Importance of Disulfide Bonds for the Expression and Ligand Binding of the Adenosine A_{2A} Receptor

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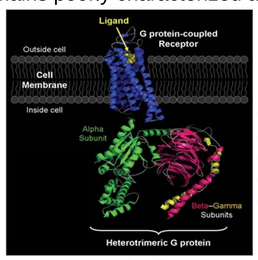
Abstract

The human adenosine A_{2A} receptor is a specific G protein-coupled receptor found in cell membranes and targeted for its role in signal transduction. Certain disulfide bonds of the A_{2A} receptor are believed to contribute to its folding and ligand-binding activity. In this project, a wildtype A_{2A} receptor containing disulfide bonds was compared to a variant lacking three disulfide bonds. The expression, trafficking, purification, and ligand-binding affinities of both receptor variants were characterized to better understand the importance of disulfide bonds in the adenosine A_{2A} receptor.

Introduction

G protein-coupled receptors (GPCRs) are a family of integral membrane proteins involved with intracellular signaling by activation of a G protein following ligand binding in the extracellular region.

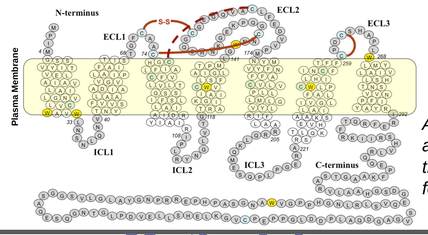
- Major **drug targets** as a result of their roles in cellular signaling
  - Nearly **half (44%)** of all drugs currently patented target GPCRs [1]
- Studied for use in **drug development** and **treatment of disease**
- **Structural biology** remains poorly characterized and limits development of pharmaceuticals [2]



Three-dimensional crystal structure of a GPCR embedded within a cell membrane along with a ligand in the extracellular region and G protein in the intracellular region [6].

The **human adenosine A_{2A} receptor ($A_{2A}R$)** is a member of the class A GPCRs and can be found in the basal ganglia of the brain. Therefore, A_{2A}Rs are being analyzed for treatment of neurodegenerative diseases such as Parkinson's [3].

- Target for both **adenosine** and **caffeine** [3]
- Three extracellular loops (ECLs) and three intracellular loops (ICLs)
- **Three disulfide bonds** formed between the cysteine amino acids of ECL1 and ECL2, and one disulfide bond formed within ECL3
  - One of the three disulfide bonds is conserved among many class A GPCRs and believed to be critical for **GPCR folding** and **ligand binding** [4, 5]



Amino acid sequence of adenosine $A_{2A}R$ denoting the four disulfide bonds found in the three ECLs [2].

Methodology

1) **DNA Propagation:** DH5α *E. coli* bacteria strain contained pITy vectors for expressing either A_{2A}R with cysteines or without cysteines in ECL1 and ECL2
   - DNA of pITy vectors extracted and linearized from DH5α
2) **Transformation:** BJ5464 *S. cerevisiae* yeast strains transformed and screened for greatest A_{2A}R expression
   - Screening conducted by Western Blotting and analysis in Fiji imaging software
3) **Expression and Trafficking:** A_{2A}R with GFP tags fluorescently imaged using confocal microscopy to determine intracellular localization of protein
4) **Purification:** A_{2A}R-10 His purified from 100 mL cultures of BJ5464 using nickel resin (IMAC)
5) **Ligand-Binding Affinities:** 1nM FITC-APEC bound to purified A_{2A}R in DCC for kinetics and equilibrium measurements
   - Anisotropy calculated from parallel and perpendicular fluorescence

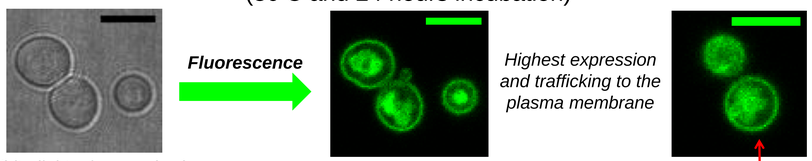
Expression and Trafficking

Since most GPCRs are located in the cell's plasma membrane, proper folding, expression, and trafficking must result for the GPCRs to be functional at the membrane. Any complications from folding or other quality control measures can lead to either harmful or inoperative proteins in the cell [7].

Expression and trafficking were influenced by cell culture conditions, such as **temperature** and **incubation time**, as well as structural characteristics of A_{2A}R, such as the presence or absence of **disulfide bonds between cysteines** of the first and second ECLs.

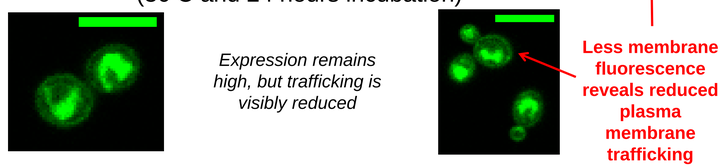
- Optimal expression denoted by cells with highest amount of **total A_{2A}R**
- Optimal trafficking denoted by cells with highest amount of **A_{2A}R at the plasma membrane**
- A_{2A}R detected by green fluorescence from GFP tagging

A_{2A}R Wildtype
(30˚C and 24 hours incubation)

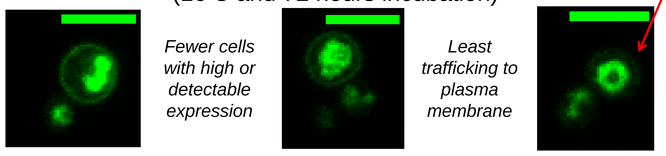


White light micrograph of BJ5464 yeast cells

A_{2A}R lacking Cysteines in ECL1 & ECL2
(30˚C and 24 hours incubation)



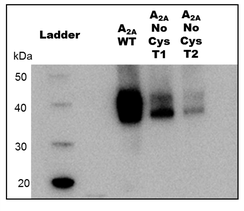
A_{2A}R lacking Cysteines in ECL1 & ECL2
(16˚C and 72 hours incubation)



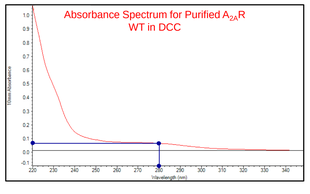
Scale Bars = 8 µm

Purification

Prior to measuring conformational changes in A_{2A}R as a result of the removal of disulfide bonds, the receptors were purified from membrane preparations. A **detergent based solubilization system** consisting of DDM, CHAPS, and CHS (DCC) enabled preservation of receptor structure and ligand-binding capabilities.



Western Blot showed bands just below 40 kDa for purified $A_{2A}R$ variants.



Absorbance at 280 nm used for calculating concentration of $A_{2A}R$ variants following purification.

Purified Receptor Concentrations			
$A_{2A}R$ Variant	A_{280}	Concentration	
		(µM)	(mg/mL)
A_{2A} Wildtype	0.061	1.1	0.05
A_{2A} No Cysteine Transformant 1	0.043	0.8	0.04
A_{2A} No Cysteine Transformant 2	0.036	0.7	0.03

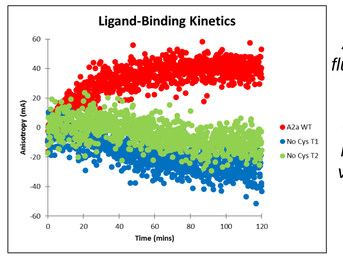
Receptor concentrations calculated based on A_{280} and amino acid sequences of variants.

Ligand-Binding Affinities

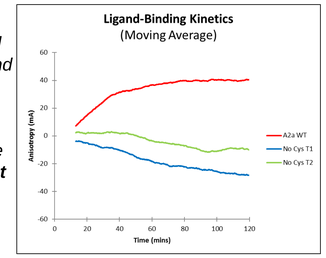
A fluorescent ligand, FITC-APEC, at a concentration of 1nM was added to purified A_{2A}R variants in DCC for studying differences in ligand-binding affinities.

Ligand-Binding Kinetics

- Measurements taken every 8 seconds for a total of 2 hours
- A_{2A}R concentrations normalized to 0.03 mg/mL (~ 0.7 µM)

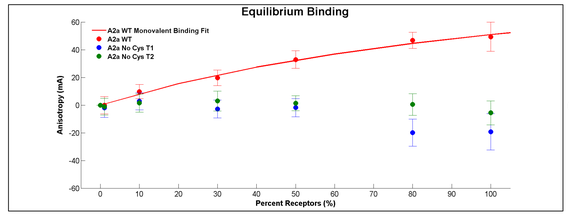


*A_{2A} WT **bound** fluorescent ligand and **reached equilibrium** within 2 hours, but no-cysteine variants **did not** exhibit ligand binding*



Ligand Binding at Equilibrium

- Receptors and ligands allowed sufficient time to reach binding equilibrium
- Anisotropy calculated as a function of receptor percentage with 100% receptors being the normalized concentration of 0.03 mg/mL (~ 0.7 µM)



Equilibrium dissociation constant (K_D) of A_{2A} WT = 132.7 nM

***Monovalent binding model** only used to fit data from A_{2A} WT. The no-cysteine variants displayed similar anisotropy trends indicating a lack of ligand binding.*

Conclusions

Removal of the cysteines, thus disulfide bonds, between ECL1 and ECL2 of the adenosine A_{2A}R suggested:

- Decreased A_{2A}R expression and trafficking to the plasma membrane as observed by confocal microscopy
- Lowered yield of purified receptors as observed by Western Blotting and A_{280} measurements
- Reduced ligand-binding affinities as observed from the fluorescence anisotropy calculations via kinetics and equilibrium studies

As a result, disulfide bonds prove relevant to the activity of adenosine A_{2A} receptors.

References

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