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ALLOSTERIC REGULATION OF GPCR ACTIVITY BY PHOSPHOLIPIDS

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Abstract

Lipids are emerging as key regulators of membrane protein structure and activity. Such effects can either be attributed to modification in bilayer properties (thickness, curvature and surface tension) or to binding of specific lipids to the protein surface. For G Protein-Coupled Receptors (GPCRs), the effect of phospholipids on receptor structure and activity remains poorly understood. Here we reconstituted purified β_2 -adrenergic receptor in High-Density-Lipoparticles to systematically characterize the effect of biologically relevant phospholipids on receptor activity. We observe that the lipid head-group type affects ligand binding (agonist and antagonist) and receptor activation. Specifically, phosphatidylglycerol markedly favors agonist binding and facilitates receptor activation while phosphatidylethanolamine favors antagonist binding and stabilizes the inactive state of the receptor. We then show that these effects can be recapitulated with detergent-solubilized lipids, demonstrating that the functional modulation occurs in the absence of a bilayer. Our data suggest that phospholipids act as direct allosteric modulators of GPCR activity.

INTRODUCTION

Protein sequences have evolved so as to optimize function within a given environment and, as such, membrane proteins have adapted to accommodate the physico-chemical properties of the lipid bilayer. As a corollary, changes in the composition of the lipid bilayer may affect

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Author Contributions R.D. expressed and purified the receptor, performed all HDL reconstitutions and functional characterizations, with the assistance of C.T. R.D. also performed all mass spectrometry analysis. M.M. also expressed and purified receptor samples. C.D. and P.V.A. performed mass spectrometry experiments. C.G. and B.K.K. provided overall project supervision and wrote the paper together with RD.

the structure and the function of membrane proteins¹. The role of lipids in such modulation has often been discussed as either “specific”, where bound lipid act as chemical partner, or “bulk”, where given physical properties of the membrane are responsible for the effect on protein function. Numerous studies have demonstrated that bilayer thickness, curvature and surface tension can significantly influence the behavior of embedded proteins^{2,3}. On the other hand, binding of given lipidic species to specific binding pockets may be required for protein stability and/or activity^{3,4}. High-resolution structures have illustrated such tight binding in a variety of cases^{5,6} and in several instances the presence of lipids was actually required for crystallogenesis^{2,7,8}. Furthermore, crystal structures of proteins obtained in the presence of bound lipids have shown conformational changes when compared to similar structures obtained in the absence of bound lipids⁴.

For G Protein-Coupled Receptors (GPCRs), earlier studies (typically based on depletion by cyclodextrin) have indicated that cholesterol is a key player in providing appropriate environment for receptor function (reviewed in⁹). This was originally viewed as a modulation of the lipid order by cholesterol itself and/or the requirement for cholesterol-rich microdomains for efficient signaling^{10,11} but direct cholesterol-receptor interactions had also been described¹². Remarkably, the effect of cholesterol on GPCR function is receptor-dependent. For example, cholesterol modulates agonist binding to oxytocin receptors¹³ and serotonin receptors¹⁴ whereas in the case of the NTS1 receptor its presence allows for dimerization¹⁵. Stability studies of detergent-solubilized receptors and high-resolution structures have demonstrated binding of cholesterol molecules to a conserved motif located between helices 1, 2, 3 and 4^{16,17}. Still, the effect of cholesterol on the order and fluidity of the membrane may be an important parameter for receptor function¹⁸.

The role of phospholipids on GPCRs has been studied by following protein function after reconstitution in given lipidic environments. Early work on rhodopsin suggested that bulk properties of the bilayer may modulate GPCR function¹⁹⁻²² while structural studies indicated that specific rhodopsin-PE interactions are also at play²³. Furthermore, addition of solubilized phospholipids to the transducin-rhodopsin complex significantly improved light-induced activation²⁴. Recent studies on NTS1 receptor reconstituted in nanodiscs have indicated that change in phospholipid composition may alter G protein coupling without affecting agonist binding²⁵.

In this context, a clear picture on how biologically relevant phospholipids affect GPCR function is lacking and, in particular, it is not known whether given lipidic species are interacting with receptors to modulate their activity. Taking advantage of the recent availability of appropriate biochemical tools, we use the β 2-adrenergic receptor (β 2R) to systematically characterize the effect of biologically relevant lipid species on receptor function. Our data show that lipids act as specific modulators of β 2R activity.

RESULTS

Purified human β 2R receptor was reconstituted in High-Density-Lipoparticles (HDLs, or nanodiscs) of defined homogenous composition. We opted for HDL reconstitution over proteoliposomes in order to avoid the critical issue of protein orientation. In addition,

previous studies have demonstrated that the $\beta 2R$ can be reconstituted as a fully functional monomer in HDL ²⁶. We focused on the main lipids observed in membranes of mammalian cells, as detected by quantitative mass-spectrometry analysis of HEK293 membranes: phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylserine (PS), phosphatidylinositol (PI) (Fig. 1a). We selected 1,2-Dioleoyl-*sn*-glycerol (two chains of 18 carbons with one unsaturated double bond) lipids because they are among the most abundant in mammalian membranes and all have transition temperatures below 0°C, allowing for efficient reconstitution. Conditions for reconstitution of $\beta 2R$ into HDL were optimized for each lipid species (see Methods and Supplementary Fig. 1&2).

For each reconstitution we tested binding of agonist and antagonist by competition with a radiolabelled ligand (³H-DHA). As shown in Fig. 1, the binding affinities are strongly modulated by the phospholipid type surrounding the receptor. The IC₅₀ for the agonist isoproterenol is about 5.9*10⁻⁷M when the receptor is reconstituted in DOPG but increases to 4.3*10⁻⁶M when the receptor is in DOPE, a 7.2 fold difference (Fig. 1b and Supplementary Table 1). Statistical analysis (see Methods) shows that pIC₅₀(DOPG)≈ pIC₅₀(DOPS)≈ pIC₅₀(DOPI)< pIC₅₀(DOPC)≈ pIC₅₀(DOPE) where “<” implies statistically significant difference with p<0.05 and ≈ implies no statistically significant difference. Remarkably, the behavior is dramatically changed when comparing agonist and antagonist binding (Fig. 1c) with DOPE providing the better environment for binding the antagonist alprenolol, and DOPG, DOPC and DOPI the least efficient. The statistical test shows that pIC₅₀(DOPE)< pIC₅₀(DOPS)< pIC₅₀(DOPG)≈ pIC₅₀(DOPC)≈ pIC₅₀(DOPI). This also demonstrates that the receptor is properly reconstituted in each one of the lipid compositions and suggests that specific lipids may favor a given functional state of the $\beta 2R$.

These results suggest that lipid head-groups have allosteric effects on the $\beta 2R$ structure. To directly examine the effect of phospholipids on receptor conformation, we monitored the fluorescence of a conformational reporter on the cytoplasmic end of TM6. Upon activation of the $\beta 2R$, TM6 opens outward by about 14Å ²⁷. This movement can be detected by monitoring the fluorescence properties of a bimane probe covalently bound to Cys265, because the outward movement of TM6 leads to an increase solvent exposure of bimane (Fig. 2a) and thus a decrease in fluorescence intensity along with a red-shift of the emission maximum ²⁸. After reconstituting bimane-labelled receptor in each lipidic composition we measured fluorescence emission spectra and compared them to the signal observed for detergent-solubilized receptor. For DOPC and DOPE, this basal signal (agonist-free) is similar to the one observed in detergent, with a λ_{max} of about 450nm (Fig. 2b) but a significant red shift is observed when the receptor is reconstituted in DOPG where the λ_{max} is about 455nm (Fig. 2b and 2c) as well as DOPI and DOPS (Fig. 2b). This increased solvent exposure of Cys265 is not due to partial destabilization of the receptor as it can be reversed upon addition of the inverse agonist ICI118,55 (Fig. 2b).

As the agonist-induced opening of TM6 will lead to a time-dependent decrease in bimane fluorescence, the intensity and kinetics of activation can be followed by monitoring the red-shift in the λ_{max} of bimane emission (Fig. 2d, the λ_{max} is highlighted with filled dots on each curve). Similarly, reversal of the TM6 opening upon addition of saturating concentration of antagonist will lead to a fluorescence recovery that results in a blue-shift in the λ_{max} (Fig.

2e). We have therefore used the time dependent changes in $\lambda_{\max}$ to directly compare the effect of the lipidic environment on receptor activation. We monitored the rate and magnitude of changes in $\lambda_{\max}$ upon addition of 200 μM of the agonist isoproterenol (Fig. 2f) to $\beta 2\text{R}$ reconstituted into different lipids. We observed the fastest rate of change and the largest shift in $\lambda_{\max}$ (relative to basal fluorescence in detergent) for DOPG, followed by DOPI and DOPS. The smallest change and slowest rate of change was observed for DOPE, with DOPC being intermediate. These results parallel the effect of lipids on the binding affinity of isoproterenol.

Testing the effect of lipids on effector coupling is complex as the G protein interacts directly with the membrane and thus changes in coupling efficiency may be due to modified affinity of the G protein for the membrane, altered receptor behavior, or combination of both. Therefore, isolating the respective contributions may be very difficult, as observed in the case of the NTS1 receptor, where PG increased G protein coupling without affecting receptor pharmacology²⁵. In the case of the $\beta 2\text{R}$, this issue can be circumvented by using nanobody Nb80, a G protein surrogate that does not interact with the bilayer but stabilizes a true active state of the receptor²⁷ and behaves as a G protein mimetic from a pharmacological standpoint²⁹. As shown in Fig. 2g, the ability of Nb80 to stabilize an active conformation is strongly influenced by the lipidic composition and here again DOPG provides the better environment for activation, reaching a $\lambda_{\max}$ shift of 13 nm after 30 min. Interestingly, DOPS and DOPI do not stabilize the Nb80-bound active state as well as DOPG, indicating that a negative charge on the headgroup is not sufficient to fully stabilize the active conformation. Considering the respective sizes of the polar head-groups ($\text{PS} \approx \text{PG} < \text{PI}$), these differences are not likely to arise from variability in charge density. In agreement with our other assays, DOPE provides the least supportive environment for Nb80-induced $\beta 2\text{R}$ activation. Interestingly, upon addition of a saturating concentration of isoproterenol, each lipidic composition allows for complete stimulation as measured by a final $\lambda_{\max}$ shift of 18nm, observed in every case, further demonstrating that the $\beta 2\text{R}$ is fully functional in the different nanodiscs.

The limited size of a HDL particle implies that only a few lipid layers surround the reconstituted beta2R, indicating that bulk effect of lipids observed in native membranes or lipid vesicles should be limited in such system³⁰. We tested this hypothesis by adding detergent-solubilized lipids on purified $\beta 2\text{R}$ in the absence of apolipoprotein. We verified using size-exclusion chromatography, dynamic light scattering and electron microscopy that no liposomes were formed under these conditions (Supplementary Fig.3).

As shown in Fig. 3a, adding increasing amounts of solubilized DOPG leads to a dose-dependent positive effect on $\beta 2\text{R}$ activation as the bimane fluorescence is affected quicker and stronger than in the absence of lipids. A dose-dependent basal $\lambda_{\max}$ shift is observed, reaching 4nm at 200 μM of solubilized DOPG. When comparing different solubilized phospholipids at this concentration, DOPG remains the strongest positive modulator; DOPS also shows a positive effect albeit not as strong, while DOPC and DOPE show little or no effect (Fig. 3b).

Stabilization of the active state by Nb80 is also modulated by lipids (Fig. 3c) in the absence of a bilayer, with DOPG facilitating a fast and large change in bimane fluorescence, reaching an almost completely active state even before addition of agonist. Here also DOPS provides a positive modulation of the activation, although not as strong as DOPG. The combination of G protein mimetic and saturating concentration of agonist leads to full activation in all conditions tested including in the absence of lipids.

While our functional data suggests a possible physiological role for PG in the activity of the $\beta 2R$, this lipid is a minor component in mammalian cell membranes, representing just over 2% of all detected phospholipids. We thus investigated whether the receptor binds preferentially this lipid. To this end, we purified $\beta 2R$ expressed in Sf9 cells with half the detergent concentration normally used to prepare the samples (0.5% vs. 1% DDM), leading to significant increase in retained lipids (see Supplementary Table 2). These bound lipids were extracted and quantitatively measured, allowing to compare the relative amounts of each phospholipid bound to the receptor with those found in Sf9 membranes (see Methods).

As shown in Supplementary Table 2, while PG constitutes about 4% of total lipids in Sf9 membranes, it represents over 16% of all $\beta 2R$ -bound lipids. In fact, the receptor appears to favor binding of specific PG species such as DOPG, constituting about 0.1% of all Sf9 lipids but over 7% of all receptor bound phospholipids (Supplementary Table 3). In total, specific PG species found on the purified receptor (18:1–18:1 and 18:1–16:1) are increased about 15 fold compared to the insect cell membrane. For PC and PE we observed modest relative enrichment. While the total amount of stably bound lipids is probably underestimated due to the inevitable use of detergent for protein solubilization, our data argue that $\beta 2R$ and phosphatidylglycerol are likely to interact even with low natural abundance of PG.

DISCUSSION

Taken together, our data demonstrate that lipids act as modulators of GPCR structure and activity, and that this effect is driven by the head-group. Both binding studies and agonist-induced conformational assays show that DOPG, DOPS and DOPI are promoting $\beta 2R$ activation, while DOPE appears to favor the inactive state. The effect of lipids on the $\beta 2R$ structure can be observed in the absence of ligand (Fig. 2c, Fig. 2g and Fig. 3c), demonstrating that they are not due to interactions between the lipids and the ligands.

A key finding is that the negatively charged lipids favor receptor activation in the absence of a bilayer and in a dose-dependent manner (Fig. 3a and 3b), thus acting as true positive allosteric modulators. The presence of these lipids enhances the constitutive activity of the receptor as detected by the redshift of bimane fluorescence in the absence of ligand (Fig. 3).

In the absence of agonist and lipids, Nb80 shows a mild effect on bimane fluorescence (ie about 4nm max shift in $\lambda_{\max}$ after 10 mins, Fig. 3c), which correlates with the limited basal activity of the receptor under these conditions as this nanobody strictly requires opening of TM6 for binding. The level of stimulation at steady state must reflect the balance between the dynamics of the conformational transition of the receptor (ie. relative time spent in the active and inactive state) and the specific off-rate of the nanobody. Both in nanodiscs and in

solution, we observe a highly cooperative effect of negatively charged lipids as their addition dramatically potentiates the ability of Nb80 to stabilize the active state (Fig 2g and Fig. 3c). This effect is lipid-specific as the magnitude correlates with the ability of each lipid species to increase constitutive activity of the receptor (Fig. 2b), thus confirming that the nanobody Nb80 acts as a sensor of basal activity. It is unlikely that the differences between the lipid species are due to differences in affinity for the receptor as we observed them in nanodiscs (Fig. 2g) where the very high local concentration of lipids ensures permanent interaction. Rather, these variations most likely indicate that the lipids differentially affect the conformational kinetics of the receptor, e.g. that binding of DOPG maintains the cytoplasmic side of the $\beta 2R$ in an open conformation for a longer (average) time than binding of other lipids, leading to a larger apparent stimulation by Nb80, with almost complete receptor activation after 10 mins (Fig. 2g and Fig. 3c). This suggests that binding of such negatively charged lipids favors TM6 opening through specific binding of the head-group to the protein, most likely on the intracellular side of the receptor.

Recent studies have shown that, even at saturating concentration of full agonist, the majority of the receptor population adopts an inactive or intermediate conformation, indicating that each agonist-bound receptor spends only a limited time in the active state^{31, 32} and that additional binding of a G protein (or G protein mimetic) is required to completely shift the receptor ensemble towards the active conformer. We observe a similar behavior here as, in several conditions (for example in absence of lipids, see Fig. 3a and 3b), only partial $\lambda_{\max}$ shifts of bimean labeled receptor are observed even after more than 30 min of incubation with 200 μM of isoproterenol; full activation is consistently observed in the presence of both agonist and Nb80 whether or not lipids are present. However the time-dependence of this activation greatly depends on the presence of lipids, thus showing that they act as allosteric modulators that primarily regulate the kinetics of the process.

In the presence of negatively charged lipids the effect of the agonist on activation of the receptor population is greatly enhanced. As this effect is observed at saturating concentrations of isoproterenol, it is unlikely that these lipids act in an agonist-like fashion on the receptor, but rather that they stabilize the active conformation of the cytoplasmic side of the $\beta 2R$. This would also be consistent with a direct interaction of the lipid at the intracellular end of the receptor. However, in contrast to what is observed with Nb80 (Fig. 2g), we do not see strong differences between the three negatively charged lipids on isoproterenol response (Fig. 2f). While this could imply a different molecular mechanism than the one potentiating Nb80 (i.e. another lipid binding site), it could also be simply due to kinetic effects (for example if the off-rate of the agonist is slower than that of Nb80).

Specific and direct lipid-protein interactions have been demonstrated for a variety of membrane proteins⁵, including mammalian ones⁷, and in a number of cases this interaction is essential for protein activity^{3, 6}. It is reasonable to expect that GPCRs may also contain phospholipid binding sites that would be coupled to the activation mechanism. The strong effect of negatively charged phospholipid would suggest ionic interactions between receptor side chains and lipid head-group. In addition the stronger effect observed with DOPG compared to DOPS or DOPI indicates a clear level of specificity, suggesting that given sequence motifs of the receptor interact directly with the phosphatidylglycerol head-group.

Identification of such binding sites typically relies on structural studies. Crystallizing the $\beta 2R$ in complex with PG could locate the binding sites involved on positive allosteric modulation, assuming that lipids will be present in the crystal and sufficiently ordered. However such studies will face practical challenges as $\beta 2R$ is typically crystallized in lipidic cubic phases that may dilute out or compete with bound lipids, or that the allosteric lipids may promote intermediate states not compatible with crystallization.

A key question that will also require detailed identification of the lipid binding site is whether the functional coupling to lipid binding is receptor-dependent. Adding lipids greatly enhanced stability and function of detergent-solubilized rhodopsin with negatively charged lipids (DOPS) having the strongest effect on the activation rate ²⁴. Work on nanodisc-reconstituted rhodopsin has also shown that the presence of PG enhances arrestin-induced active state (meta-II) formation ³³, but direct interaction between the effector and the lipids could possibly be responsible for the effect. For the NTS1 receptor, no difference in ligand affinity was observed upon changes in the lipidic composition, including variations in the amount of PG, in contrast to what we observed with the $\beta 2R$. ²⁵ Note however that G protein coupling to the NTS1 receptor was increased in PG nanodiscs, although this effect could be due to G protein-PG interactions.

While our data show that direct lipid binding affects receptor function, they do not rule out additional modulation of the $\beta 2R$ by bulk properties of the membrane. Early studies on rhodopsin have focused on identifying global properties of the bilayer as determinant of receptor state or conformational transition. As increasing the relative amount of PE appeared to favor the meta-II state, Brown and colleagues have proposed that membrane curvature stress is a key element in supporting the activation of the photoreceptor ²⁰. In contrast, our data show that phosphatidylethanolamine tends to stabilize the inactive state of the $\beta 2R$. As the lipidic composition of rod outer segment (ROS) is different from most plasma membranes ³⁴, rhodopsin may show specific adaptation to the physico-chemical properties of its surrounding membrane. On the other hand, reconstitution of rhodopsin in HDL particles have demonstrated native-like transducing activation ³⁵ and photointermediates ³⁶, indicating that bulk effects are not required for rhodopsin function.

Note that testing bulk effects on the $\beta 2R$ receptor would require reconstitution in larger structures than HDLs but, as mentioned earlier, the lipidic composition of proteoliposomes may strongly affect the orientation of the receptor which, unlike for rhodopsin, will alter ligand access and thus void comparisons of pharmacological profiles.

Considering the magnitude of the effects observed in vitro, it is legitimate to ask how they will translate in vivo. As different tissues will typically display distinct lipidic compositions, it is reasonable to hypothesize that the actual pharmacology of a given receptor may vary from one organ to another due to change in lipid composition. Not only the apparent affinity of a given ligand for its target receptor could be different from tissue to tissue, our data suggest that the kinetics of response would also be modulated by the lipidic composition. Variations in ligand response with cellular background have been described in the past for several GPCRs ³⁷ but are typically attributed to difference in signaling or regulatory

partners. Our study suggests that the phospholipid composition of the cell type or lipid domain might play a key role.

ONLINE METHODS

All lipids used in this study were purchased from Avanti[®] polar lipids inc. β 2AR cold ligands Isoproterenol hydrochloride I6504, Alprenolol hydrochloride A7686, ICI-118,551 I127 and Monobromobimane 69898 were purchased from Sigma-Aldrich[®].

Cell culturing and β 2R expression

The constructs of β 2R used in this study are 365N and PN1. β 2AR-365N contains the human wild-type coding sequence of the β 2AR with a FLAG epitope tag at the aminoterminal. A sequence containing a TEV cleavage site was introduced between the FLAG epitope and the start of the receptor. The third glycosylation site was removed by mutating N187E and the receptor sequence was terminated after Gly365. β 2AR-PN1 contains the human wild-type coding sequence of the β 2AR with a FLAG epitope tag at the aminoterminal. A sequence containing a TEV and 3C protease cleavage site were introduced respectively between Val24 and Thr25 and Gly365 and Tyr 366 of the receptor. The third glycosylation site was removed by mutating N187E and the receptor sequence was terminated after Gly365. Four mutations were introduced to the construct M96T, M98T, C378A and C406A. The sequences were cloned into the pFastBac1 Sf9 insect cell expression vector (Invitrogen). Expression of β 2R for purification was achieved in SF9 cells. 4L of cells were infected with baculovirus encoding for one of the β 2R constructs (365N or PN1) and grown in ESF 921 protein free medium from Expression system at 27°C with 130 rpm agitation for 48-50h. Cells were then harvested and pellets stored at -80°C for purification.

β 2R purification and bimane labelling

β 2R was purified from Sf9 cells using a three-step purification procedure as described previously³⁸. Briefly, cell pellets were lysed in lysis buffer (10mM Tris pH 7.4, 1mM EDTA, 1 μ M alprenolol, protease inhibitors) and centrifuged (15 min at 30,000g). Lysed cells were resuspended in solubilization buffer (20mM HEPES pH 7.4, 100mM NaCl, 1% dodecylmaltoside, 1 μ M alprenolol, protease inhibitors) and homogenized with 10 rounds of potter and then stirred for 1 h at 4 °C. Solubilized receptor was purified by chromatography using M1 Flag antibody affinity resin (sigma). The eluate from the M1 anti-Flag column was further purified on an alprenolol-sepharose affinity column and finally through a second M1 flag antibody affinity resin purification step. For bimane labeling, Flag pure receptor (50-70nmol) was incubated on ice with 100 μ M TCEP then 20 μ M mBBR in presence of protease inhibitors for at least 2h, then 2mM Iodoacetamide were added for 30min and 5 mM cysteine were added to quench Iodoacetamide before proceeding to Alprenolol-M1 Flag affinity purification as described above.

HDL reconstitutions

ApoAI used for HDL reconstitutions was purified as described³⁹. Reconstitution of β 2R in HDL particles of different lipid compositions was done based on methods previously published²⁶. Briefly, the reconstitution mix consisted of the following: 3.6mM detergent

(cholate), 1.2mM lipids, and 72-100 μ M apoA-I (depending on the lipid composition) and 5 μ M purified β 2R. Lipids were solubilized with 20mM Hepes, pH 8/100 mM NaCl/1 mM EDTA plus 50 mM detergent. Purified apoA-I was mixed (at least 10-fold excess) to receptor then solubilized lipids added. ApoAI/lipid ratio was optimized for each lipidic composition in order to have homogeneous HDL population; we used a range of 1/30 to 1/50. Reconstitution mix incubated O.N. on ice, and then samples were subjected to BioBeads (BioRad, Hercules, CA) to remove detergents, resulting in the formation of rHDL particles. rHDLs were then injected on a Superdex200 300GL (GE Healthcare) size exclusion column in HNE buffer (20mM HEPES, 150mM NaCl, 1mM EDTA) and the main homogeneous peak kept. Samples were stored on ice until used. If necessary, β 2R rHDL particles from receptor-free rHDL were subsequently purified by M1-anti-FLAG immunoaffinity chromatography. Purified β 2R rHDL particles were eluted with 1mM EDTA plus 200 μ g/ml FLAG peptide and stored on ice.

Electron Microscopy

rHDL formation and homogeneity were further checked by electron microscopy. Grids were treated in a glow discharge system over 1 min in a low current regime, 5 mA, 2 μ l of β 2R rHDL sample at 10 μ g/ml were used per grid and stained with Uranyl Formate 0.75%. TEM data was collected at magnification 60000 and pixel size 1.9 $\text{\AA}$ /px in a Jeol Jem-1400 operating at 120 kV with a LaB6 filament and equipped with a CMOS TEMCAM-416 4016 $\times$ 4016.

Radioligand binding affinities

Competition binding assays were performed using [3H]-dihydroalprenolol (DHA) on reconstituted β 2R. Concentrations of [3H]-DHA used was 1 nM. Competing ligands were added at following concentrations: 10^{-11} to 10^{-4} M for cold antagonist (alprenolol) and 10^{-13} to 10^{-1} M for cold agonist (isoproterenol). The assays were carried out for 60 min at room temperature with shaking at 130 rpm. Reactions were harvested on a Brandel haverster M-24T on GF/B filters.

All data points were obtained in duplicates and experiments were repeated at least three times. Data were normalized and fit to theoretical one-site competition binding models using Prism (GraphPad Software, San Diego, CA). A one-way ANOVA plus a posteriori Scheffe statistical test was used to compare pIC₅₀ values. All statistical analyses were performed with SPSS version 21, with $p < 0.05$ considered as statistically significant.

Bimane fluorescence spectroscopy on solubilized or reconstituted in HDL particles β 2R

To compare lipids effect on receptor conformation β 2R was labelled with the environmentally sensitive fluorescent probe monobromobimane (Invitrogen) at cysteine 265 located in the cytoplasmic end of TM6. Fluorescence spectroscopy was performed on a Photon Technology International (PTI) fluorometer, digital mode, using excitation and emission bandpass of 6nm. Excitation wavelength was set at 370nm and emission was collected from 430 to 510nm 1nm increments with 0.5 scm^{-1} . Spectra were registered at 25°C as mean of two. 1 μ M of bimane labeled receptor in 0.025% DDM or in rHDL particles was used for each measure. The lipid effect on basal activity of rHDLs β 2R was quantified

as the red-shift of bimeane $\lambda_{\max}$ compared to the detergent solubilized receptor. For basal activity inhibition, the receptor was treated with 100 μ M inverse agonist ICI-118,551 and $\lambda_{\max}$ blue shift monitored. A two-way ANOVA plus a posteriori Scheffe statistical test was used to compare $\lambda_{\max}$ values. All statistical analyses were performed with SPSS version 21, with $p < 0.05$ considered as statistically significant. Conformation changes upon ligand binding were assayed using saturating concentrations of agonist Isoproterenol (200 μ M) and antagonist Alprenolol (100 μ M). Effect on coupling was measured in presence of 4 μ M (4 molar excess) Nb80 (G protein-mimetic nanobody²⁹) followed by the addition of 200 μ M Isoproterenol. Kinetics of maximum wavelength ($\lambda_{\max}$) Shift was monitored for each condition with at least 6 time points. The initial value was established as the $\lambda_{\max}$ of condition without ligand - $\lambda_{\max}$ of receptor in DDM without ligand nor lipid. For the effect of lipids on DDM solubilized receptor, 25, 100, or 200 μ M lipids in sodium cholate were added to the receptor. Ratios of lipids/receptor/detergent were as following:

2 μ M lipids/1 μ M β 2R/75 μ M cholate/500 μ M DDM; 100 μ M lipids/1 μ M β 2R /300 μ M cholate/500 μ M DDM; 200 μ M lipids/1 μ M β 2R /600 μ M cholate/500 μ M DDM; and (non-lipidated control) 0 μ M lipids/1 μ M β 2R/600 μ M cholate/500 μ M DDM.

Kinetic curves were drawn using Prism (GraphPad Software, San Diego, CA) as mean of at least 3 independent experiments.

Mass spectrometry identification and quantification of lipids in SF9 membranes and bound to purified β 2R

Lipid extraction and mass spectrometry analysis of lipids from SF9 membranes and SF9 purified β 2R was performed as following: Briefly, cell pellets of 6×10^7 SF9 cells were transferred into hypotonic lysis buffer (10mM Tris pH7.4, 1mM EDTA) and stirred for 15min for cell breaking. Lysed cells were centrifuged at 25000g for 10 min and the pellet containing the membranes was resuspended in PBS. Standards for each phospholipid class and cholesterol-¹³C3 were added to the SF9 membrane or 1-2mg purified receptor and lipids were then extracted from membranes by methanol/chloroform extraction as described in ⁴⁰ with the following modifications: 10 μ L HCl 6N were added to the mixture and extraction was repeated 3 times on the same sample to have maximum recovery of all types of lipids; corresponding organic phases were pooled together, the solvent evaporated under nitrogen stream and lipids then dissolved in dichloromethane:Isopropanol (1:4) prior to mass spectrometry analysis. Samples were injected in a rapid resolution liquid chromatography system (RRLC 1200 series from Agilent Technologies, CA, USA) fitted with a XDB Eclipse Plus C18, 4.6 $\times$ 150 mm, 1.8 μ m. A 6520 series electrospray ion source (ESI) - quadrupole time-of-flight (Q-TOF) from Agilent Technologies was used for the MS/MS analyses in positive and negative mode. Data were acquired by the Mass Hunter Acquisition[®] (Agilent Technologies). For quantification, single MS analyses were performed and data were acquired by the Mass Hunter Acquisition[®] (Agilent Technologies). For data analysis, first fragment based searching mode analysis by Mass Hunter Qualitative Analysis[®] (Agilent Technologies, CA, USA) was applied on MS/MS spectra in positive and negative mode to identify lipid species. Then for quantification, samples were run in simple MS mode. Extracted ion chromatograms were extracted based on the exact masses of the lipids

observed during auto MSMS analyses. Phospholipids were quantified by the standard curves run during the set of experiments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Lipids modulate ligand affinity of β_2R

a. Chemical structure of the lipids used for in this study. For clarity the acyl chains are not shown and replaced by R1 and R2 labels.

b-c. Ligand binding curves for the agonist Isoproterenol and the antagonist Alprenolol competing against [³H]-dihydroalprenolol ([³H]-DHA) for β_2R reconstituted in rHDL particles of different lipid compositions.

Figure 2. β_2 R activation is regulated by phospholipids

a. Molecular surface representation of the active (PDB id: 3SN6) and inactive (PDB id: 4GBR) structures of the β_2 R with TM6 highlighted (colored ribbon) and the position of Cys265 (in yellow), showing the increased exposure of the latter in the active state.

b. Wavelength of the emission maximum fluorescence ($\lambda_{\max}$) in the absence of ligand (basal) and after addition of 100 μ M of inverse agonist ICI-118,551 for bimane-labelled receptors reconstituted in rHDLs of different lipid compositions compared with DDM solubilized receptor. Asterisk denotes statistically significant difference ($p < 0.05$).

- c.** Basal fluorescence emission spectra for bimane-labelled receptors in rHDL of DOPC, DOPG and DOPE in comparison with DDM solubilized receptor. The effect of the inverse agonist (ICI-118,551) on $\beta 2R$ in DOPG rHDL is represented by dashed red curve. Blue shift in $\lambda_{\max}$ is highlighted by an arrow.
- d.** Time dependent fluorescence change on $\beta 2R$ rHDL in DOPC upon addition of saturating concentration of 200 μ M agonist (Isoproterenol). The $\lambda_{\max}$ positions are highlighted on each curve.
- e.** Fluorescence recovery of agonist-stimulated receptor shown in **d** upon the addition of saturating 100 μ M antagonist (Alprenolol).
- f.** Time dependent changes in bimane $\lambda_{\max}$ upon addition of Isoproterenol (200 μ M) followed by Alprenolol (100 μ M) for labelled receptor reconstituted in the different rHDLs. Data are the mean $\pm$ SEM of at least 3 independent experiments.
- g.** Time dependent changes in bimane $\lambda_{\max}$ upon addition of 4 μ M Nb80 followed by agonist activation (200 μ M Isoproterenol). Data are the mean $\pm$ SEM of at least 3 independent experiments.

Figure 3. Modulation of β 2R function by lipids does not require a bilayer

a. Time dependent changes in bimane $\lambda_{\max}$ of solubilized bimane-labelled β 2R (1 μ M receptor, 500 μ M DDM) upon addition of saturating concentration of 200 μ M Isoproterenol in the presence of increasing concentration of solubilized DOPG (with 600 μ M Sodium Cholate). No lipids condition was performed in presence of 600 μ M sodium cholate. Data are the mean $\pm$ SEM of at least 3 independent experiments.

b. Time dependent changes in bimane $\lambda_{\max}$ upon addition of saturating concentration of 200 μ M Isoproterenol in the presence of 200 μ M of either DOPC, DOPG, DOPE and DOPS

(detergent concentrations are identical to those in panel a). Data are the mean $\pm$ SEM of at least 3 independent experiments.

c. Time dependent changes in bimane λ_{max} upon addition of 4 μ M Nb80 followed by agonist activation (200 μ M Isoproterenol). Data are the mean $\pm$ SEM of at least 3 independent experiments.