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The lipid phase preference of the adenosine A_{2A} receptor depends on its ligand binding state

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Abstract

Giant unilamellar protein vesicles (GUPs) were formed with the adenosine A_{2A} receptor (A_{2A}R) incorporated in the lipid bilayer and observed protein partitioning into liquid ordered and liquid disordered phases. When no ligand is bound, A_{2A}R partitions preferentially into the liquid disordered phase of GUPs, while ligand-bound A_{2A}R partitions into the liquid ordered phase.

Lipid rafts are hypothesized areas on the cell plasma membrane where cholesterol, sphingolipids, and proteins may aggregate in a manner that modulates cellular signaling.^[1–4] In recent decades, lipid rafts have been modeled extensively using phase-separating giant unilamellar vesicles (GUVs).^[5, 6] GUVs allow for stringent control of membrane composition and direct observation of micron-scale lipid phase separation that mimics the hypothetical rafts.^[4, 6, 7] GUVs of certain ternary lipid compositions can undergo liquid-liquid (l-l) phase separation where the liquid ordered (l_o) phase (analogous to lipid rafts) is rich in cholesterol, saturated lipids and sphingolipids, and the liquid disordered phase (l_d) is enriched in unsaturated lipids.^[8, 9] Protein incorporated GUVs, known as giant unilamellar protein-vesicles (GUPs), offer a means to directly observe protein partitioning in phase-separating GUP compositions. Our group has previously reported on the partitioning of spinach aquaporin SoPIP2;1 and the human serotonin receptor 5-HT_{1A}R in phase-separating GUPs.^[10, 11] Here we report on the phase-separating behavior of the G protein-coupled receptor (GPCR) adenosine A_{2A} receptor (A_{2A}R) in GUPs and observe changes in its partitioning due to ligand binding.

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Conflicts of interest

There are no conflicts to declare

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GPCRs are the largest family of proteins in the human genome and are the target of over half of medical therapies on the market.^[12] They participate in intracellular signaling cascades and are implicated in behavioral and psychiatric disorders. In particular, A_{2A}R is implicated in addiction, in respiratory diseases such as chronic obstructive pulmonary disease (COPD) and is a target for Parkinson's disease therapies.^[13, 14] Furthermore since A_{2A}R participates in immunological feedback loops, this receptor is being targeted for anti-tumor immunotherapies.^[15] Assisted by the growing number of structural studies on ligand-bound A_{2A}R, novel drug candidates are being developed and it has been shown that A_{2A}R has a cholesterol consensus motif.^[16, 17] Biochemical analysis and crystal structures of A_{2A}R and other GPCRs have revealed significant changes in helix conformation depending on binding partners including ligands, G proteins, ions, and lipids.^[18–23] Despite this, studies regarding the biophysical interactions of A_{2A}R and the lipid bilayer of the plasma membrane remain scant.^[24, 25] To better understand the biophysics of A_{2A}R phase partitioning, we incorporated A_{2A}R in phase separating vesicles made of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and cholesterol (chol) using a hydrogel-assisted method.^[10, 11] This work focuses on a single membrane protein, A_{2A}R, and uniquely addresses lipid-protein interactions. We investigated ligand-bound and ligand-unbound A_{2A}R in purified and crude form and observed that A_{2A}R not bound to ligand partitioned to the l_d phase while agonist- or antagonist-bound A_{2A}R partitioned into the l_o phase. Further we observed that ligand-bound A_{2A}R protein concentration in the l_o phase was unaffected by the area of l_o domains (A_o) in GUPS.

Two forms of A_{2A}R were used in our investigations: purified protein (pA_{2A}R) expressed in Sf9 insect cells and protein from crude membranes fragments (cA_{2A}R) expressed in HEK-293 cells and purchased from Perkin Elmer (see SI).^[18] Throughout this work, agonist-bound protein will be identified with (Ag) and antagonist-bound protein will be identified with (An). Protein purified from Sf9 cells (as described in the SI) was solubilized in a 0.01%/0.002% dodecyl-β-D-maltoside (DDM)/ cholesterol hemisuccinate (CHS), 25 mM HEPES buffer at pH 7.5 and then subsequently bound to 100μM ZM241385 antagonist (pA_{2A}R(An)). pA_{2A}R purity were determined using analytical aSEC, and proper protein incorporation in GUPS was confirmed by radioligand binding (Figure S1). Unbound apo cA_{2A}R, agonist bound (cA_{2A}R(Ag), final concentration N-ethyl-carboxyadenosine (NECA) 100μM) and antagonist bound (cA_{2A}R(An), final concentration ZM241385 100μM) were used to identify ligand effects on protein partitioning. Both forms of A_{2A}R were tagged using a rhodamine-labeled monoclonal antibody prior to incorporation in unilamellar GUPS for observations (fluorescence intensity controls, phase partitioning, antibody binding controls and apo and ligand controls and other controls are shown in Figure S2, Figure S3, Figure S4, Figure S5, Figure S6, Figure S7 and Table S1 and Table S2). Antibody-labeled A_{2A}R was incorporated in GUPS made from lipid compositions at various DOPC:DPPC:chol ratios (Table S2). All compositions are known to phase separate into l_o and l_d phases.^[8] In all cases, the protein:lipid molar ratio was ~1:155 (See SI). The concentration of chol was increased over several GUP trials to identify l_o and l_d areas; since l_o regions are chol-rich, the total l_o area will increase with increasing chol concentration. GUPS were formed using the agarose hydration method for protein incorporation as described in the SI. Upon formation, GUPS were harvested by gentle pipetting, settled in an

isosmotic glucose buffer and then transferred to observation chambers for viewing using confocal microscopy and subsequently analyzed (Figure S8). Vesicles were anchored to glass microscope slides via biotinylation for ease of imaging (see SI).

In all cases, the antibody-labeled protein segregated preferentially to certain regions of the GUP, indicating that it was participating in phase separation (See Figure 1). The only dye in this system was conjugated to the A_{2A}R antibody. To identify the l_o and l_d phases of GUPs with pA_{2A}R(An), apo cA_{2A}R, cA_{2A}R(An), and cA_{2A}R(Ag), we quantified the ratio of bright area versus dark area in Z-stack projections of GUP confocal images while varying chol concentration (see SI Figure S3 for analysis). DOPC:DPPC:chol GUPs with concentrations of chol from 20 to 40 mol% were prepared. In GUPs containing A_{2A}R bound to ligand, the bright areas increase with increasing chol content while in those containing apo cA_{2A}R the bright areas decrease with increasing chol (Figure 1). The micrographs in Figure 1 show phase separating GUPs with varying amounts of chol. Since chol concentration positively correlates with percent area of the l_o phase (A_o) we concluded that ligand-bound pA_{2A}R(An), cA_{2A}R(An), and cA_{2A}R(Ag) partitions to the l_o phase while ligand-unbound cA_{2A}R partitions to the l_d phase. Furthermore, protein partitioning is independent of CHS or DDM because cA_{2A}R solubilized in either CHS or CHS/DDM (similar conditions to pA_{2A}R) consistently partitions to the l_d phase (Figure S9 and Figure S10).

Ligand-bound A_{2A}R partitioning to the l_o phase of phase-separating mixtures of GUPs could be due to changes in protein conformation or dynamics when ligand is bound to the GPCR. While previous reports on GPCR protein partitioning have prominently shown partitioning into the l_d phase of phase separating lipid bilayers,^[10, 11] some results have suggested that ligand binding with GPCRs causes co-segregation into lipid rafts.^[26, 27] For example, in investigations isolating caveolae (which tend to associate with raft-like lipid compositions) from plasma membranes, subfractionation of caveolae and immunofluorescence have shown the G protein subunits G_{ai} and G_{as} in these lipid rafts,^[28] and the adenosine A1A GPCR has been shown to move into and out of these lipid rafts.^[29] The lipid raft hypothesis suggests that proteins and signaling components aggregate into dynamic lipid rafts to strengthen signaling pathways and to avoid signaling protein cross-talk and degradation.^[27, 30] While protein behavior in a cell plasma membrane will depend on protein-protein and protein-cytoskeleton interactions, GPCRs are known to undergo conformational changes during ligand binding and activation that change the receptor's microenvironment in the plasma membrane.^[31–33] Our results suggest and support theories on the dynamic aggregation of protein signaling components into cellular lipid rafts, which may be triggered by agonist binding.

GPCR functionality and membrane elasticity also support liquid ordered phase partitioning of A_{2A}R. Most of this research has been done on rhodopsin and Garwisch et al. showed that rhodopsin could induce lipid order by hydrophilic matching phenomenon,^[34] and this was further supported by MD simulations performed by Grossfield and coworkers.^[35] Further research by Garwisch and coworkers has shown that elastic curvature stress of the lipid bilayer favors the active meta-II state of rhodopsin.^[36, 37] A similar effect was observed by Brown and coworkers, who argued that the conformation energetics of the protein are linked to the continuum mechanical properties of the lipid bilayer.^[38]

We have also observed that 5-HT_{1A}R, is more active in ordered membranes.^[39] Therefore it is not surprising that ligand-bound A_{2A}R is observed here to partition into ordered domains in phase separating membranes.

To further assess protein partitioning, a partition coefficient K was calculated: $K = i_o/i_d$ where i_o is the fluorescence intensity (a.u.) of the l_o area and i_d is the fluorescence intensity (a.u.) of the l_d area. As shown in Figure 2, K plotted against A_o has a negative slope for all samples. As A_o increased, the ratio of i_o/i_d decreased, indicating a decrease in the fluorescence intensity of the l_o area (Figure 2). Furthermore for all ligand-bound samples, K values were greater than 1, whereas for ligand-unbound apo cA_{2A}R, the K values were less than 1. The observed K values for ligand-unbound cA_{2A}R is due to its opposite partitioning behavior. Without ligand bound, the protein partitions into the liquid disordered phase as described above and in the SI (SI control images). K values for ligand-unbound crude protein controls in 0.01% CHS or 0.01%/0.002% CHS/DDM are shown in Figure S10 and have the same trends as cA_{2A}R in Fig 2. These results show that ligand-bound protein preference for the l_o phase decreases as chol concentration increases in GUPs and because the trends in Figure 2 do not cross $y=1$ suggests that GPCR partitioning is independent of the size of the l_o phase, A_o . In serotonin, rhodopsin, and β -adrenergic receptors, chol modulation and binding affects the orientation and population of receptor oligomers.^[40–42] Thus spatial arrangement of GPCRs, including dimerization and oligomerization, in the plasma membrane may be mediated by lipid interactions. The decrease observed in K with increasing chol concentration may be related to such spatial organizational effects. Note that this effect is least pronounced (to the point of being nearly unobservable) for the cA_{2A}R(An) sample. This could be due to interactions with endogenous lipids introduced in the crude membrane fragments.

To demonstrate that membrane-incorporated A_{2A}R is biochemically active, we measured the activity of A_{2A}R incorporated from crude membrane fragments into GUPs. The activity assay was performed as described in Gutierrez et al 2016,^[39] where GUPs were formed to encapsulate BODIPY-GTP γ S. Upon formation and settling, GUPs were incubated with the agonist NECA and fluorescent intensity increase due to receptor-catalyzed BODIPY-GTP γ S binding to the G α subunit of the G protein was tracked over time via microplate reader (Figure 3). This experiment was performed in GUPs made from two ratios of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) to chol. The protein is active at both chol concentrations, but receptor-catalyzed nucleotide exchange is faster at the higher chol concentration. The fact that the receptor is capable of catalyzing GDP-GTP exchange subsequent to reconstitution in GUPs demonstrates that the phase segregation phenomenon reported here reflects the behavior of a correctly folded, biologically active protein.

The results here show that the behavior of the GPCR A_{2A}R depends on lipid composition. Effects of lipid composition on GPCR function have been reported for rhodopsin, β_2 -adrenergic receptors, and serotonin receptors.^[1, 22, 34, 36, 39, 43] For example, increases in chol concentration in lipid bilayers have been reported to increase the functional activity of the serotonin receptor 5-HT_{1A}R and initial work on the A_{2A}R receptor shows similar behavior (Figure S11).^[39] Differences in lipid bilayer thickness, ordering, geometry, and pressure differences between l_o and l_d phases have also been shown to alter the activity of

rhodopsin.^[34, 36, 37] Differential partitioning into lipid regions with different bulk properties may therefore modulate the functional activity of A_{2A}R. This is in addition to the potential of phase segregation to promote the aggregation of signaling components and regulate GPCR degradation and activity.^[1, 43] Apart from signaling mechanisms linked to putative raft localization, the results here support the idea that lipid ordering preferentially supports ligand-bound configurations of A_{2A}R.

Phase separating compositions of GUPs incorporated with purified and crude GPCR A_{2A}R display I_o partitioning when ligand is bound and I_d partitioning when unbound. Preferential partitioning of ligand-bound receptor supports the hypotheses on transient dynamic lipid raft assemblies in the plasma membrane that aggregate for protein signaling and regulation. The interplay between ligand-mediated alterations in protein geometry and preferential location to membrane subcompartments may represent an important signal control mechanism for GPCRs and membrane proteins in general.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A) GUPs with incorporated A_{2A}R show protein segregation to certain regions of vesicles. The top set of micrographs shows pA_{2A}R(An) tagged with rhodamine-labeled antibody. The bottom set of micrographs shows apo cA_{2A}R tagged with rhodamine-labeled antibody. For pA_{2A}R(An), as mol% chol increases, the bright area of the GUP increases, while for cA_{2A}R, as mol% chol increase, the dark area of the GUP increases. All scale bars are 5 μm. B) %Light area of GUP versus mol% of chol. On average 8 GUPs were analyzed per sample with each experiment being repeated 4 times. The error bars indicate standard error of the mean. In rhodamine-labeled antibody-tagged GUP samples with ligand-bound A_{2A}R, %light area increases with increasing mole percent chol. However, in unbound protein, cA_{2A}R, %light area decreases with increasing mole percent chol. This shows a protein partitioning dependence on ligand binding state.

Figure 2.

Partition coefficient (K) versus area of liquid ordered domain. All proteins investigated, bound and unbound, have a negative slope. The shaded areas around the lines show the goodness of fit.

Figure 3.

Fluorescence intensity change as a function of time for $A_{2A}R$ -incorporating GUPs incubated with a fluorescent GTP analog and stimulated by agonist. The $A_{2A}R$ was reconstituted from crude membrane fragments which also include the relevant G protein. The experiment was performed at two chol concentrations; higher activity was observed at higher chol concentration. These data show single exponential rates and the standard error mean of 6 replicates at each chol concentration.