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Impact of purification conditions and history on A_{2A} adenosine receptor activity: The role of CHAPS and lipids

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ABSTRACT

The adenosine A_{2A} receptor ($A_{2A}R$) is a much-studied class A G protein-coupled receptor (GPCR). For biophysical studies, $A_{2A}R$ is commonly purified in a detergent mixture of dodecylmaltoside (DDM), 3-(3-cholamidopropyl) dimethylammoniopropane sulfonate (CHAPS), and cholesteryl hemisuccinate (CHS). Here we studied the effects of CHAPS on the ligand binding activity and stability of wild type, full-length human $A_{2A}R$. We also tested the cholesterol requirement for maintaining the active conformation of the receptor when solubilized in detergent micelles. To this end, the receptor was purified using DDM, DDM/CHAPS, or the short hydrocarbon chain lipid 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC, di-6:0PC). After solubilization in DDM, DDM/CHAPS, or DHPC micelles, although $A_{2A}R$ was found to retain its native-like fold, its binding ability was significantly compromised compared to DDM or DDM/CHAPS with CHS. It therefore appears that although cholesterol is not needed for $A_{2A}R$ to retain a native-like, α-helical conformation, it may be a critical component for high affinity ligand binding. Further, this result suggests that the conformational differences between the active and inactive protein may be so subtle that commonly used spectroscopic methods are unable to differentiate between the two forms, highlighting the need for activity measurements. The studies presented in this paper also underline the importance of the protein's purification history; *i.e.*, detergents that interact with the protein during purification affect the ligand binding properties of the receptor in an irreversible manner.

1. Introduction

Membrane proteins are essential biomolecules needed for a vast array of cellular processes including cellular signaling, ion and metabolite transport, adhesion, and migration [1], to name a few. G protein-coupled receptors (GPCRs) are integral membrane proteins consisting of seven α-helical segments that span the plasma membrane and respond to different extracellular stimuli (e.g. peptides, neurotransmitters, and small molecules). Upon binding to a ligand, GPCRs transmit a cellular signal mainly through intracellular G proteins and arrestins [2].

Structural studies typically require milligrams of purified protein [3,4], and unlike water-soluble proteins, membrane proteins require a membrane mimetic system to stabilize their hydrophobic transmembrane regions. However, obtaining substantial amounts of active GPCRs remains a challenge. This is in part due to their low expression in native tissues, structural flexibility, and instability when in detergent solutions [5]. Because of these challenges, structure-function studies of GPCRs using their native, full-length sequence, are not commonly carried out; modifications to their native sequences have been used to stabilize the receptors, facilitating crystal formation [6,7].

Detergents with a hydrophobic tail of 6–12 carbon atoms are commonly used to solubilize, stabilize and crystallize GPCRs [8]. In the case of GPCR X-ray crystal structures, the membrane protein was first solubilized in detergent prior to crystallization in detergent micelles, lipidic cubic phases, or bilayered micelles (also referred to as bicelles), without exception.

Even though many GPCRs are stable and retain functionality in

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detergent micelles, GPCRs and other membrane proteins often unfold and aggregate when solubilized in detergents [9], altering their native structure and eliminating or suppressing their biological function(s). At this point, selecting a detergent that retains membrane protein stability and function is typically a matter of trial and error [10]. In the case of the wild type, full-length human A_{2A} adenosine receptor ($A_{2A}R$), a class A GPCR, purification with the detergent dodecylmatoside (DDM) requires the presence of a cholesterol analog (cholesteryl hemisuccinate, CHS) in order for the receptor to retain its ligand binding activity [11,12]. It should be pointed out that three cholesterol interaction sites have been identified from molecular dynamic simulations [13], and cholesterol's presence was observed in one $A_{2A}R$ crystal structure [14]. However, it remains unclear whether cholesterol stabilizes the high affinity conformation of the receptor due to changes to the membrane properties, direct lipid-protein interactions, or a combination of both.

When purifying $A_{2A}R$, the zwitterionic detergent 3-(3-cholamidopropyl) dimethylammoniopropane sulfonate (CHAPS) is often used to solubilize CHS into micelles. In the current studies, we employed the wild type, full-length human $A_{2A}R$ to investigate the effects of CHAPS on the ligand binding activity and stability of this receptor. We also solubilized $A_{2A}R$ in micelles, with and without CHS, to further test the notion that a cholesterol analog is needed to retain the native fold and ligand binding activity of $A_{2A}R$. Finally, we tested the efficacy of 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC), a short chain phospholipid that self-assembles into micelles, and which acts as a biologically relevant detergent [9,15]. DHPC has been shown to minimally perturb membrane proteins [16] and has been studied extensively in conjunction with the long hydrocarbon chain lipid, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC, di-14:0PC) [16–19]. This lipid mixture (i.e., DHPC/DMPC) is tunable, and forms a number of different morphologies (e.g. bilayered micelles, unilamellar vesicles, multilamellar vesicles, perforated lamellae, ribbon-meshed lamellae) depending on the total lipid concentration, the molar ratio of DMPC-to-DHPC, net charge of the system, and temperature [20,21].

2. Materials and methods

2.1. Expression and purification of $A_{2A}R$ from yeast membrane preparations

$A_{2A}R$ was expressed in *Saccharomyces cerevisiae* cells, BJ5464, using the multi-integrating plTy-$A_{2A}R$-His_{10} plasmid, as previously described [11]. In order to improve protein purity and reproducibility between purifications, a previous purification protocol [11,22] was adapted to employ membrane preparations instead of crude cell lysis [23]. Briefly, cell pellets were collected via centrifugation 24 h post-induction. 600 ml of liquid culture with a total OD_{600} of 22 was separated into 50 ml aliquots, centrifuged at 3,220*g*, and cooled to −80 °C. Both lipid and detergent purifications started from cell pellets, with cell pellets consistently collected at an OD close to 22, which reduced batch-to-batch variability.

For details regarding the purification protocol refer to [23]. For these studies, additional detergent and lipid mixtures were used as follows. The homogenized membrane preparations were resuspended in 22 ml Buffer A, composed of 10% glycerol, 50 mM sodium phosphate monobasic, and 300 mM sodium chloride at pH 8, supplemented with 1 mM phenylmethanesulfonyl fluoride (PMSF) and complete EDTA-free protease inhibitor tablets (Roche Applied Science, Indianapolis, IN), and the appropriate detergent or lipid: 1) 2% (w/v) DDM + 1% (w/v) CHAPS + 0.2% (w/v) CHS (all from Anatrace, Maumee, OH); 2) 2% (w/v) DDM + 0.2% (w/v) CHS; 3) 2% (w/v) DDM + 1% (w/v) CHAPS; 4) 2% (w/v) DDM; or 5) 6.25% (w/v) DHPC (Avanti Polar Lipids Alabaster, AL). 6.25% (w/v) DHPC corresponds to 138 mM, approximately 10 times the critical micellar concentration (CMC) of DHPC (11–16 mM) [15]. The concentrations of DDM and CHAPS used correspond to approximately 200 and 3 times their CMC (i.e., approximately 0.2 mM and 6 mM, respectively) [24].

The elution buffers contained the appropriate detergent or lipid mixtures: 1) 0.1% (w/v) DDM + 0.1% (w/v) CHAPS + 0.02% (w/v) CHS; 2) 0.1% (w/v) DDM + 0.02% (w/v) CHS; 3) 0.1% (w/v) DDM + 0.1% (w/v) CHAPS; 4) 0.1% (w/v) DDM; or 5) 0.8% (w/v) DHPC. Purified $A_{2A}R$ samples were stored at 4 °C and used within one week of preparation to ensure maximal ligand binding activity.

2.2. Protein purity, concentration and biophysical characterization

Samples were separated via electrophoresis on 12% SDS-PAGE, and protein bands were detected via staining with Sypro Ruby (Life Technologies, Carlsbad, CA). For Western blotting mouse anti-A_{2A} (Santa Cruz Biotechnology, Santa Cruz, CA, catalogue # 32261) primary antibody was used at a 1:5000 dilution. Alexa Fluor 488 goat anti-mouse (Life Technologies, Carlsbad, CA, catalogue # A11029) was used as the secondary antibody at a 1:5000 dilution. Fluorescence was detected using a BioSpectrum Imaging System (UVP, Upland, CA). Protein concentration was determined using UV absorbance at 280 nm as described in Ref. [11], and protein purity was quantified from the Sypro Ruby stained gel images using FIJI [25].

For characterizing the protein's secondary structure, circular dichroism (CD) measurements were conducted using a Jasco J-810 spectropolarimeter (Jasco, Easton, MD), as previously described in Ref. [11]. Measurements were performed at 25 °C, and spectra collected with 1 nm resolution, with at least 3 integrations per spectrum. Reference spectra containing the appropriate buffer were collected and subtracted from their respective spectra. Fluorescence spectra were collected using a PC-1 spectrofluorimeter (ISS, Champaign, IL), as described previously in Ref. [11]. To minimize light scattering effects, measurements were collected with the excitation polarizer set to 90° and the emission polarizer set to 0°. Intrinsic fluorescence was measured at 15 °C, and excitation was set to 280 nm. CD and fluorescence measurements were taken at a protein concentration of 0.05–0.06 mg/ml.

To estimate particle sizes, Dynamic Light Scattering (DLS) measurements were taken using a Brookhaven Instruments 90Plus Particle Analyzer (Brookhaven Instruments, Holtsville, NY). A 50 μL micelle solution was loaded into Eppendorf UVette small volume cuvettes (Fisher Scientific, Pittsburgh, PA) and mounted on the instrument's small-volume cuvette adapter pedestal.

2.3. Radioactive ligand binding

Ligand binding of purified receptors was carried out as described previously [11], with minor modifications. Briefly, approximately 1 ml of purified receptor was incubated with a Ni-NTA Superflow resin (approximately 15 μl of settled resin) overnight. If necessary, samples were centrifuged briefly to allow the resin to settle, the elution buffer volume was removed, and the sample volume adjusted to achieve a target concentration of 55 μg/ml. 180 μl of the protein/resin mix was added per well, to enable loading of 10 μg of $A_{2A}R$-His_{10} per well in a 96-well plate (glass fiber type B filters, Millipore, Billerica, MA) for saturation and point measurement ligand binding analysis. For saturation binding experiments, protein samples were incubated with increasing amounts of [^{3}H] CGS 21680 (Perkin Elmer, Waltham, MA) for 4 h. Non-specific binding was determined using 10 μM of the cold ligand, CGS 21680, which was added to the samples at each value of