400 Hz after agonist addition, which can be interpreted as a reduction in exchange between active and inactive states (or possibly other activation intermediates). In contrast, despite the increased mass of the HDL particles, the line width of the active state for unliganded HDL–$\beta_2$AR is much narrower (250 Hz) than for MNG–$\beta_2$AR, and it further decreases with the addition of agonist (200 Hz) and agonist plus Nb80 (∼120 Hz). Importantly, these findings are in qualitative agreement with prior spectroscopic studies (7, 8). Taken together, these data indicate the lipid bilayer provides greater stabilization of the active state while slowing the exchange rate between active and inactive conformations. Agonist and Nb80 likely further stabilize the active state lifetime, thereby reducing exchange broadening.

**Discussion**

The differences in the biochemical and biophysical properties of MNG- and HDL–$\beta_2$AR have profound implications: first, for our interpretation of **in vitro** experiments with purified...
receptors, and second, for our understanding of the physiological regulation of GPCR activity. As with rhodopsin (6), the conformational distributions of β2AR reconstituted in detergent and HDL particles exhibit major differences. Here, we have further shown that this altered conformational distribution is associated with drastic differences in the β2AR’s two core functions—agonist binding and transducer activation. Moreover, differences have also been previously observed even among the conformational distributions of receptors reconstituted in different detergents (10). Together, these data underscore how acutely sensitive GPCRs are to their environment.

β2AR reconstituted in a lipid bilayer via HDL particles—presumably a more physiological environment than a detergent micelle—shows unexpectedly high constitutive activity. This finding is at odds with the low levels of agonist-independent signaling observed in the cell for the β2AR and most other GPCRs (15). This is generally explained on the grounds that unliganded receptors reside in a quiescent state, with exceedingly low populations of active conformations. The discrepancy between the low levels of constitutive activity in cells and our biophysical and functional data on HDL–β2AR raises intriguing questions as to how the basal activity of receptors in a phospholipid bilayer could be exploited or regulated physiologically. Basal GPCR activation and subsequent recycling can be an important factor in maintaining homeostasis, and inhibiting this process has been shown to cause up-regulation of the β2AR (16) and other GPCRs (17). In addition, having conformational equilibria poised on the verge of efficient transducer activation could help maximize the sensitivity of GPCRs to very low concentrations of endogenous agonists.

Another possibility is that GPCR constitutive activity could be kept in check by numerous cellular regulatory mechanisms. First, total signaling flux is a product of constitutive activity and receptor abundance, and most GPCRs are expressed at very low levels. The constitutive activity of most GPCRs does in fact become apparent upon receptor overexpression. For example, when massively overexpressed in the cardiac tissue of transgenic mice, the basal activity of the β2AR is sufficient to increase myocardial function to a similar extent as isoproterenol stimulation of endogenous levels of receptor (18). Second, GPCR activity can be tuned in the cellular environment by many factors such as phospholipid composition, ion concentrations, and interacting proteins. In this context, one obvious interaction that would restrain constitutive activity in the native cell membrane is GPCR kinase-mediated phosphorylation of the constitutively active receptors and subsequent binding of β-arrestins (i.e. constitutive desensitization). We cannot yet say with certainty where these receptors’ conformational equilibria lie in native cell membranes, but these allosteric regulators could create diverse patterns of regulatory potential in different tissues or membrane compartments. An additional implication is that “inverse agonists,” which reduce basal receptor signaling, and the more “neutral” antagonists, which minimally alter basal signaling, could have very different physiological effects from each other, potentially in a tissue-specific manner. This possibility should be considered as antagonist drug candidates are moved from in vitro assays to animal models.

In conclusion, the drastic differences in the functional properties and conformational distribution of the β2AR when in detergent or a lipid bilayer sound an important cautionary note that the choice of the reconstitution system for GPCRs can profoundly alter experimental results and conclusions. As technical advances permit, the goal should be to mimic the physiological environment as faithfully as possible.

Experimental procedures
β2AR expression and purification

For GTPase and radioligand-binding assays, human β2AR with an N-terminal FLAG tag, C-terminal His6-tag, and the N187E glycosylation mutation was expressed in SF9 insect cells using the BestBac expression system (Expression Systems). The minimal cysteine variant of the β2AR (β2ARΔ4) used in 19F NMR experiments contained an N-terminal FLAG tag and amino acid substitutions at four endogenous cysteines, C77V, C327S, C378A, and C406A. Importantly, ligand affinity and activation of G protein is unchanged between WT β2AR and β2ARΔ4 (19). The β2ARΔ4 was cloned into pcDNA-Neo-TetO and stably expressed in the mammalian Expi293F cell line with a stably integrated tetracycline repressor (20). Receptor was solubilized from cell membranes using DDM (Anatrace) and purified using FLAG-M1 chromatography as described previously (21). An additional alprenolol affinity chromatography step was included for β2AR purified from SF9 cells to remove nonfunctional receptor.

Detergent exchange and HDL reconstitution

To exchange into MNG detergent, DDM–β2AR immobilized on M1-FLAG resin was washed with a gradient of 0.5% MNG containing 0.05% cholesterol hemisuccinate (CHS). Receptor was subjected to size-exclusion chromatography (SEC) on a Superdex 200 Increase column, and the final buffer composition was 20 mM HEPES (pH 7.4), 100 mM NaCl, and 0.01% MNG with 0.001% CHS. The reconstitution of DDM–β2AR (5 μM) into HDL particles was conducted using a 3:2 mixture of 8 mM POPC/POPG and 200 μM MSP1D1H5 (22). Reactions were incubated for 1 h at 4 °C and subsequently rotated overnight with Biobeads (Bio-Rad) to promote HDL formation. Reconstituted HDL–β2AR WT or Δ4 was separated from HDL particles lacking receptor by FLAG-M1 chromatography (Fig. S4).

Nanobody and G-protein purification

Nb60 and Nb80 were purified as described previously (13). Nanobodies were collected from the periplasm of BL21 Escherichia coli, purified using Ni-NTA resin (GoldBio), and subjected to size-exclusion chromatography in 20 mM HEPES (pH 7.4) and 100 mM NaCl. Heterotrimeric Gαi protein was expressed as described elsewhere (23). In brief, the human short Gαi splice variant was co-infected with a bicistronic vector encoding Gβi–His6 and Gγ7 in Hive Five cells using the BestBac expression system. Cells were harvested 48 h post-infection, and heterotrimeric Gαi was purified from solubilized membranes using nickel-nitrilotriacetic acid chromatography and HiTrap Q-Sepharose anion–change chromatography (GE Healthcare).
**GPCR activity: detergent vs lipid**

**GTPase assay**

Activation of heterotrimeric G protein by MNG- or HDL–β2AR was measured using the GTPase Glo assay (Promega), as described previously (20). In brief, the final reaction buffer consisted of 20 mM HEPES (pH 7.4), 100 mM NaCl, 10 mM MgCl2, 1 mg/ml BSA, and 0.01% MNG (omitted for HDL–β2AR). The indicated concentration of MNG- or HDL–β2AR was preincubated with 10 μM isoproterenol for 15 min at room temperature. Receptor was added to heterotrimeric G protein (0.5 μM) and rGTP (2.5 μM) for 1 h at room temperature before the addition of Glo Reagent and ADP as described by the manufacturer’s protocol.

**Radioligand binding**

Competition radioligand-binding assays were conducted in a volume of 200 μl and a final buffer composition consisting of 20 mM HEPES (pH 7.4), 100 mM NaCl, 1 mg/ml BSA, 1 mM ascorbic acid, and 0.01% MNG (excluded with HDL–β2AR). HDL- or MNG–β2AR was added with a dose response of isoproterenol, 60 pm [125I]-cyanopindolol, or 1 nM [3H]dihydroalprenolol and 1 μM Nb60 or Nb80. Reactions were incubated at room temperature for 90 min, and receptor was captured on glass-fiber filters (GFB) soaked with 0.3% polyethyleneimine using a 96-well Brandel harvester.

**NMR receptor labeling**

For NMR experiments with the MNG receptor, DDM-β2AR Δ4 was exchanged into MNG detergent as described above. The receptor was treated with 100 μM tris(2-carboxyethyl)phosphine for 15 min at 4 °C, and a 5-fold excess of 2-bromo-N-[4-(trifluoromethyl)phenyl]acetamide (BTFMA, Apollo Scientific) (relative to β2AR) was added. After the reaction was allowed to proceed overnight at 4 °C, a second bolus of 5-fold excess BTFMA was added, and the reaction was incubated at room temperature for another 1 h before it was quenched with a 2-fold excess of L-cysteine (relative to the total amount of BTFMA added). The receptor was further purified by SEC and concentrated for use in NMR experiments. For NMR experiments with the HDL receptor, DDM-β2AR Δ4 was labeled with BTFMA as described above for MNG receptor. After SEC purification, the labeled receptor was reconstituted into HDL particles as described above, purified via M1 affinity chromatography, and concentrated for use in NMR experiments.

**NMR sample preparation**

Concentrated BTFMA-labeled receptor was diluted with buffer for a final concentration of 30–50 μM. It was added to a stock solution of bendroflumethiazide or sodium trifluoroacetate for 25 μM final concentration and D2O for 10% final concentration. The resulting solution was filtered through a 0.2-μm micron spin filter for sterilization.

**NMR acquisition**

Spectra were acquired on Varian Unity Inova 500- or 600-MHz spectrometers equipped with 5-mm H/F/X and H-E/C/N-Cryo probes, respectively. A typical spectrum was acquired at 20 °C with a spectral window from −20 to −110 ppm, a 0.8-s acquisition time, and a 0.2-s recycle delay, acquiring 10,000 scans using a 45° excitation pulse. The excitation pulse was set to be shorter than the Ernst angle accounting for T1 of the BTfMA label (0.9 s) and reference (2.2 s). Between 4 and 12 spectra were acquired for every sample and added together to increase signal-to-noise. The resulting spectrum was processed using MestReNova version 12 applying 2× zero filling, 50 Hz of line broadening, 5 point back linear prediction, and polynomial baseline correction. Chemical shift was referenced using benzoflavumethiazide, set to −59.05 ppm, which was later switched to TFA, set to −75.6 ppm. Peaks were fit using MestReNova to Lorentzian line shape using automated fitting and manual adjustments to give peak chemical shifts, areas, and half-height line widths.

**Author contributions**—D. P. S., L. M. W., and R. S. P. conceptualization; D. P. S., L. M. W., and D. P. data curation; D. P. S., L. M. W., and D. P. formal analysis; D. P. S., L. M. W., and D. P. writing—original draft; D. P. S., L. M. W., D. P., R. S. P., and R. J. L. writing—review and editing; R. S. P. and R. J. L. supervision; R. S. P. and R. J. L. funding acquisition.

**Acknowledgments**—We are grateful to Darrell Capel and Xinrong Jiang for technical assistance and to Quivetta Lennon, Jan Taylor, and Victoria Ronk for administrative assistance.

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