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

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Giant unilamellar protein vesicles (GUPs) were formed with the adenosine A2A 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.\cite{1-4} In recent decades, lipid rafts have been modeled extensively using phase-separating giant unilamellar vesicles (GUVs).\cite{5, 6} GUVs allow for stringent control of membrane composition and direct observation of micron-scale lipid phase separation that mimics the hypothetical rafts.\cite{5, 6} Giant unilamellar protein vesicles (GUPs),\cite{10, 11} 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.\cite{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.

GPCRs are the largest family of proteins in the human genome and are the target of over half of medical therapies on the market.\cite{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.\cite{13, 14} Furthermore since A_{2A}R participates in immunological feedback loops, this receptor is being targeted for anti-tumor immunotherapies.\cite{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.\cite{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.\cite{18-23} Despite this, studies regarding the biophysical interactions of A_{2A}R and the lipid bilayer of the plasma membrane remain scant.\cite{14, 24} 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-di-palmitoyl-sn-glycero-3-phosphocholine (DPPC) and cholesterol (cholesterol) using a hydrogel-assisted method.\cite{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_0 phase while agonist- or antagonist-bound A_{2A}R partitioned into the l_0 phase. Further we observed that ligand-bound A_{2A}R protein concentration in the l_0 phase was unaffected by the area of l_0 domains (A_{l_0}) 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).\cite{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 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 100 µM ZM241385 antagonist (pA<sub>2A</sub>R(An)). pA<sub>2A</sub>R purity were determined using analytical aSEC, and proper protein incorporation in GUPs was confirmed by radioligand binding (Figure S1). Unbound apo cA<sub>2A</sub>R, agonist bound (cA<sub>2A</sub>R(Ag), final concentration N-ethylcarboxyadenosine (NECA) 100 µM) and antagonist bound (cA<sub>2A</sub>R(An), final concentration ZM241385 100 µM) were used to identify ligand effects on protein partitioning. Both forms of A<sub>2A</sub>R were tagged using a rhodamine-labeled monoclonal antibody prior to incorporation in unilamellar GUPs for observations (fluorescence intensity controls, phase partitioning, cantibody 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<sub>2A</sub>R were made in GUPs made from lipid compositions at various DOPC:DPPC:chol ratios (Table S2). All compositions are known to phase separate into l<sub>o</sub> and l<sub>d</sub> phases. In all cases, the protein:lipid molar ratio was ~1:155 (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<sub>2A</sub>R antibody. To identify the l<sub>o</sub> and l<sub>d</sub> phases of GUPs with pA<sub>2A</sub>R(An), apo cA<sub>2A</sub>R, cA<sub>2A</sub>R(An), and cA<sub>2A</sub>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<sub>2A</sub>R bound to ligand, the bright areas increase with increasing chol content while in those containing apo cA<sub>2A</sub>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<sub>o</sub> phase (A<sub>o</sub>) we concluded that ligand-bound pA<sub>2A</sub>R(An), cA<sub>2A</sub>R(An), and cA<sub>2A</sub>R(Ag) partitions to the l<sub>o</sub> phase while ligand-unbound cA<sub>2A</sub>R partitions to the l<sub>d</sub> phase. Furthermore, protein

![Figure 1](image-url)

**Figure 1.** A) GUPs with incorporated A<sub>2A</sub>R show protein segregation to certain regions of vesicles. The top set of micrographs shows pA<sub>2A</sub>R(An) tagged with rhodamine-labeled antibody. The bottom set of micrographs shows apo cA<sub>2A</sub>R tagged with rhodamine-labeled antibody. For pA<sub>2A</sub>R(An), as mol% chol increases, the bright area of the GUP increases, while for cA<sub>2A</sub>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<sub>2A</sub>R, %light area increases with increasing mole percent chol. However, in unbound protein, cA<sub>2A</sub>R, %light area decreases with increasing mole percent chol. This shows a protein partitioning dependence on ligand binding state.
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 in the plasma membrane, subfractionation of caveolae and Gαs in these lipid rafts, and the adenosine A1A GPCR have prominently shown partitioning into the liquid disordered phase as described above and in the SI (SI Figure S10 and have the same trends as cA_{2A}R). Our results suggest and support theories on 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 Gαi and Gαs 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 hydrophobic 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: \( K = \frac{i_o}{i_d} \) 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 \( \beta \)-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,[19] where GUPs were formed to encapsulate BODIPY-GTPyS. Upon formation and settling, GUPs were incubated with the agonist NECA and fluorescent intensity increase due to receptor-catalyzed BODIPY-GTPyS binding to the $\alpha_3$ 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, $\beta_2$-adrenergic receptors, and serotonin receptors.[1, 2, 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$_2A$ 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 $l_p$ 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 $l_o$ partitioning when ligand is bound and $l_p$ 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.

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Conflicts of interest
There are no conflicts to declare

Notes and references
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As cholesterol fraction increases, ligand-bound receptor occupies more vesicle surface area, demonstrating co-localization with the cholesterol-rich phase.