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Conserved disulfide bond is not essential for the adenosine A_{2A} receptor: Extracellular cysteines influence receptor distribution within the cell and ligand-binding recognition

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ABSTRACT

G protein-coupled receptors (GPCRs) are integral membrane proteins involved in cellular signaling and constitute major drug targets. Despite their importance, the relationship between structure and function of these receptors is not well understood. In this study, the role of extracellular disulfide bonds on the trafficking and ligand-binding activity of the human A_{2A} adenosine receptor was examined. To this end, cysteine-to-alanine mutations were conducted to replace individual and both cysteines in three disulfide bonds present in the first two extracellular loops. Although none of the disulfide bonds were essential for the formation of plasma membrane-localized active GPCR, loss of the disulfide bonds led to changes in the distribution of the receptor within the cell and changes in the ligand-binding affinity. These results indicate that in contrast to many class A GPCRs, the extracellular disulfide bonds of the A_{2A} receptor are not essential, but can modulate the ligand-binding activity, by either changing the conformation of the extracellular loops or perturbing the interactions of the transmembrane domains.

1. Introduction

G protein-coupled receptors (GPCRs) are heptahelical, integral membrane proteins involved in signal transduction. Because of their location at the plasma membrane and their importance in cellular signaling, GPCRs constitute major drug targets. Approximately 36% of drugs on the market are known to interact with GPCRs [1]. The adenosine receptors (A_1, A_3, A_{2B} and A_{2A}) are members of the family A GPCRs, and are ubiquitously expressed throughout the human body. This subfamily is one of the main targets for the treatment of neurodegenerative diseases, diabetes, cancer and heart disease [2].

The adenosine receptors (ARs) maintain a sequence homology of approximately 40%, with the highest sequence identity between A_{2A} and A_{2B} (46%), and A_1 and A_3 (46%) [3]. Despite the high sequence homology, the ARs have distinct affinities for various ligands and couple to different G proteins, whose activation regulates different membrane and intracellular proteins (e.g. adenylyl cyclase, Ca^{2+} channels, K^+ channels, and phospholipase C) [4]. Out of the four ARs, only the A_{2A} receptor ($A_{2A}R$) expresses at high levels in heterologous systems [5] and has been extensively studied in biophysical and structural studies [6–13].

The crystal structure of $A_{2A}R$ identified three disulfide bonds between extracellular loop 1 (ECL1) and ECL2 of the receptor [10]. It is speculated that this extensive disulfide bond network forms a rigid structure exposing the ligand-binding pocket [10]. One of the three disulfide bonds is highly conserved among many class A GPCRs [3,10,14], and numerous studies indicate that this disulfide bond is critical for the structural stability, expression, and function of GPCRs [14,15]. Mutations to the conserved cysteines have shown that this covalent linkage between ECL1 and ECL2 is critical for maintaining the high-affinity ligand-binding conformation of the thyrotropin-releasing hormone receptor [16], rhodopsin [17,18], μ opioid receptor [19], β_2 adrenergic receptor [20,21], and A_1 adenosine receptor [22], to name a few. For some GPCRs, mutating the extracellular cysteines also resulted in lower protein expression levels or reduced/abolished trafficking of

Abbreviations: GPCRs, G protein-coupled receptors; ARs, adenosine receptors; ECL, extracellular loop; DTT, dithiothreitol; CFP, cyan fluorescent protein; $A_{2A}R$, A_{2A} receptor; WT, wild type; ER, endoplasmic reticulum; M, plasma membrane; HR, Hausdorff ratio; HEK cells, human embryonic kidney

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the receptor to the plasma membrane. For example, mutations to the conserved cysteines in μ opioid receptor reduced the number of receptors present at the plasma membrane compared to the wild type [19]. In contrast, mutations of the cysteines in ECL1 or ECL2 of the A_1 adenosine receptor resulted in a loss of receptors at the cell surface [22]. In the aforementioned examples, the conserved disulfide bond is the only covalent link between ECL1 and ECL2, and disruption of this link likely affected the topology of the ECLs and thus the ligand-binding affinity.

Previously, the role of the disulfide bonds in the A_{2A} adenosine receptor has been investigated using reducing agents. Dithiothreitol (DTT) treated A_{2A}R displayed reduced activity compared to the wild type A_{2A}R [6,23]. In this study, we used a mutational approach to evaluate the role of the disulfide bond network of the human A_{2A}R for contributing to the ligand-binding capability and the exceptional expression levels that have been previously described [7,24,25]. To this end, systematic mutations of the cysteines were conducted, revealing that the conserved disulfide bond was not essential for the trafficking and ligand-binding activity of this receptor. On the contrary, mutations to the cysteines in the ECLs of the A_{2A}R resulted in a range of ligand-binding affinities and trafficking patterns.

2. Results

Disulfide bonds have been shown to have a critical role in protein stability, trafficking and function for many GPCRs [14–21]. To characterize the role of the specific disulfide bonds in A_{2A}R, we created Cys-to-Ala mutations in the three disulfide bonds that join ECL1 and ECL2 (Fig. 1).

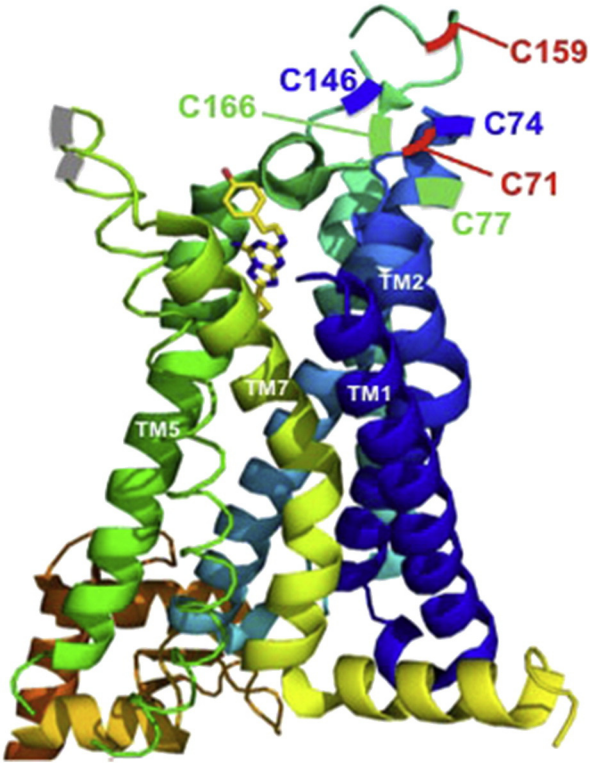

Fig. 1. Crystal structure of A_{2A}R bound to an antagonist, ZM 241385 [10]. The cysteines that form the disulfide bonds are color coded in green, red and blue. Adapted using PyMOL (The PyMOL Molecular Graphics System, Version 1.3 Schrödinger, LLC), Protein Data Bank identification code 3EML. (For interpretation of the references to colors in this figure legend, the reader is referred to the web version of this article.)

Table 1
List of the Cys-to-Ala constructs created to test the role of the disulfide bonds in the A_{2A} adenosine receptor.

Cysteine-to-alanine constructs		
Single Cys-to-Ala constructs		Double Cys-to-Ala constructs
ECL1	ECL2	ECL1 and ECL2
C71A	C146A	C71A–C159A
C74A	C159A	C74A–C146A
C77A	C166A	C77A–C166A

Six single Cys-to-Ala and three double Cys-to-Ala constructs were created as outlined in Table 1.

2.1. Trafficking patterns and fluorescent-ligand binding of A_{2A}R wild type and Cys-to-Ala variants

The constructs listed in Table 1 were transfected and expressed in HEK-293 cells as described in the Materials and methods section to test how the Cys-to-Ala mutations affected the trafficking of the receptor and ligand-binding activity. Trafficking refers to the receptor movement within the cell, including insertion of new receptors into the plasma membrane, internalization, recycling, and sorting of internalized receptors to lysosomes for degradation [26]. For these studies, the A_{2A}R constructs were C-terminally tagged with the cyan fluorescent protein (CFP), and trafficking to the plasma membrane was analyzed by CFP fluorescence detection at the periphery of the cell via confocal microscopy. Fig. 2 displays the typical trafficking pattern of the wild type (WT) A_{2A}R; a strong halo is seen at the periphery of the cell, indicating that the receptor trafficked well to the plasma membrane.

The typical expression patterns of all the Cys-to-Ala variants are displayed in Fig. 3. From these images, it appears that all A_{2A}R variants trafficked to the plasma membrane. However, it is also evident that the internally-localized receptor population differs between the variants and the WT A_{2A}R.

From these images, it is not clear whether receptors present within the cell are retained in the endoplasmic reticulum (ER), in lysosomes for degradation, or are en route to the plasma membrane. However, as our focus was on proper localization of active receptor to the plasma membrane, we used the following methods to characterize the distribution of the receptors between the plasma membrane versus ER, and the activity of the receptor once it reached the cell surface:

1) Comparison of the distribution of the A_{2A}R variants at the plasma membrane and at the ER using plasma membrane and ER dyes
2) Fluorescent–ligand (FITC-APEC) binding to further test if the receptor was at the plasma membrane and in its active form

Cells were stained with plasma membrane (M) and ER dyes to compare localization of A_{2A}R WT and the A_{2A}R variants. At least twenty images for each A_{2A} variant were analyzed using the Hausdorff ratio (HR), which is defined as the directed Hausdorff distance between the CFP tagged receptor and the plasma membrane divided by the directed Hausdorff distance between the ER and the plasma membrane, as described in the Materials and methods section. When this ratio is low (<0.5), it indicates that the receptor was localized primarily at the plasma membrane. Ratios close to one indicate that there was a higher ER-localized receptor population. Fig. 4 displays two examples of disparate receptor trafficking; the top image represents a cell where most of the receptor trafficked to the plasma membrane, and the bottom image represents a cell with higher levels of ER localized receptor. This difference can be seen by the clear outline of CFP at the cell periphery in Fig. 4A compared to the diffuse CFP fluorescence throughout the ER network in Fig. 4D. Comparison of the CFP fluorescence (Fig. 4A and 4D) to that of the plasma membrane dye (Fig. 4B or 4E, respectively) versus that