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

M. Gertrude Gutierrez , Jacob Deyell, Kate White, Lucia C. Dalle Ore, Vadim Cherezov, Raymond C. Stevens, and Noah Malmstadt

Abstract: 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.

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Experimental Procedures

Materials

DOPC, DPPC, cholesterol, and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-(cap biotinyl) were acquired from Avanti Polar Lipids. All reagents such as, but not limited to, low melt-temperature agarose, phosphate buffered saline (PBS), dimethyl sulfoxide (DMSO), chloroform (CHCl₃), methanol (MeOH), sucrose, glucose, ZM, and avidin were of analytical grade from Sigma Aldrich, USA. Crude membrane fragments containing A_{2A}R or 5-HT_{1A}R (Perkin Elmer, USA), A_{2A}R monoclonal antibodies (Millipore, USA), ZM2413185 (Sigma Aldrich), NECA (Tocris, UK), ATTO-488-DPPE (Sigma Alrich, USA) and [³H] ZM241385 (American Radiolabelled Chemicals, Inc.) were used without further purification. Sykes-Moore chambers (Bellco, USA), standard 25 mm no. 1 glass coverslips (ChemGlass, USA), and flat bottom 96-well plates (BD Biosciences, USA) were used throughout all experiments. 18.2 MΩ-cm Milli-Q water was used in all experiments (EMD Millipore, USA). Protein desalting micro spin columns (Thermo Scientific, USA) and NHS-rhodamine (Thermo Scientific, USA) were used as per the manufacturer's instructions. BODIPY-GTPγS and QSY7 were obtained from Life Technologies, USA and used as directed by manufacturer's instructions. NHS-rhodamine was dissolved in DMSO at 25 mg/ml. Solutions of ligands were made to 10 mM in DMSO.

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A_{2A}R Crude Membrane Preparations (cA_{2a}R)

Adenosine A_{2A} , GPCR A_{2A} R, crude membrane preparations were from Perkin Elmer. Product number RBHA2AM400UA. Lot number 1869208. The same lot was used throughout all experiments presented in this work. According to the manufacturer, the membrane preparations are from HEK293-EBNA cell lines and are suspended in a storage buffer consisting of 50 mM Tris-HCI (pH 7.4), 0.5 mM EDTA, 10 mM MgCl₂, 10% sucrose with no additional surfactant. The cell line has been stably transfected to express the receptor and key impurities are other proteins endogenously expressed by the cell. B_{MAX} and K_d are determined using radioactive saturation binding assays and protein concentration is determined using the BCA method, according to the manufacturer's product description. Product specifications report protein concentration at 9 μ g/ μ l, a B_{MAX} of 14.4 pmol/mg membrane protein, and K_d for [³H]-

CGS 21680 of 8.6 nM. When included in hydrogel assisted GUP formation as described below, the crude membrane preparations are diluted in low-melt temperature agarose. Protein in crude membrane preparations was mixed with agarose so as to achieve a total protein concentration of 0.25 nM on each coverslip (calculation based on $A_{2a}R$ MW of 45 kDa). This corresponds to a lipid-to-total protein molar ratio of ~155:1 in the system, where lipid molar amount is equal to the amount of lipid deposited as a thin film in the rehydration system described in the next section.

A2AR Purified Protein (pA2AR)[1]

Receptor construct design, expression and purification were described in Liu *et al*, 2012. Briefly, the adenosine A_{2A} receptor was expressed in Sf9 cells using a baculovirus expression system. The receptor also has a BRIL fusion partner in ICL3 loop for enhanced stability, the C-terminus is truncated at residue 317 and there are N-terminal FLAG and C-terminal 10xHis tags. Cells were lysed with lysis buffer (10 mM HEPES pH 7.5, 10 mM MgCl2, 20 mM KCL) in a 100 mL homogenizer and spun for 35 minutes at 42K RPM. The cell pellet was washed twice in a high salt wash buffer (10 mM HEPES pH 7.5, 10 mM MgCl2, 20 mM KCL) and spun at 45K RPM for 35 minutes. A protease cocktail was used during all membrane washes that consists of Aprotinin, Leupeptin, E-64, and AEBSF (AGScientific and GoldBio). Cell membranes were solubilized for three hours at 4 °C on a rotator (25 mM HEPES, 1600 mM NaCl, 1%/0.2% w/v DDM/CHS, 10% v/v Glycerol). The sample was then centrifuged at 45K RPM for 1 hour. The lysate was then incubated with TALON resin overnight in the presence of 20mM imidazole. After overnight binding the resin was washed with 10 column volumes of Wash 1 (50 mM HEPES pH 7.5, 800 mM NaCl, 10 mM MgCl2, 0.1%/0.02% w/v DDM/CHS, 10% u/v DDM/CHS, 100 µM ZM241385). The receptor was eluted with elution buffer (25 mM HEPES pH 7.5, 800 mM NaCl, 0.01%/w/v DDM/CHS, 100 µM ZM241385). The receptor was eluted with elution buffer (25 mM HEPES pH 7.5, 800 mM NaCl, 0.01%/w/v DDM/CHS, 100 µM ZM241385). The receptor was eluted with elution buffer (25 mM HEPES pH 7.5, 800 mM NaCl, 0.01%/w/v DDM/CHS, 10% w/v Glycerol, 20 mM Imidazole, 100 µM ZM241385). Receptor purity and monodispersity was measured using SDS-PAGE and analytical size-exclusion chromatography (aSEC). Final receptor concentration was roughly 1 mg/ml. (Figure S1).

Fabrication of Giant Unilamellar Vesicles and Protein Incorporation^[2]

Giant unilamellar vesicles and protein incorporation were performed using methods previously described by Gutierrez *et al*, 2014. Briefly, 25 mm no. 1 coverslips were cleaned via sonication in MeOH for 30 minutes at 35 °C. Coverslips were dried and were further plasma treated in a PDC-32G benchtop plasma cleaner (Harrick Plasma, USA) for 15 minutes. Coverslips were held in Sykes-Moore chambers for vesicle formation. Protein-incorporated giant unilamellar protein-vesicles (GUPs) were formed using methods similar to those previously reported.² Briefly, a 1:3 v/v mixture of crude membrane fragment suspension and low-melt temperature agarose (3% w/v) or 1:1 v/v mixture of purified protein and low-melting temperature agarose (3% w/v) was drop casted onto the coverslips and a thin film was formed and allowed to gel. A lipid film was made on the protein-agarose from a 3.3 mg/mL solution in CHCl₃. Solvent was evaporated using a stream of N₂ gas. Protein in membrane preparations was mixed with agarose so as to achieve a total protein concentration of 44 nM after rehydration with buffer. For control giant unilamellar vesicle (GUV) fabrication without protein, protein was omitted from the hydrogel film and only 1.5% w/v agarose was used. The system was hydrated with 200 mM sucrose in PBS (pH 7.4). The films were swollen for 20 minutes above the transition temperatures of the lipid used (~45 °C). Bilayer vesicles were harvested from the coverslip and settled in 3x of an isoosmotic glucose solution (200 mM glucose in PBS, pH 7.4) and then transferred to observation chambers. Prior to observation, GUPs were allowed to equilibrate to 25 °C for 30 minutes to allow for domains to further coalesce as more hemispherical domains were easier to analyze and negated the possibility of photooxidation by fluorescence excitation.

Antibody Labeling

 $A_{2A}R$ monoclonal antibodies were equilibrated to room temperature and conjugated to NHS rhodamine in aqueous buffer (PBS, pH 7.4, DMSO < 5%) at 10x molar excess of NHS-rhodamine. Sodium bicarbonate was added as per manufacturer's instructions to raise the solution pH to 8.0. The solution was allowed to react for one hour at room temperature and then overnight at 5 °C. Rhodamine-labeled antibodies were subsequently desalted using spin columns according to the manufacturer's instructions. Labeled antibody UV-vis absorbance was read on a NanoDrop ND-1000 (Thermo Fisher, USA). Rhodamine labeled $A_{2A}R$ monoclonal antibody concentration was 3.4 µM with 0.81 labeling efficiency.

A2A Receptor Identification via Antibody Binding

An aliquot of $A_{2A}R$ crude membrane fragment and purified protein was incubated with 1:1000 labeled antibody at 37 °C for 1 hour. Labeled protein was used in the agarose film for GUP formation. GUPs were formed as described above. Lipid solutions included 0.2% 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-(cap biotinyl) for anchoring and ease of imaging. Vesicles were harvested, settled, and anchored to observation chambers using BSA-Biotin modification with avidin. GUPs were imaged at 561 nm excitation, corresponding to 575 nm emission for the rhodamine-labeled-antibody-tagged-A_{2A} receptor.

Ligand binding of A2AR in GUPs

Rhodamine antibody labeled $cA_{2A}R$ and $pA_{2A}R$ were incubated with 100 μ M of agonist or anatagoinst and then incorporated into GUPs are described above. For apo $pA_{2A}R$ GUPs, protein was not prebound to ligand. Upon incorporation into lipid vesicles, GUPs were incubated with their agonist or antagonist at a final concentration of 100 μ M. Excess ligand was removed and GUPs were imaged.

Radioligand binding of pA2AR GUPs

Radioligand binding assays were performed as previously described.^[3] Briefly, ligand binding was measured using $A_{2A}R$ with a fusion protein inserted in intracellular loop 3 (BRIL) and reconstituted in GUVs. This $A_{2A}R$ was produced in Sf9 cells using baculovirus expression system. Binding assays were carried out in a total volume of 0.125 ml in 96-well plates with a binding buffer (50 mM Tris HCl, 1 U/ml adenosine deaminase, pH 7.4) containing 1 nM [³H] ZM241385 (American Radiolabelled Chemicals, Inc.) for 60 min at room temperature. GUVs were harvested over 0.3% w/v polyethyleneimine-treated, 96-well double thick glassfibre filter mats (PerkinElmer, 1450-521) using a 96-well Filtermate harvester (Perkin Elmer) and washed three times with cold buffer (50 mM Tris HCl, pH 7.4). Filter mats were dried, wax scintillant was melted onto each filter, and radioactivity was counted in a MicroBeta2 TriLux plate scintillation counter (Perkin Elmer). ZM241385 (K_D) binding affinities was determined using homologous competition binding, and NECA (K_i) binding affinity was determined using competition binding with [³H] ZM241385. The data were analyzed by Prism 6.05 (GraphPad Software) to give K_D and K_i values and reported as the mean \pm S.D. and done three times or more in triplicate.

Microscopy

Imaging was done on a TI-Eclipse inverted microscope (Nikon, Japan) equipped with a spinning-disc CSUX confocal head (Yokogawa, Japan) and a 16-bit Cascade II 512 EMCCD camera (Photometrics, USA). Confocal excitation of fluorophores was done using 50 mW solid-state lasers at 561 nm for rhodamine (Coherent Inc., Germany). All confocal images were taken using a Plan-Apo 60x NA 1.43 oil immersion Nikon objective. Temperature control during imaging was performed using a heating-cooling stage with a stability and accuracy of 0.1°C (Bioscience Tools, USA). Z-stack projections of all vesicles were using at 0.2 µm slices and all experiments were performed at 20°C.

Image Processing & Data Processing

All images were processed and analyzed using ImageJ. Particle analysis and measurements were performed using ImageJ Analyze Tools. All images are presented without any further processing adjustments or corrections and are scaled from minimum to maximum intensity.

To determine the area of liquid ordered and liquid disordered phase, Z-stack projections of GUPs were first converted to binary. The number of white pixels (1) and black pixels (0) were calculated for each vesicle and the ratio of white pixels to total vesicle area was calculated to determine A_0 . To calculate the fluorescence intensity of I_0 to I_d , the same binary map was used to identify the two areas, and the fluorescence intensity (a. u.) of each of the areas was computed using ImageJ.

Unless otherwise specified, an average of 8 GUPs were analyzed for each sample group with each experiment being repeated at least four times. The error bars presented indicate the standard error of the mean and analysis was performed using JMP statistical software.

Results and Discussion



Figure S1. Analysis of purified A_{2A}R. (a) Representative analytical SEC trace of apo A_{2A}R showing minimal aggregation and a pure monomeric peak (b) SDS-PAGE gel of purified protein with two bands typical for A_{2A}AR. (c) Representative competition curves for both K_D and K_i calculations are shown. Binding data for ZM241385 and NECA were done in triplicate and are represented as the mean \pm S.D.



Figure S2. Fluorescence intensity increases linearly as a function of concentration of Rhodamine-conjugated anti- A_{2A} antibody. Anti- A_{2A} antibody was conjugated to NHS-Rhodamine according to the protocol provided by Thermo-Fisher. The labelled antibody was then suspended in 1X PBS pH 7.4 in linearly decreasing concentrations and subsequently imaged on a microplate reader to determine fluorescence intensity. The microplate reader settings were as follows: excitation/emission wavelengths of 554/575 nm, 5 seconds of shaking to ensure adequate mixing prior to a single endpoint read. For reference, the GUPs in this manuscript are labelled with a final concentration of 0.64 μ M Rhodamine-conjugated anti- A_{2A} antibody.



Figure S3. Antibody control images show no nonspecific binding of antibody to GUVs or 5-HT_{1A}R-GUPs. Both images are scaled to the maximum and minimum fluorescence intensity of the micrograph in A. A) GUVs without protein and without any fluorescent tags were fabricated using the described methods. Upon formation and settling, GUVs were incubated with rhodamine labelled $A_{2A}R$ antibodies. For visualization purposes, GUVs were not washed and immediately imaged after incubation with antibodies. GUVs do not show any increased fluorescence at their surface as indicated by the plot under the micrograph in A. This indicates that the rhodamine labelled $A_{2A}R$ antibodies used do not display significant GUV bilayer interaction. B) To test for antibody non-specificity interactions, GUPs were fabricated with 5-HT_{1A}R. As described in the methods section, 5-HT_{1A}R was incubated with rhodamine labelled $A_{2A}R$ antibodies then used in GUP formation. While GUPs were present in bright field imaging (not shown), under 561 nm

excitation, no fluorescence is observed. This indicated that the antibody is not present in the GUPs, and therefore does not exhibit non-specific binding to a different GPCR.



Figure S4. Images of lipid dye-labelled GUVs with fluorescently labeled antibody loaded into the hydrogel. The GUVs were prepared according to the method described above, with the exception that the protein was excluded from the preparation. Instead, we suspended the same amount of antibody in an equivalent amount of 1X PBS pH 7.4 and was allowed to incubate at 45°C for an hour prior to suspending in agarose for incorporation into GUVs. The top set of images are for GUVs that were allowed to phase separate. The bottom set of images are GUVs that were not allowed to phase separate, to allow for ease of viewing the intensity and plotting. The left images (A) were taken at 491 nm excitation, to visualize the lipid dye (ATTO-488-DPPE) and the right images (B) were taken at 561 nm, the excitation wavelength for the rhodamine-labelled antibodies. (B) images show no signal, thus this shows that the localization behavior is not an artifact of antibody aggregation or denaturation during hydrogel incubation and vesicle formation. Scale bars are 10 micrometers.



Figure S5. Denatured A_{2A} does not contribute to the phase separation behavior of GUPs. Crude membrane fragments containing A_{2A} were denatured for an hour at 80°C prior to antibody binding and agarose suspension. The lipid dye that was used is 0.2 mol% ATTO-488-DPPE. GUPs were swelled and harvested and imaged at 491 nm and 561 nm excitation. Image (A) is a GUP imaged at 491 nm showing successful GUV formation and image (B) was imaged at 561 nm, showing no signal. This shows that denatured protein cannot be labelled by the antibody and/or incorporate into the membrane. Scale bars are 10 micrometers.



Figure S6. Images of GUPs before and after adding 2 μ L of QSY-7 fluorescence quencher suspended in 200 mM sucrose in 1X PBS pH 7.4 to a final QSY-7 concentration of 75.82 μ M. Since the quencher is membrane-impermeable, an approximate 50% decrease in fluorescence intensity (FI) demonstrates that the GUPs are unilamellar. GUPs were imaged immediately prior to the addition of QSY-7 and then allowed to incubate for 10 minutes at room temperature in the dark before imaging again. Image (A) was taken at 491 nm excitation prior to the addition of the quencher. (B) shows the subsequent ~50% decrease in lipid dye FI after being allowed to incubate with QSY-7 in the dark for 10 minutes. The plot shows the FI of the line profiles in the pre- and post-quenching images after subtracting the minimum intensity values.

Table S1. Lipid vesicles were formed with apo $pA_{2A}R$ and without protein (GUV (no protein) +ATTO-488-DPPE lipid dye)) and then evaluated for phase separation and protein partitioning. GUPs formed with rhodamine antibody labeled apo GUP $pA_{2A}R$ displayed increase in bright regions as percent cholesterol decreased indicating that apo GUP $pA_{2A}R$ without ligand bound partitions to the liquid disordered phase. These GUPs were then subsequently incubated with either antagonist ZM241385 or agonist NECA for 1 hr at 25 °C in observations chambers. After incubation, apo GUPs with ZM or NECA were imaged and displayed increase in bright regions with increasing cholesterol content indicating liquid ordered phase partitioning. These results are consistent with proteins prebound to ligands (antagonist ZM241385 ($pA_{2A}R(An)$), main Figure 1) or agonist NECA ($pA_{2A}R(Ag)$), Table S1) which display partitioning into the liquid ordered phase. GUVs without protein and labeled with ATTO-488-DPPE were evaluated as a phase separation control, in which, as previously reported, the labeled lipid partitions to the liquid disordered phase.²

% Cholesterol	40%	33%	25%	20%	Paritioning
Apo GUP pA _{2a}	50 ± 3%	53 ± 5 %	57 ± 5%	61 ± 4%	Liquid Disordered
Apo GUP (pA _{2a}) incubated with ZM	66 ± 7%	59 ± 5%	53 ± 6%	49 ± 2%	Liquid Ordered
Apo GUP (pA _{2a}) incubated with NECA	49 ± 2%	45 ± 2%	43 ± 9%	38 ± 7%	Liquid Ordered
pA _{2a} (Ag)	51 ± 4%	48 ± 5 %	46 ± 5%	44 ± 4%	Liquid Ordered
GUV (no protein) + ATTO-488	54 ± 6%	56 ± 7%	58 ± 5 %	61 ± 4%	Liquid Disordered

Table S2. Lipid membrane compositions by components are listed in the table below by percent of each of the three lipid species used in throughout this work. Throughout the text, the compositions are referenced according to the percent cholesterol.

Membrane Compositions by							
Component							
_	%Chol	%DOPC	%DPPC				
	33	33	34				
	25	50	25				
	20	40	40				
	40	20	40				



Figure S7. Images of GUPs tagged with fluorescently labeled proteins in GUPs with lipid dye (ATTO-488-DPPE at 0.4%mol). These images represent lipid labeling examples that support the non-use of lipid dyes for the work presented here. Images on the left show fluorescence from the lipid dye; images on the right show fluorescence from antibody-labeled $A_{2A}R$. When lipid dye was added to the GUPs, proteins displayed some partitioning in the liquid ordered phase as shown in the top set of images (A) and most GUPs displayed protein co-localizing with the lipid dye in the liquid disordered phase (B). The lipid dye used here is ATTO-488-DPPE and both GUPs have 25% cholesterol content. Because of the discrepancy in GUP images collected when both dyes were present, the results in this work are only based on GUPs without lipid dye. The co-localization of protein in to the liquid disordered phase is thought to be due to dye-dye interactions and oxidation driven membrane rearrangement. All scale bars are 5 µm.



Figure S8. GUP micrographs were made binary for area mapping. GUPs were fabricated as described in the Methods and Materials section. GUPs were allowed to equilibrate at 25°C for 30 minutes after formation to allow domains to coalesce, resulting in hemispherically phase separated GUPs. Allowing GUP domains to ripen and coalesce also ensured that domains did not dynamically form upon visualizing due to photooxidative triggered events. The first column of micrographs shows Z-stack confocal micrographs of selected GUPs. The second column outlines the phase separation boundaries on the micrograph. The third column shows micrographs made binary with phases outlined for visual aid. The number of white pixels (1) and black pixels (0) were calculated for each vesicle and the ratio of white pixels to total vesicle area was calculated to determine A_o All scale bars are 5 µm.



Figure S9. Identification of liquid phase partitioning in $A_{2A}R$ membrane fragments suspended in 0.002% w/v CHS and 0.002%/0.01% w/v CHS/DDM. The mean percent of light area of on average 8 GUPs are presented with error bars as standard error of the mean. For both types of GUPs, as mol% of cholesterol increases, % light area decreases. This indicates that the unbound $cA_{2A}R$ incorporated GUPs reside in the liquid disordered phase. Representative confocal micrographs are shown above each data point. All scale bars are 5 µm. The star symbol indicates use the membrane fragments with CHS and the plus symbols indicate membrane fragments with CHS/DDM. The intensity of color of the symbol corresponds to cholesterol concentration, with darker symbols corresponding to higher cholesterol concentrations.



Figure S10. Partitioning coefficient (K) versus area of liquid ordered domain for unbound apo $A_{2A}R$ membrane fragments incorporated GUPs. A linear fit on points is presented with the shaded area showing the goodness of fit. Both samples have a negative slope. Thus as A_o increases the partitioning coefficient (K) decreases. These results indicate that as chol concentration increases more protein resides in the I_d phase which may be indicative of protein de-oligomerization events. The star symbol indicates use the membrane fragments with CHS and the plus symbols indicate membrane fragments with CHS/DDM. The intensity of color of the symbol corresponds to cholesterol concentration, with darker symbols corresponding to higher cholesterol concentrations; compare to Figure S9.

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Author Contributions

MGG led work and collected, analyzed data. DY, KW, and LCDO collected and analyzed data. MGG and KW wrote manuscript. VC, RS, NM reviewed work.