Title: Local membrane charge regulates β_2 adrenergic receptor coupling to G_i

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Abstract

G protein coupled receptors (GPCRs) are transmembrane receptors that signal through heterotrimeric G proteins. Lipid modifications anchor G proteins to the plasma membrane; however, little is known about the effect of phospholipid composition on GPCR-G protein coupling. The β_2 adrenergic receptor (β_2 AR) signals through both G_s and G_i in cardiac myocytes where studies suggest that G_i signaling may be cardioprotective. However, G_i coupling is much less efficient than G_s coupling in most cell-based and biochemical assays, making it difficult to study β_2 AR- G_i interactions. To investigate the role of phospholipid composition on G_s and G_i coupling, we reconstituted β_2 AR in detergent/lipid mixed micelles and found that negatively charged phospholipids (PS and PG) inhibit β_2 AR- G_{i3} coupling. Replacing negatively charged lipids with neutral lipids (PC or PE) facilitated the formation of a functional β_2 AR- G_{i3} interaction that activated G_{i3} . Ca^{2+} , known to interact with negatively charged PS, facilitated β_2 AR- G_{i3} interaction in PS. Mutational analysis suggested that Ca^{2+} interacts with the negatively charged EDGE motif on the carboxyl-terminal end of the αN helix of G_{i3} and coordinates an EDGE-PS interaction. These results were confirmed in β_2 AR reconstituted into nanodisc phospholipid bilayers. β_2 AR- G_{i3} interaction was favored in neutral lipids (PE and PC) over negatively charged lipids (PG and PS). In contrast, basal β_2 AR- G_s interaction was favored in negatively charged lipids over neutral lipids. In negatively-

charged lipids, Ca^{2+} and Mg^{2+} facilitated $\beta_2\text{AR}$ - G_{13} interaction. Taken together, our observations suggest that local membrane charge modulates the interaction between $\beta_2\text{AR}$ and competing G protein subtypes.

Introduction

A third of all FDA approved pharmaceutical drugs function by modulating the activity of G protein coupled receptors (GPCRs)¹, a large receptor superfamily. GPCRs catalyze the activation of heterotrimeric G proteins, which in turn initiate a multitude of signaling cascades that alter cellular function.

G proteins are also a large superfamily, grouped into 4 subfamilies (G_s , $\text{G}_{i/o}$, $\text{G}_{q/11}$, $\text{G}_{12/13}$) encoded by 16 different genes². Each subfamily activates distinct signaling pathways, and functional effects are cell-type specific. Most GPCRs can signal through more than one G protein subfamily, and ongoing research attempts to identify mechanisms that regulate G protein selectivity within a cell².

Here we investigate the dual G protein selectivity of the β_2 adrenergic receptor ($\beta_2\text{AR}$), a prototypical GPCR that mediates the fight-or-flight response. The dual G protein selectivity of $\beta_2\text{AR}$ is best characterized in heart muscle (cardiac myocytes) where activation of G_s increases contraction rate and activation of G_i decreases it. $\beta_2\text{AR}$ activity is stimulated by the hormone epinephrine. In healthy neonatal cardiac myocytes, epinephrine stimulated $\beta_2\text{AR}$ immediately activates G_s , but after 10-15 minutes $\beta_2\text{AR}$ signals predominantly through G_i ³. Of interest, G_i activation is impaired if $\beta_2\text{AR}$ internalization is blocked⁴. Also, G_i does not interact with a modified $\beta_2\text{AR}$ that internalizes but does not recycle to the plasma membrane⁵, and WT $\beta_2\text{AR}$ that internalizes but is pharmacologically blocked from recycling⁶. Taken together, these

observations demonstrate that β_2 AR- G_i interaction is regulated spatially and temporally, and interaction occurs after epinephrine stimulated trafficking of β_2 AR.

β_2 AR- G_i signaling plays a complex role in heart disease. While its anti-apoptotic effects^{7,8} may prevent ischemic-reperfusion injury⁹, G_i activation comes at the price of decreased contractility, which is problematic in diseases where the heart is already sufficiently weakened, such as in heart failure¹⁰, Takotsubo syndrome^{11,12}, and ischemia¹³. Notably, in heart failure G_{i2} is upregulated¹⁴⁻¹⁶, β_1 AR (strictly G_s coupled) is downregulated¹⁷⁻²⁰, while G_s ¹⁶, G_{i3} ¹⁶, and β_2 AR¹⁸ expression levels are unchanged. These changes may increase β_2 AR- G_{i2} coupling relative to the β_2 AR- G_s coupling.

The mechanism that initiates β_2 AR- G_i signaling in the healthy heart is not fully understood. Multiple biochemical mechanisms may play a role. PKA phosphorylation of β_2 AR has been reported to increase G_i coupling *in vitro*²¹ and in HEK cells²², but in cardiac myocytes, β_2 AR- G_i coupling is PKA independent³. In addition, GRK2 phosphorylation of β_2 AR has been suggested to increase G_i coupling²³, but other investigators have reported that dephosphorylation is critical for β_2 AR recycling to the plasma membrane, and β_2 AR- G_i interactions⁶. Therefore, we sought to discover mechanisms that modulate β_2 AR- G_i coupling.

The epinephrine-stimulated trafficking of β_2 AR (internalization and plasma membrane recycling) may influence the composition of phospholipids surrounding the β_2 AR. It has been shown that negatively charged phospholipids stabilize an active conformation of the β_2 AR and enhance its affinity for epinephrine²⁴. Here we examine the effect of phospholipid charge on β_2 AR interactions with G_s and G_i .

Results

Epinephrine activates β_2 AR by stabilizing a conformation that is recognized by G protein. This conformation is partially stabilized by epinephrine and fully stabilized by the addition of G protein²⁵⁻²⁷. The conformational change can be detected using a modified β_2 AR labeled on Cys265 at the cytoplasmic end of TM6 with an environmentally sensitive fluorophore, monobromobimane (mB- β_2 AR, see methods)²⁸. Epinephrine and G protein interaction red-shifts the emission maximum (λ_{max} , the wavelength where fluorophore emission intensity is greatest) and decreases the intensity of mB- β_2 AR (Fig. 1a). Because λ_{max} is independent of mB- β_2 AR concentration, it is a more reliable indicator of β_2 AR conformation than fluorescence intensity. We therefore monitored changes in λ_{max} of mB- β_2 AR to detect G protein coupling.

Negatively charged phospholipids decrease β_2 AR coupling to G_i

In the presence of epinephrine, we observe a change in intensity and $\lambda_{\max}$ of mB- β_2 AR following the addition of G_s in a detergent mixture containing n-dodecyl- β -D-maltopyranoside (DDM) and cholesteryl hemisuccinate (CHS) that is commonly used for biochemical study of GPCR/G protein complexes (Fig. 1a). In contrast, the coupling efficiency of mB- β_2 AR- G_{i3} was relatively weak (Fig. 1a). Next, we compared the coupling efficiency of mB- β_2 AR- G_i in DDM-CHS mixtures with different phospholipids incorporated (Fig. 1b,c). While we were unable to detect interactions of mB- β_2 AR with G_{i1} , G_{i2} or G_{i3} in the presence of negatively charged lipids (POPS and POPG), we observed a weak interaction with G_{i1} and G_{i3} in neutral lipids (POPE and POPC) (Fig. 1c,d). This result suggested that negatively charged lipids may repel G_{i1} and G_{i3} interaction with β_2 AR, despite the fact that negatively charged lipids enhance epinephrine binding affinity, as previously reported²⁴. Given that mB- β_2 AR- G_{i1} and mB- β_2 AR- G_{i3} interaction appeared comparable, we narrowed our focus on mB- β_2 AR- G_{i3} interaction because G_{i3} (not G_{i1}) is expressed in the heart¹⁶.

We also observed that negatively charged CHS decreased mB- β_2 AR- G_{i3} coupling (Supplementary Fig. 1). This effect was minimized in acidic buffers known to protonate (and neutralize) CHS²⁹, supporting our hypothesis that negatively charged lipids decrease β_2 AR- G_i coupling. Given reports that PKA phosphorylation of β_2 AR increases β_2 AR- G_i interaction *in vitro*²¹, we also tested the effect of PKA phosphorylation, but no mB- β_2 AR- G_{i3} enhancement was observed (Supplementary Fig. 2), suggesting that phosphorylation does not potentiate β_2 AR- G_{i3} interaction under our experimental conditions, and that other mechanisms may enhance β_2 AR- G_{i3} interaction.

In subsequent experiments, we omitted negatively charged CHS in order to assess the effect of phospholipid charge on mB- β_2 AR- G_{i3} interactions. We tested whether the increased mB- β_2 AR- G_{i3} interaction we observed in neutral lipid represented functional interaction. Indeed, β_2 AR stimulated GTP turnover was detected in DDM micelles containing POPE (net neutral lipid) but not in DDM micelles containing POPS (net negative) (Fig. 1e). This effect on β_2 AR mediated turnover was significant, even though the lipid:DDM molar ratio was only 1:4. The lipid environment (POPS vs. POPE) did not affect basal GTP turnover by G_{i3} (Supplementary Fig. 3). Taken together, these results indicate that the charge property of phospholipids regulates G_i activation by β_2 AR.

Ca²⁺ promotes β_2 AR- G_{i3} coupling in negatively charged phospholipids

Ca²⁺, a ubiquitous second messenger, plays an important role in cardiac myocytes; Ca²⁺ waves, magnified by G_s activation, drive the cardiac myocyte contraction machinery. Recently Ca²⁺ was reported to regulate T cell receptor activation by modulating the charge property of lipids³⁰. Given that Ca²⁺ interaction with negatively charged phospholipids screens the negative charge, we tested whether Ca²⁺ improves mB- β_2 AR- G_{i3} coupling efficiency in negatively charged DDM-POPS. Indeed, Ca²⁺ improved coupling efficiency in DDM-POPS micelles (Fig. 2a), and this effect required POPS (Fig. 2b). Moreover, Ca²⁺ had little effect on mB- β_2 AR- G_s interaction, implicating differences in G_s and G_{i3} surface charge.

Ca^{2+} interacts with the amino terminal helix of G_{13}

Next, we sought to determine the mechanism by which Ca^{2+} -POPS interactions increase mB- $\beta_2\text{AR}$ coupling to G_{13} but not to G_s . Given that the amino terminal helix (αN) of G protein is adjacent to the membrane when coupled to the $\beta_2\text{AR}$ ²⁷, and polybasic residues

effect of Ca^{2+} on G_{i3} αN . Taken together, our results imply that Ca^{2+} coordinates an interaction between the negatively charged EDGE motif on αN of G_{i3} and the headgroup of POPS. In the absence of Ca^{2+} , like-charge repulsion decreases mB- $\beta_2\text{AR}$ coupling to G_{i3} .

We also constructed a G_{i3} mutant (“ $\text{G}_{i3}\text{-pos.}$ ”) containing the positively charged motif of G_s (EDGE $\rightarrow$ KDKQ). The mutations only partially removed the effect of Ca^{2+} (Fig. 3b), indicating the effect of Ca^{2+} on G_{i3} extends beyond an effect on αN (see discussion).

Bilayer charge is a tunable modulator of the G protein subtype selectivity

To examine the effects of phospholipids in a more native environment, we reconstituted mB- $\beta_2\text{AR}$ into nanodisc bilayers and purified the nanodiscs to homogeneity using size-exclusion chromatography (Supplementary Fig. 4).

First, we compared the influence of lipid composition in the absence of Ca^{2+} . Negatively charged bilayers (DOPG and DOPS bilayers) red-shifted the emission spectra of mB- $\beta_2\text{AR}$ alone, suggesting negatively charged bilayers stabilize mB- $\beta_2\text{AR}$ in an active conformation, as has been previously reported²⁴. While negatively charged bilayers increased mB- $\beta_2\text{AR}$ coupling to G_s (Fig. 4), negatively charged bilayers (especially DOPS bilayers) decreased mB- $\beta_2\text{AR}$ coupling to G_{i3} (Fig. 4).

In fact, in negatively charged bilayers without epinephrine, G₁₃ unexpectedly *blue-shifted* the emission spectra of mB-β₂AR. While this may indicate that G₁₃ stabilizes the β₂AR in an inactive conformation in negatively charged lipids, it may represent a non-specific interaction of inactive G₁₃ with the β₂AR or the lipid bilayer.

Next we examined the effect of Ca²⁺ and Mg²⁺. In the absence of G₁₃, both Ca²⁺ and Mg²⁺ reversed the active-state stabilizing effect of negatively charged DOPS and DOPG bilayers (Fig. 5a). In contrast, Ca²⁺ and Mg²⁺ increased mB-β₂AR coupling to G₁₃ in negatively charged DOPS bilayers, but only Ca²⁺ was efficacious at concentrations below 1 mM (Fig. 5a). Ca²⁺ similarly affected mB-β₂AR-G₁₃ interaction in negatively charged DOPG bilayers (Fig. 5a), but the magnitude of the effect in DOPG bilayers was less than observed in DOPS bilayers due to the higher baseline effect of DOPG on β₂AR conformation.

We compared the effect of Ca²⁺ on mB-β₂AR interactions with G_s and G_{i3} in DOPS bilayers. As observed in micelles, Ca²⁺ increased mB-β₂AR coupling to G_{i3} but not to G_s (Fig. 5b). Ca²⁺ also improved mB-β₂AR-G_{i3} coupling efficiency in negatively charged DOPG bilayers (Fig. 5c). Only a minor effect of Ca²⁺ was observed in neutral DOPE and

DOPC bilayers (Fig. 5c & Supplementary Fig. 5), which could be attributable to weaker Ca^{2+} /DOPE and Ca^{2+} /DOPC interactions that have been reported³². Taken together, our results strongly suggest that Ca^{2+} facilitates mB- $\beta_2\text{AR}$ - G_{i3} interaction but not mB- $\beta_2\text{AR}$ - G_s interaction. Moreover, these observations provide biochemical proof-of-concept that divalent cation/membrane interactions can increase G_i coupling to the $\beta_2\text{AR}$.

Discussion

We observed that local membrane charge regulates β_2 AR-G protein interaction.

Negatively charged membrane promotes β_2 AR- G_s coupling and suppresses β_2 AR- G_{i3} coupling. However, G_s bias is reduced in neutral membrane and in negatively charged membrane in the presence of divalent cations (see model in Fig. 6).

We have begun to explore the mechanism by which Ca^{2+} increases mB- β_2 AR coupling to G_{i3} in PS phospholipids. Although G proteins are membrane tethered via lipidation, the lipid anchor of G_{i3} is not sufficient for optimal interaction with β_2 AR in negatively charged bilayers, possibly due to repulsion of the carboxyl terminal end of the αN helix. We propose that Ca^{2+} helps orient the carboxyl terminal end of the αN helix of G_{i3} near the membrane, thereby facilitating β_2 AR- G_{i3} interactions. More specifically, we propose that Ca^{2+} facilitates the interaction of PS with the negatively charged EDGE motif on the αN helix of G_{i3} . Ca^{2+} may stabilize the PS-EDGE interaction by coordinating a like-charge interaction between the carboxylate groups on G_{i3} and the carboxylate group on PS (not present on PG) (refer to structures in Fig 1b). Alternatively, Ca^{2+} might coordinate a like-charge interaction between the carboxylate groups on G_{i3} and the phosphate group present on PS and PG, or Ca^{2+} might coordinate an intramolecular interaction between the phosphate group and the carboxylate group on PS, “freeing” the amino group (NH_3^+) on PS to interact with the carboxylate groups on G_{i3} . Notably, the amino terminal helix (αN) of G_{i1} , G_{i2} , and G_{i3} are similarly charged, and all three sequences contain the EDGE motif.

We have previously shown that negatively charged lipids, particularly PG, stabilize the β_2 AR in an active-like conformation as revealed by changes in mB- β_2 AR fluorescence and an increased affinity for agonists²⁴. These effects are likely due to interactions between the lipids and positively charged amino acids on the β_2 AR. Here we observed that the effect of DOPG and DOPS on mB- β_2 AR can be reversed by both Ca^{2+} and Mg^{2+} (Fig. 5a). Yet, these divalent cations do not appear to reduce coupling to G_s .

β_2 AR signals from caveolin-rich rafts^{33,34} within T-tubules³⁵. While β_2 AR preferentially interacts with PG in insect cell membrane²⁴, the phospholipid composition immediately adjacent to β_2 AR in T-tubules, and how it changes during β_2 AR trafficking, is currently unknown. Net-neutral PC and PE are the major phospholipids in T-Tubules³⁶⁻³⁸. However, negatively charged PS is enriched in T-tubules relative to other membrane fractions (7.5-12.3% of total phospholipid)³⁶⁻⁴⁰. While cytosolic Ca^{2+} concentrations are typically less than 1 mM⁴¹, concentrations of Ca^{2+} in the mM range may be observed in cardiac myocytes (discussed below).

Investigators have long speculated about the functional role of Ca^{2+} in the cleft between T-tubule membrane (where β_2 AR is localized) and juxtaposed sarcoplasmic reticulum (SR)⁴²⁻⁴⁴. During each action potential, extracellular Ca^{2+} flows into the cleft through L-type Ca^{2+} channels (LTCCs) on the plasma membrane and through ryanodine receptors (RyRs) on the sarcoplasmic reticulum⁴¹. Cleft Ca^{2+} concentrations spark to > 100 μM in the absence of epinephrine and >1 mM^{45,46} following epinephrine stimulation, a consequence of G_s activation. Computational models show that negatively charged phospholipids buffer approximately half the Ca^{2+} released into the cleft⁴⁵, and experiments have shown that 80% of inner-leaflet bound Ca^{2+} is bound to negatively-

charged phospholipids⁴⁷. Additionally, biochemical investigations show that Ca^{2+} can cluster negatively charged PS⁴⁸ and PIP2^{49,50} lipids.

$\beta_1\text{AR}$ and $\beta_2\text{AR}$ signaling through G_s alters calcium handling in the cardiac myocyte, and increases the magnitude of Ca^{2+} currents and Ca^{2+} transients, which stimulate cardiac contraction^{41,51}. However, elevated Ca^{2+} concentrations also activate the Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII), which is implicated in structural remodeling that ultimately results in cardiac dysfunction⁵²⁻⁵⁶. Several lines of evidence suggest $\beta_2\text{AR-G}_i$ signaling keeps $\beta_2\text{AR-G}_s$ signaling in check via negative feedback: $\beta_2\text{AR-G}_i$ signaling occurs minutes after $\beta_2\text{AR-G}_s$ signaling³, $\beta_2\text{AR-G}_i$ signaling suppresses changes in calcium handling^{51,57}, and $\beta_2\text{AR-G}_i$ signaling is anti-apoptotic^{7,8}. While the mechanism that triggers $\beta_2\text{AR-G}_i$ signaling is unknown, our biochemical observations suggest Ca^{2+} concentrations could directly regulate $\beta_2\text{AR}$ coupling to G_i . It is notable that overexpression of the Ca^{2+} /sodium exchanger facilitates $\beta_2\text{AR-G}_i$ suppression of $\beta_1\text{AR-G}_s$ signaling⁵⁸, and overexpression has been cited to increase the inward LTCC Ca^{2+} current⁵⁹.

It is also notable that intracellular Ca^{2+} ^{60,61} and Mg^{2+} ⁶¹ concentrations rise during ischemia and rise even higher during reperfusion. Whether the rising concentrations affect the G protein subtype specificity of $\beta_2\text{AR}$ is unknown. However, laboratory-controlled bouts of ischemia and reperfusion have been cited to evoke therapeutic $\beta_2\text{AR-G}_i$ signaling, i.e. reduce necrosis during a heart attack⁹.

Whether negatively charged phospholipids affect G_i interaction with other G_i -coupled GPCRs is not known. The observation that Ca^{2+} sensing receptor (CaSR) switches from G_q to G_i after cytosolic Ca^{2+} increases⁶² is potentially relevant to our findings.

In conclusion, we show that local membrane charge differentially modulates β_2 AR interaction with competing G protein subtypes (G_s and G_i). This discovery expands our knowledge of mechanisms that regulate the G protein coupling selectivity of GPCRs.

Online Methods

Heterotrimeric G protein and β_2 AR purification. All G proteins were heterotrimeric G proteins. G_s heterotrimer (WT $G_{\alpha_s \text{ short}}$, his6-3C- β_1 , WT γ_2) and G_i heterotrimer (WT $G_{\alpha_{i1-3}}$, his6-3C- β_1 , WT γ_2) and all G protein mutants were expressed and purified as previously described⁶³. The G_{i3} - G_s chimera was constructed by replacing residues 1-38 of WT G_{α_s} with residues 1-31 of WT $G_{\alpha_{i3}}$. The G_s -neg. mutant was constructed by replacing the sequence EDGE (residues 25-28) of WT G_{α_s} with the sequence KDKQ. The G_{i3} -pos. mutant was constructed by replacing the sequence KDKQ (residues 32-35) of WT $G_{\alpha_{i3}}$ to the sequence EDGE. The β_2 AR construct was PN1. The construct, expression, purification, and monobromobimane labeling have been described²⁴. Labeling efficiency was ~80-100%, as determined by spectroscopic analysis. Purified β_2 AR and G protein were dephosphorylated using Lambda Protein Phosphatase (Lambda PP, New England BioLabs, NEB). Where indicated, β_2 AR was phosphorylated with Protein Kinase A (PKA, NEB) in purification buffer supplemented with 0.1 mM EDTA and 20 μ M ATP. (Subsequently, EDTA/ATP were removed by dialysis). Phosphorylation was assessed using the Pro-Q Diamond Phosphoprotein Gel Stain (ThermoFisher Scientific), per the manufacturer's instructions.

Micelle Composition. n-dodecyl- β -D-maltopyranoside (DDM), Cholesteryl hemisuccinate (CHS), and 1-palmitoyl-2-oleoyl-(PE,PC,PG,PS) lipids (Avanti Polar Lipids) were mixed in the indicated ratios and solubilized in chloroform. Chloroform was evaporated, and films were re-suspended in 20 mM HEPES (pH 7.4), 100 mM NaCl.

Fluorescence Spectroscopy. In experiments examining β_2 AR in micelles, mB- β_2 AR was pre-incubated (30 min room temperature) in micelle stock prior to dilution with other reaction components. Buffer (containing 100 mM NaCl, +/- ligand, +/- CaCl₂ or MgCl₂), and G protein were sequentially included. Mixtures were incubated 2.5 – 3.0 h at room temperature. Final mB- β_2 AR concentration was 100-500 nM. Emission spectra were read at 22 degrees Celsius using Flurolog-3 or Spex FluoroMax-3 spectrofluorometers (Horiba Jobin Yvon Inc.). (Bandpass = 4 nm; Excitation = 370 nm; Emission = 420-500 nm.) Raw S1c/R1c spectra were smoothed using Prism (GraphPad Software) (n=15 neighbors, 2nd order polynomial). Lambda max is defined as the wavelength at which fluorescence emission is maximum. To determine the EC₅₀, curves were fit to the “agonist vs. response” model in Prism 7.0d software.

GTP Turnover. Samples were prepared as they were for fluorescence spectroscopy. Following the incubation with G protein, 1 μ M GTP/5 μ M GDP mixtures were added. Reactions contained: 20 mM HEPES (pH 7.4), 100 mM NaCl, 0.25 μ M G_{i3}, 0.5 or 1.0 μ M β_2 AR (per legend), 200 μ M epinephrine, and 4:1 DDM:Lipid. 12 minutes after GTP was added, free GTP was assessed using the GTPase-Glo assay (Promega), which reports free GTP concentration using a luminescence readout. Luminescence was detected using a SpectraMax Paradigm plate reader equipped with a TUNE SpectraMax detection

cartridge (Molecular Devices). Background luminescence was subtracted from experimental reactions.

Statistics. Two-sided parametric paired student's *t*-tests were performed using Graphpad Prism7.0d. When comparing lambda max +/- CaCl₂, *t*-test calculation assumed all data within a panel were sampled from populations with the same scatter.

Electrostatic Modeling: Structural views and mutant models were generated using PyMOL (Schrödinger, LLC). We selected rotamer positions that most closely matched those seen in PDB 3SN6 (for "G₁₃-pos." model) and PDB 1GP2 (for "G_s-neg." model). Continuum electrostatics models were calculated using the APBS⁶⁴ plugin (MG Lerner, University of Michigan, Ann Arbor) for PyMOL. Atomic charge and radii were calculated using the online PDB2PQR server⁶⁵ (pH 7.4, PARSE force field, hydrogen bond optimization, clash avoidance).

Nanodisc Reagents. 1,2-dioleoyl-(PE,PC,PG,PS) lipids (Avanti Polar Lipids) were used because of their low phase transition temperature. MSP1E3D1 (Addgene #20066) was expressed and purified as described⁶⁶.

Nanodisc Reconstitution: Reconstitution was performed as described²⁴ with the following modifications: Nanodiscs were formed with one lipid type (i.e. 100% DOPS, DOPG, DOPE, or DOPC). The Lipid-to-MSP1E3D1 ratio was 35:1. The MSP1E3D1-to-mB-β₂AR ratio was 1:10. Empty nanodiscs were separated from nanodiscs containing mB-β₂AR using M1-anti-FLAG immunoaffinity chromatography in the presence of 2 mM CaCl₂, which captures the FLAG-tagged PN1 mB-β₂AR construct. Eluate was incubated with 5 mM EDTA > 1.5 h at 4 degrees Celsius to remove divalent cations. Subsequently,

samples were injected into a Superdex 200 10/300GL size-exclusion column (GE Healthcare) and the main peak was harvested. The concentration of nanodisc β_2 AR was approximated by SDS-PAGE, using detergent purified β_2 AR of known concentration as a reference.

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Author contributions

M.J.S designed, performed, and interpreted the research, and wrote the manuscript. B.K.K. championed the investigation, advised on the project, and edited the manuscript. M.J.S performed the purification, labeling, nanodisc reconstitution, and all of the experiments, and S.M., D.H., M.M., and Y.D. provided valuable technical assistance as described: S.M. advised on cloning and provided G protein for pilot experiments. D.H. advised on G protein purification and the GTP turnover assay. M.M. advised on nanodisc reconstitution and provided MSP1E3D1 protein. Y.D. collaborated on pilot experiments not included.

Competing Interests Statement

None declared

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