Molecular Determinants of CGS21680 Binding to the Human Adenosine A_{2A} Receptor[§]

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ABSTRACT

The adenosine A_{2A} receptor (A_{2A}R) plays a key role in transmembrane signaling mediated by the endogenous agonist adenosine. Here, we describe the crystal structure of human A_{2A}R thermostabilized in an active-like conformation bound to the selective agonist 2-[*p*-(2-carboxyethyl)phenylethyl-amino]-5'-*N*-ethylcarboxamido adenosine (CGS21680) at a resolution of 2.6 Å. Comparison of A_{2A}R structures bound to either CGS21680, 5'-*N*-ethylcarboxamido adenosine (NECA), UK432097 [6-(2,2-diphenylethylamino)-9-[(2*R*,3*R*,4*S*,5*S*)-5-(ethylcarbamoyl)-3,4-dihydroxy-tetrahydrofuran-2-yl]-*N*-[2-[[1-(2-pyridyl)-4-piperidyl]carbamoylamino]ethyl]purine-2-carboxamide], or adenosine shows that the adenosine moiety of the ligands binds to the receptor in an identical fashion. However, an extension in CGS21680 compared with adenosine, the (2-carboxyethyl)phenylethylamino group, binds in an extended vestibule formed from transmembrane regions 2 and 7 (TM2 and TM7) and extracellular loops 2 and 3 (EL2 and EL3).

The (2-carboxyethyl)phenylethylamino group makes van der Waals contacts with side chains of amino acid residues Glu169^{EL2}, His264^{EL3}, Leu267^{7.32}, and Ile274^{7.39}, and the amine group forms a hydrogen bond with the side chain of Ser67^{2.65}. Of these residues, only Ile274^{7.39} is absolutely conserved across the human adenosine receptor subfamily. The major difference between the structures of A_{2A}R bound to either adenosine or CGS21680 is that the binding pocket narrows at the extracellular surface when CGS21680 is bound, due to an inward tilt of TM2 in that region. This conformation is stabilized by hydrogen bonds formed by the side chain of Ser67^{2.65} to CGS21680, either directly or via an ordered water molecule. Mutation of amino acid residues Ser67^{2.65}, Glu169^{EL2}, and His264^{EL3}, and analysis of receptor activation either in the presence or absence of ligands implicates this region in modulating the level of basal activity of A_{2A}R.

Introduction

Innovative strategies in protein engineering, such as the construction of T4 lysozyme (T4L) fusion proteins and the identification of thermostabilizing point mutations (Tate and Schertler, 2009; Tate, 2012), have led to the structure determination of over 25 G protein-coupled receptors (GPCRs) bound to ligands with a wide range of efficacy (Venkatakrishnan et al., 2013). The first human adenosine A_{2A} receptor (A_{2A}R) structure was determined after engineering a T4L fusion and crystallizing it in lipidic cubic phase in complex with the inverse agonist 4-(2-[7-amino-2-(2-furyl) [1,2,4]-triazolo[2,3-*a*][1,3,5] triazin-5-ylamino]ethyl)phenol (ZM241385; PDB ID [3EML](#);

Jaakola et al., 2008). Subsequently, the structure of a thermostabilized version of A_{2A}R was determined, also with ZM241385 bound (PDB ID [3PWH](#); Dore et al., 2011), and the high similarity between the structures [root mean square deviation (RMSD) 0.6 Å for 284 residues and 1648 atoms aligned in PyMOL (Schrödinger, San Diego, CA)] showed that neither the thermostabilizing mutations nor the T4L fusion had any significant impact on the structure of the receptor (Tate, 2012). A total of 12 X-ray structures are now available for A_{2A}R, bound to the following ligands: inverse agonist ZM241385 (Jaakola et al., 2008; Dore et al., 2011; Hino et al., 2012); the full agonists adenosine (Lebon et al., 2011b), 5'-*N*-ethylcarboxamido adenosine (NECA; Lebon et al., 2011b), and UK432097 [6-(2,2-diphenylethylamino)-9-[(2*R*,3*R*,4*S*,5*S*)-5-(ethylcarbamoyl)-3,4-dihydroxy-tetrahydrofuran-2-yl]-*N*-[2-[[1-(2-pyridyl)-4-piperidyl]carbamoylamino]ethyl]purine-2-carboxamide] (Xu et al., 2011); the neutral antagonists caffeine and xanthine amine congener (Dore et al., 2011); and to novel compounds developed as preclinical candidates for the treatment of Parkinson's disease (Congreve et al., 2012; Langmead et al.,

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ABBREVIATIONS: A_{2A}R, adenosine A_{2A} receptor; A_{2A}R-GL31, thermostabilized A_{2A}R; CGS21680, 2-[*p*-(2-carboxyethyl)phenylethyl-amino]-5'-*N*-ethylcarboxamido adenosine; CHO, Chinese hamster ovary; DM, *n*-decyl-β-D-maltopyranoside; EL, extracellular loop; GPCR, G protein-coupled receptor; LCP, lipidic cubic phase; NECA, 5'-*N*-ethylcarboxamido adenosine; RMSD, root mean square deviation; Ro20-1724, 4-(3-butoxy-4-methoxybenzyl)imidazolidin-2-one; T4L, T4 lysozyme; TM, transmembrane region; UK432097, 6-(2,2-diphenylethylamino)-9-[(2*R*,3*R*,4*S*,5*S*)-5-(ethylcarbamoyl)-3,4-dihydroxy-tetrahydrofuran-2-yl]-*N*-[2-[[1-(2-pyridyl)-4-piperidyl]carbamoylamino]ethyl]purine-2-carboxamide; ZM241385, 4-(2-[7-amino-2-(2-furyl) [1,2,4]-triazolo[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl)phenol.

2012). Recently, the mechanism of the negative allosteric modulation of A_{2A}R by sodium for agonist binding was elucidated following the determination of a 1.8-Å resolution structure of the receptor (Liu et al., 2012). The crystal structures have revealed a common set of amino acid residues that interact with ligands—namely, Leu85^{3.33}, Phe168^{EL2}, Glu169^{EL2}, Met177^{5.38}, Trp246^{6.48}, Leu249^{6.51}, Asn253^{6.55}, and Ile274^{7.39} (superscripts refer to the Ballesteros Weinstein nomenclature; Ballesteros and Weinstein, 1995). Both Asn253^{6.55} and Phe168^{EL2} make strong interactions with the adenine moiety in agonists and the equivalent triazotriazine moiety in inverse agonists (see Fig. 1 for ligand structures) via two hydrogen bonds and by π -electron stacking, respectively. All of the structures of A_{2A}R bound to agonists (NECA, adenosine, UK432097) identified hydrogen bonds between the ribose and the side chains of Ser277^{7.42} and His278^{7.43} in transmembrane region 7 (TM7; Lebon et al., 2011b; Xu et al., 2011). Residues in TM3, Val84^{3.32}, and Thr88^{3.36} are also important for both agonist binding and receptor activation. Interestingly, structure-based drug design led to the development of new triazine derivatives that are inverse agonists, but which interact with an amino acid residue, His278^{7.43}, previously identified as interacting with the ribose moiety of the agonist adenosine (Congreve et al., 2012).

Most of the residues of the orthosteric binding site engaged by adenosine are conserved between the different receptor subtypes (Lane et al., 2011; Lebon et al., 2011b). It is consequently difficult to design selective ligands for an adenosine receptor subtype, unless extensions are made into regions of the receptor that are less well conserved, although recently structure-based drug design has facilitated the development of A_{2A}R-specific ligands that bind deeply in the orthosteric binding pocket (Congreve et al., 2012). Before structures were available, a large

number of ligands were developed with modifications at position 2 of the adenine ring (see Fig. 1), some of which display high potency and selectivity toward one receptor subtype (Jacobson and Gao, 2006). 2-[p-(2-carboxyethyl)phenylethyl-amino]-5'-N-ethylcarboxamido adenosine (CGS21680) was the first highly selective ligand allowing the discrimination of A_{2A}R from the A_{2B} and A₁ receptors in rat brain (Jarvis et al., 1989; Jarvis and Williams, 1989). CGS21680 was predicted to extend out of the adenosine binding pocket into the extracellular loops (ELs) of the receptor, which represents a region of significant sequence diversity and the proposed site of binding of allosteric ligands in the adenosine A₁ receptor (Peeters et al., 2012). In the structures of A_{2A}R bound to adenosine or NECA, EL2 and EL3 are in close proximity with a hydrogen bond between His264^{EL3} and Glu169^{EL2}. Different conformations of EL2 and EL3 are observed in A_{2A}R bound to the agonist UK432097, where the bulky extensions from position 2 on the adenosine ring sterically block close interactions between EL2 and EL3, resulting in His264^{EL3} and Glu169^{EL2} being 3.8 Å further apart (distance between C α atoms) than observed in the structures bound to adenosine or NECA (Lebon et al., 2011b; Xu et al., 2011).

In efforts to understand the molecular determinants of CGS21680 binding and to provide a foundation for understanding subtype specificity, we report here the high-resolution crystal structure of the subtype-selective agonist CGS21680 bound to the thermostabilized human A_{2A} receptor, A_{2A}R-GL31.

Materials and Methods

Adenosine deaminase, ZM241385, CGS21680, NECA, and 4-(3-butoxy-4-methoxybenzyl)imidazolidin-2-one (Ro20-1724) were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). *n*-Decyl- β -D-maltopyranoside (DM) was purchased from Anatrace (Maumee, OH). Monoolein was from Nu-Chek Prep (Elysian, MN). Cholesteryl hemisuccinate and cholesterol were purchased from Sigma-Aldrich (Dorset, England). Chloroalkane-Lumi4Tb and tag light reagent were from Cisbio Bioassays (Codolet, France). Lipofectamine 2000 was purchased from Invitrogen–Life Technologies (Saint Aubin, France).

Receptor Constructs. Pharmacology of wild-type A_{2A}R was determined using the full-length human A_{2A}R cDNA cloned into pcDNA3.1 (pcDNA3.1-WT-A_{2A}R; Bennett et al., 2013) provided by Kirstie A. Bennett from Heptares Therapeutics (Welwyn Garden City, England). A_{2A}R mutants S67A, E169A, H264A, and L267A were produced by introducing single-point mutations into pcDNA3.1-WT-A_{2A}R using the QuikChange II site-directed mutagenesis kit (Agilent Technologies, Stockport, England). Receptor expression at the cell surface was assessed using A_{2A}R with a HaloTag at the N-terminus of the receptor, HALO-A_{2A}, provided by Cisbio Bioassays. Construction of the thermostabilized agonist-bound conformation of the human adenosine receptor, A_{2A}R-GL31, was described previously (Lebon et al., 2011a,b).

Cell Culture, Transient Transfections, and cAMP Assays. Chinese hamster ovary (CHO) cells were grown to 80% confluence in Glutamax Dulbecco's modified Eagle's medium Ham's F12 medium (Invitrogen, Paisley, UK) complemented with 10% fetal bovine serum (Labtech International, Uckfield, UK). For transfection, cells were seeded at a density of 12,500, 25,000, 50,000, or 100,000 cells/well in a volume of 100 μ l in a 96-well plate and transfected with 50 ng of either wild-type A_{2A}R or the mutants S67A, E169A, H264A, or L267A using Lipofectamine 2000 (Life Technologies, Paisley, UK). Dose-response curves were obtained by transfecting 25,000 cells/well with 50 ng of plasmid DNA. In brief, 50 ng of plasmid DNA was mixed with Lipofectamine 2000 in a total volume of 50 μ l and incubated 30 minutes at room temperature, according to the manufacturer's guidelines, and then added to the required number of cells. After 48 hours of receptor expression, cAMP production was measured using

Fig. 1. Structures of selected ligands for the adenosine A_{2A} receptor. The structure of the endogenous agonist adenosine is shown in light blue, and structural elements conserved between adenosine, CGS21680, and ZM241385 are likewise in blue. The (2-carboxyethyl)phenylethylamino group of CGS21680 is depicted in black, whereas the *N*-ethylcarboxamido group is cultured green. The structure of the agonist NECA is composed of the blue and green portions of CGS21680. Regions in ZM241385 that differ from adenosine are shown in red.

the Dynamic 2 cAMP HTRF kit (Cisbio Bioassays), according to the manufacturer's protocol. In brief, the cells were washed in phosphate-buffered saline, then stimulated using different compound concentrations in a total volume of 50 μ l, complemented with the phosphodiesterase inhibitor Ro20-1724 (50 μ M) and adenosine deaminase (1 U/ml). Compounds were diluted in Dulbecco's modified Eagle's medium Ham's F12 medium, without serum. After 30-minute incubation, 25 μ l of cAMP-d2 conjugate and 25 μ l of anti-cAMP-cryptate diluted in lysis buffer were added per well. The plates were incubated at room temperature for 1 hour, and then the fluorescence per well was recorded using the Pherastar 96-well time-resolved fluorescence plate reader (BMG Labtech, Champigny sur Marne, France). Data were analyzed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA). Functional concentration response data were fitted to a four-parameter logistic equation. The pEC₅₀ values are expressed as the mean $\pm$ S.E.M. Statistical analyses were performed using a one-way analysis of variance with Dunnett's post test. Values were stated as significantly different at $P < 0.05$.

Expression, Purification, and Crystallization. Cell-surface receptor expression levels in human embryonic kidney 293 cells were measured using the HALO-A_{2A}R construct, which were labeled according to the manufacturer's protocol (Cisbio Bioassays). In brief, cells were washed with 150 μ l of Tag-Lite buffer (Cisbio Bioassays), incubated for 1 hour (37°C) with 100 nM Chloroalkane-Lumi4Tb (Cisbio Bioassays) diluted in Tag-Lite buffer (50 μ l/well), washed three times with 100 μ l of Tag-Lite buffer, and the fluorescence measured using a Pherastar 96-well time-resolved fluorescence plate reader.

The A_{2A}R construct, A_{2A}R-GL31, is an engineered version of human A_{2A}R. A_{2A}R-GL31 contains four thermostabilizing mutations, L48A, A54L, T65A, and Q89A, that improve thermostability of the agonist-bound conformation of human A_{2A}R (Lebon et al., 2011a). In addition, to facilitate crystallization, the C-terminus was deleted after Ala316, and the point mutation N154A in EL2 was introduced to remove the N-linked glycosylation site. A proteolytically cleavable His₁₀ tag was introduced at the C-terminus to facilitate purification. Baculovirus expression and purification were performed exactly as previously described, except that the detergent DM was used throughout at a final concentration of 0.15%. Purified A_{2A}R-GL31 used for crystallization was in a buffer containing 25 mM Hepes (pH 7.4), 0.1 M NaCl, 100 μ M CGS21680, and 0.15% DM. Purified receptor fractions were concentrated to 15–25 mg/ml in a final volume of 50–60 μ l. Determination of the receptor concentration was performed using the amido black assay (Schaffner and Weissmann, 1973).

The A_{2A}R-GL31-CGS21680 complex was crystallized using the lipidic cubic phase (LCP) crystallization method (Cherezov et al., 2006; Caffrey and Cherezov, 2009). LCP crystallization setups contained a 2:3 (v/v) receptor-to-monoolein ratio, which was dispensed in 25-nl aliquots using a lipid-handling instrument, the mosquito-LCP from TTP Labtech (Melbourn, England). High-quality diffraction crystals were obtained in two different crystallization conditions, leading to two different crystal forms, monoclinic and orthorhombic. Monoclinic crystals were grown in 0.05 M acetate-HCl (pH 4.8), 6% sucrose, 21.9% pentaethylene glycol with the monoolein supplemented with 10% (w/w) cholesterol before mixing with the purified receptor. Orthorhombic crystals were grown in 0.1 M ADA (pH 7.0) and 21.6% PEG 600, with cholesterol hemisuccinate added to the purified receptor [10% (w/w) cholesteryl hemisuccinate: A_{2A}R] before mixing with the monoolein. All LCP crystallization trials contained 800 μ l of precipitant per well (lipidic cubic phase kit; Molecular Dimensions Ltd., Newmarket, UK). Crystals were grown at 22°C, harvested with cryo-loops, and cryo-cooled in liquid nitrogen.

Data Collection, Structure Solution, and Refinement. Diffraction data for the CGS21680 complex were collected at the European Synchrotron Radiation Facility (Grenoble, France) on the microfocus beamline ID23-2 (wavelength 0.8726 Å) using a 10- μ m focused beam and a Mar 225CCD detector. The microfocus beam was essential for the location of the best diffracting parts of single crystals and the collection of several wedges of data from different positions on

the crystal. Images were processed with MOSFLM (Leslie, 2006) and SCALA (Evans, 2006). The CGS21680 complex was solved by molecular replacement with PHASER (McCoy et al., 2007) using the adenosine-bound A_{2A}R-GL31 crystal structure (PDB ID 2YDO) as a search model after removal of all solvent molecules and the ligand. Two protein chains were found per asymmetric unit in the orthorhombic crystal form and one protein chain per asymmetric unit in the monoclinic crystal form. Refinement and rebuilding were carried out using REFMAC (Murshudov et al., 1997) and COOT (Emsley and Cowtan, 2004), respectively. Validation of the final refined models was performed using Molprobity. All figures in the manuscript were generated using either PyMOL or CCPmg (Potterton et al., 2004).

Results

Crystallization of the Selective Agonist CGS21680 Bound to Thermostabilized A_{2A}R-GL31. A_{2A}R-GL31 is an engineered version of the human A_{2A}R with improved thermostability in the agonist-bound conformation through the introduction of four point mutations, L48A, A54L, T65A, and Q89A (Lebon et al., 2011a). In addition, to facilitate crystallization, the C-terminus was deleted after Ala316, and the point mutation N154A in EL2 was introduced to remove the N-linked glycosylation site. A_{2A}R-GL31 has been successfully cocrystallized with the endogenous agonist adenosine and the synthetic agonist NECA (Lebon et al., 2011b) (Fig. 1). Pharmacological characterization was previously performed showing that the affinity of A_{2A}R-GL31 for agonist is identical to wild-type A_{2A}R, although the signaling properties of the receptor are impaired due to the orthosteric binding pocket being uncoupled from the G protein binding site (Lebon et al., 2011b).

CGS21680-bound A_{2A}R-GL31 was purified as described previously for NECA-bound A_{2A}R-GL31, and the purified receptor was of high quality with a monodisperse gel filtration profile. No crystals were obtained using vapor diffusion, even when crystallization conditions were used similar to those previously identified for the crystallization of A_{2A}R-GL31 bound to either NECA or adenosine. However, crystallization trials in LCP were successful and yielded two different crystal forms, with space groups P2₁ (monoclinic) and P2₁2₁2₁ (orthorhombic). Complete data sets to 2.6 Å resolution were obtained for each crystal form at European Synchrotron Radiation Facility using the microfocus beamline ID23-2. The structures were solved by molecular replacement using the adenosine-bound conformation of A_{2A}R-GL31 (PDB ID 2YDO) (Table 1).

The monoclinic crystal form contained one molecule of A_{2A}R-GL31 per asymmetric unit, whereas the orthorhombic crystal form contained two molecules of A_{2A}R-GL31 (referred to as molecule A and molecule B) arranged as a nonphysiologic antiparallel dimer. Both structures are very similar to the adenosine-bound and NECA-bound structures with overall RMSDs of 0.4 Å for all residues. Overall, the electron density was well defined for receptors crystallized in both space groups, with the exception of the extracellular surface. In the orthorhombic form, electron density for EL2, EL3, and CGS21680 was clearly resolved (Fig. 2), although density for residues 148–164 in EL2 of molecule A was very weak, and this region was not modeled. In the monoclinic form, residues 155–157 in EL2 and 263 in EL3 could not be modeled, and no density was observed for the (2-carboxyethyl)phenylethylamino group of CGS21680 (Supplemental Fig. 1). In addition, in the monoclinic space group, the electron density for the Glu169^{EL2} side chain was weak, and TM2 appears to be in a conformation

TABLE 1
Data collection and refinement statistics

	A _{2A} R-GL31 CGS21680 Monoclinic (Two Crystals)	A _{2A} R-GL31 CGS21680 Orthorhombic (Two Crystals)
Data collection ^a		
Space group	P2 ₁	P2 ₁ 2 ₁ 2 ₁
Cell dimensions		
<i>a</i> , <i>b</i> , <i>c</i> (Å)	60.4, 59.2, 65.1	57.2, 105.9, 125.9
α , β , γ (°)	90.0, 92.8, 90.0	90.0, 90.0, 90.0
Resolution (Å) ^b	45.34–2.60 (2.74–2.60)	54.10–2.60 (2.74–2.60)
<i>R</i> _{sym} or <i>R</i> _{merge}	0.100 (0.560)	0.194 (1.039)
<i>I</i> / σ <i>I</i>	7.4 (1.9)	9.6 (2.3)
Completeness (%)	95.1 (97.0)	99.2 (99.0)
Redundancy	3.4 (3.4)	9.7 (9.8)
Refinement		
Resolution (Å)	64.9–2.6	125.8–2.6
No. reflections	13562	23995
<i>R</i> _{work} / <i>R</i> _{free}	0.259/0.312	0.239/0.271
No. atoms		
All	2446	4542
Proteins	2401	4404
Ligands	36	72
Lipids	0	50
Waters	9	16
B factors		
All	55.0	39.6
Proteins	54.9	39.6
Ligands	62.9	33.3
Lipids	n/a	49.1
Waters	43.0	33.6
R.m.s. deviations		
Bond lengths (Å)	0.006	0.006
Bond angles (°)	1.02	1.02

n/a, not applicable; R.m.s., root mean square.

^aStructures were determined from data collected from two crystals for each space group.^bHighest resolution shell is shown in parentheses.

similar to the NECA-bound A_{2A}R-GL31 structure. As the model for molecule A of the orthorhombic form was the most complete, and as the structures from the two different space groups were very similar (overall RMSD 0.25 Å), further discussion will focus on molecule A of the CGS21680-bound A_{2A}R-GL31 structure determined from the orthorhombic space group, as this was considered to be the most representative structure of A_{2A}R-GL31 bound to CGS21680.

The overall conformation of CGS21680-bound A_{2A}R-GL31 is the same, within experimental error, as the structures of A_{2A}R-GL31 bound to either adenosine or NECA, which, with the exception of the extracellular loops, are virtually identical to the conformation of the wild-type receptor bound to UK432097. This conformation of the receptor is best described as “active-like” (Lebon et al., 2011b). The major internal rearrangements of side chains and α -helices characteristic of agonist binding are observed in the receptor core within all of these structures, but the opening of the cytoplasmic face to allow G protein coupling has not occurred to the same extent as observed in the β_2 -adrenergic receptor bound to a heterotrimeric G protein (Kobilka, 2011; Rasmussen et al., 2011b; Lebon et al., 2012). Thus, the structures of agonist-bound A_{2A}R represent a thermodynamically stable conformation primed to bind G proteins upon movement of the cytoplasmic end of TM6, presumably through either thermal fluctuations or by induced fit.

The Binding Mode of CGS21680. The synthetic agonist CGS21680 is an analog of the natural agonist adenosine with a (2-carboxyethyl)phenylethylamino group on the C2 position of adenine and an *N*-ethylcarboxyamido group on C5' of the ribose (Fig. 1). CGS21680 is a selective agonist of human A_{2A}R that shows weak binding and activation of the human A₁ and A_{2B}

receptors (Jacobson and Gao, 2006). The *N*-ethylcarboxyamido adenosine moiety of CGS21680 occupies the same binding site as described for NECA, with hydrogen bonds between Ser277^{7.42} and His278^{7.43} and the 3' and 2' hydroxyls of the ribose moiety, respectively. N6 of the adenosine moiety makes hydrogen bonds with Asn253^{6.55} and Glu169^{EL2}, whereas Phe168^{EL2} makes strong π -stacking interactions with the adenine ring. Further hydrophobic interactions involving Leu85^{3.33}, Met177^{5.38}, Trp246^{6.48}, Leu249^{6.51}, Met270^{7.35}, and Ile274^{7.39} complete the orthosteric ligand binding pocket (Fig. 3; Supplemental Table 1). The binding mode of the adenosine moiety in CGS21680 is thus identical to the mode of binding of the analogous regions of the agonists adenosine, NECA (Lebon et al., 2011b), and UK432097 (Xu et al., 2011).

Despite the overall similarity in the binding mode of the adenosine moieties of A_{2A}R agonists, the shape of the binding pocket at the extracellular surface differs significantly between the various ligand-bound structures (Fig. 4). In the NECA-bound A_{2A}R structure, seven ordered water molecules form a hydrogen bond network in a wide opening at the extracellular entrance to the ligand binding pocket (Lebon et al., 2011b). In contrast, only a single water molecule resides in the equivalent position of the CGS21680 binding site, which, along with significant van der Waals interactions between the receptor and the (2-carboxyethyl)phenylethylamino moiety, causes the entrance of the ligand pocket to narrow. This is the opposite of that observed in the UK432097 structure, where the bulky extensions to the adenosine moiety cause the entrance to the ligand binding pocket to widen appreciably compared with the NECA-bound structure (Xu et al., 2011). The entrance to the ligand binding pocket is also very open when the inverse agonist

Fig. 2. Electron density for the CGS21680 ligand binding site in the orthorhombic crystal form. 2Fo-Fc electron density map of molecule A viewed from two different perspectives: viewed parallel to the membrane plane to show the density for CGS21680 (A) and viewed from the extracellular side to show Glu169^{EL2} and His264^{EL3} (B). Side chains are shown in stick representation (carbon, green; oxygen, red; nitrogen, blue; sulfur, yellow). Figures were made using CCP4mg and the contour level is 1.2 σ .

ZM241385 is bound, particularly in the structure determined of A_{2A}R in detergent (3PWH; Dore et al., 2011) in comparison with the structure determined in LCP (3EML; Jaakola et al., 2008), which may reflect the different poses of the phenylethylamino moiety observed between the two structures (Fig. 4).

The (2-carboxyethyl)phenylethylamino group of CGS21680 makes van der Waals interactions with mainly three side chains—namely, Leu267^{7.32} at the top of TM7, Glu169^{EL2}, and His264^{EL3} in EL3 (Fig. 3). His264^{EL3} also makes a hydrogen bond with Glu169^{EL2}, thus potentially stabilizing the extracellular structure of the receptor. It is interesting to observe in the

structure of A_{2A}R-GL31 in the monoclinic crystal form that the geometry of the His264^{EL3}-Glu169^{EL2} hydrogen bond is much less favorable, and there is only weak density for His264^{EL3}, Glu169^{EL2}, and the (2-carboxyethyl)phenylethylamino group of CGS21680 (Supplemental Fig. 1). It is clear that the extracellular region can adopt a number of different conformations in different crystal structures (Fig. 5). It is not unknown for structures of GPCRs to exhibit slightly different conformations, even in the same crystal (Moukhametzianov et al., 2011), and it is also known that the extracellular region of many GPCRs is the site at which allosteric modulators of receptors can bind (Wheatley et al., 2012). Thus, the subtle differences in structure observed between CGS21680-bound A_{2A}R in the two different crystal forms may be of some biologic relevance in affecting the equilibrium between the inactive and active states of the receptor.

There are three water molecules (W1, W2, and W3) in the CGS21680-bound A_{2A}R-GL31 structure that have clear electron density and low B factors (Figs. 2 and 3; Supplemental Fig. 3). The water molecule W1 forms hydrogen bonds to the side chain of Ser67^{2.64} and the ribose and adenine moieties of CGS21680 (Fig. 3), and forms a weaker polar interaction with the backbone carbonyl of Ala63, thus forming a bridge between the receptor and the ligand. The top of TM2 is tilted toward the ligand in comparison with the adenosine-bound A_{2A}R structure, with the C α of Ser67^{2.65} shifted by 2 Å. In addition, the rotamer of Ser67^{2.65} has changed with respect to the adenosine-bound structure so that the hydroxyl group is oriented toward the ligand. The water molecule W1 is not resolved in the structure solved from the monoclinic crystal form, in which Ser67^{2.65} adopts a similar position seen in the adenosine-bound structure. The water molecule W2 forms hydrogen bonds with the side chains of amino acid residues Glu169^{EL2}, Asn253^{6.55}, and Thr256^{6.58}, whereas the water molecule W3 forms hydrogen bonds with Met177^{5.38}, His250^{6.52}, and Asn253^{6.55}. W2 and W3 both form hydrogen bonds with Asn253^{6.55}, which probably helps to orient the side chain and position it optimally to form hydrogen bonds with the adenine moiety of the ligand. This is supported by mutagenesis data, which suggest that Asn253^{6.55} is one of the most important amino acid residues for the binding mode of ligands to human A_{2A}R (Kim et al., 2006; Lane et al., 2012). The positions of these three water molecules are also conserved between the structures of A_{2A}R bound to either adenosine, NECA, or ZM241385 (Jaakola et al., 2008; Lebon et al., 2011b) (Supplemental Fig. 3), supporting their importance in ligand binding.

Functional Characterization of the CGS21680 Ligand Binding Site. The structure of CGS21680-bound A_{2A}R-GL31 identified the residues that make direct interactions with the ligand (Fig. 3), which is consistent with the mutagenesis of amino acid residues Leu85^{3.33}, Thr88^{3.36}, Phe168^{EL2}, Leu249^{6.51}, His250^{6.52}, Asn253^{6.55}, Ile274^{7.39}, Ser277^{7.42}, and His278^{7.43}, which significantly reduced the affinity of CGS21680 and/or impaired G protein coupling (Kim et al., 1995; Jiang et al., 1997; Gao et al., 2000; Jaakola et al., 2010). However, the binding mode of the (2-carboxyethyl)phenylethylamino group of CGS21680 was previously unknown. The structure reported here identified contacts between this region of the selective agonist CGS21680 and Ser67^{2.65}, Glu169^{EL2}, His264^{EL3}, and Leu267^{7.32}. These residues were subsequently mutated and the signaling properties of A_{2A}R analyzed to define the contribution of each residue to the activity of CGS21680 (Fig. 6). Mutants of the

Fig. 3. Ligand binding mode of the selective agonist CGS21680. (A) Structure of the extracellular portion of A_{2A}R-GL31 bound to the selective agonist CGS21680. Elements of the structure are depicted as follows: A_{2A}R-GL31, illustration (blue ribbons); specific amino acid side chains, sticks (carbon, blue; oxygen, red; nitrogen, dark blue; sulfur, yellow); CGS21680, sticks (carbon, yellow; nitrogen, dark blue; oxygen, red); ordered water molecules, red spheres; favorable hydrogen bonds, red dashed line; weak hydrogen bonds, blue dashed line (discussed later). (B) Superposition of CGS21680-bound A_{2A}R-GL31 (blue) with NECA-bound A_{2A}R-GL31 (gray). Note the differences at the extracellular end of TM2 and the different rotameric positions of Ser67^{2,64}. (C) Polar and nonpolar interactions between the receptor and CGS21680 are shown for molecule A (see Supplemental Fig. 2 for the comparison with molecule B). Amino acid residues within 3.9 Å of the ligands are depicted and make the following interactions: van der Waals contacts (blue rays), potential hydrogen bonds with favorable geometry (red dashed lines, as identified by HBPLUS (<http://www.ebi.ac.uk/thornton-srv/software/HBPLUS/>); see *Materials and Methods*), hydrogen bonds with unfavorable geometry (blue dashed lines, donor acceptor distance more than 3.6 Å). Where the amino acid residue differs between human A_{2A}R and human A₁R, A_{2B}R, or A₃R, the equivalent residue is shown highlighted in orange, purple, or green, respectively. (A) and (B) were generated using PyMOL.

full-length, wild-type A_{2A}R (S67A^{2,65}, E169A^{EL2}, H264A^{EL3}, and L267A^{7,32}) were transiently transfected into CHO cells, and the level of basal activity was determined in the absence of ligand and the level of G protein coupling was determined in the presence of the full agonists NECA and CGS21680 and the inverse agonist ZM241385. Of the four mutations tested, the mutant L267A^{7,32} showed the biggest reduction (24-fold) in CGS21680 potency, with a nearly 6-fold reduction in potency observed in the mutant H264A^{EL3} (Table 2). There was no significant effect on CGS21680 potency in the mutants S67A^{2,65} or E169A^{EL2}, which was particularly surprising given the reduction in potency observed upon NECA binding (3-fold and 15-fold, respectively). Only the mutant H264A^{EL3} gave similar reductions in potency for both NECA and CGS21680.

A_{2A}R expressed in CHO cells displayed basal activity that was totally inhibited by binding of the potent inverse agonist ZM241385 (Fig. 6). In contrast, the mutants S67A^{2,65}, E169A^{EL2}, and H264A^{EL3} all showed impaired basal activity similar to the levels observed for ZM241385-bound A_{2A}R (Fig. 6). The basal activity of A_{2A}R observed in CHO cells is expected to be directly proportional to the expression levels of cell surface receptors; thus, increasing the number of receptors per cell increased the basal activity measured (Fig. 6; Supplemental Figs. 4 and 5). However, when the expression levels of the mutants S67A^{2,65}, E169A^{EL2}, and H264A^{EL3} were increased, there was no detectable increase in basal activity, and it remained at levels similar to that observed for ZM241385-bound A_{2A}R (Fig. 6; Supplemental Fig. 5). The effect of the mutation

Fig. 4. Comparison of the ligand binding site of the human A_{2A}R bound to various ligands (PDB IDs are shown in parentheses): NECA (2YDV) (A), CGS21680 (B), UK432097 (3QAK) (C), and ZM241385 (3EML and 3PWH, respectively) (D and E). The receptor is viewed as a slice perpendicular to the membrane plane through the binding pocket, with surfaces shown in color and water molecules represented as red spheres. Figures were generated using PyMOL.

Fig. 5. Comparison of receptor-ligand interactions in EL2 and EL3. Structures shown are a portion of the extracellular region of A_{2A}R (gray illustration) and the bound ligand (sticks: carbon, yellow; nitrogen, blue; oxygen, red) (PDB IDs are shown in parentheses): CGS21680 (A); NECA (2YDV) (B); adenosine (2YDO) (C); UK432097 (3QAK) (D); ZM241385 (3EML) (E); and ZM241385 (3PWH) (F). Selected amino acid residues in van der Waals contact with CGS21680 are shown in all of the structures (Ser67^{2.65}, Leu267^{7.32}, His264^{EL3}, Glu169^{EL2}). His264^{EL3} and Glu169^{EL2} interact through a hydrogen bond that is absent in UK432097-bound A_{2A}R, due to the steric effects of the large ligand, and in one of the ZM241385-bound structures (3PWH). Figures were generated using PyMOL.

L267A^{7.32} was less pronounced than that observed for the other three mutations, but was still significant. These data suggest that residues in the extracellular region of the A_{2A}R play an important role in dictating the basal activity of the receptor.

Discussion

The structure of CGS21680-bound A_{2A}R was determined by crystallization of the thermostable mutant A_{2A}R-GL31 using the LCP technique. T4L or BRIL fusion proteins were not required, as was demonstrated previously for the crystallization of the β_1 -adrenergic receptor in LCP (Miller-Gallacher et al., 2014). The chemical structure of CGS21680 is very similar to NECA, except that there is a (2-carboxyethyl)phenylethylamino extension that, when the ligand is bound to A_{2A}R, protrudes out

of the adenosine binding pocket into a pocket formed by EL2, EL3, and the extracellular portions of TM2 and TM7. The extracellular regions are the most divergent in sequence between adenosine receptor subtypes (Supplemental Fig. 6), so it is not entirely surprising that CGS21680 may gain at least some of its subtype specificity from binding to amino acid residue side chains that are nonconserved (Ser67^{2.65}, Glu169^{EL2}, His264^{EL3}, and Leu267^{7.32}). This is highlighted by the high selectivity of CGS21680 for A_{2A}R compared with A_{2B}R, which is due mainly to differences in the amino acid sequences of EL2. Remarkably, replacement of EL2 in A_{2B}R by EL2 from A_{2A}R allowed the recovery of some binding affinity for CGS21680 and led to activation of heterotrimeric G protein (Seibt et al., 2013). However, defining the relative roles of individual amino acid residues in determining subtype specificity is not clear cut.

Fig. 6. Effects of the agonists CGS21680 and NECA and the inverse agonist ZM241385 on cAMP production in CHO cells transiently transfected with A_{2A}R. Each panel shows the response of the wild-type receptor (circles, dashed lines) compared with the mutant receptor specified (squares, continuous lines): S67A^{2.65} (A), E169A^{EL2} (B), H264A^{EL3} (C), and L267A^{7.32} (D). In each panel, the dose-response curves for the mutant are compared with the wild-type receptor for the following ligands: NECA (green), CGS21680 (red), and the inverse agonist ZM241385 (blue). Data are normalized as a percentage of the maximum and minimum responses observed in the NECA-fitted dose-response curve.

TABLE 2
Comparison of the cAMP dose response induced by NECA and CGS21680 in A_{2A}R mutants

	NECA		CGS21680	
	pEC ₅₀ ^a	Change in EC ₅₀ Relative to WT ^b	pEC ₅₀ ^a	Change in EC ₅₀ Relative to WT
WT	9.05 ± 0.05	1	8.27 ± 0.29	1
S67A	8.50 ± 0.14	3.4	8.40 ± 0.23	0.62
E169A	7.87 ± 0.18****	15.1	8.07 ± 0.18	1.58
H264A	8.18 ± 0.17**	7.4	7.52 ± 0.20	5.62
L267A	8.81 ± 0.08	1.7	9.66 ± 0.19**	0.04

WT, wild type.

^apEC₅₀ values are expressed as the mean ± S.E.M. calculated from four independent experiments, each performed in triplicate. Values are stated as significantly different from the wild-type receptor using a one-way analysis of variance with Dunnett's post test.

P < 0.005; **P < 0.0001.

The mutations S67A^{2.65} and E169A^{EL2} did not affect the potency of CGS21680, although there were 3-fold and 24-fold reductions in potency for the mutations H264A^{EL3} and L267A^{7.32}, as previously reported for the H264A^{EL3} and E169A^{EL2} mutants (Lane et al., 2012). In addition, the results were different when the effect on the potency of NECA was measured: S67A^{2.65}, E169A^{EL2}, and H264A^{EL3} all affected potency, whereas L267A^{7.32} did not. The different results obtained for CGS21680 and NECA are probably partly a reflection of the subtly different conformations of the extracellular region of A_{2A}R when bound to either ligand, such as the inward tilt of the extracellular region of TM2 when CGS21680 is bound that narrows the entrance to the ligand binding pocket. However, the dynamics of the whole extracellular region may have a greater influence.

The dynamic nature of the extracellular region was suggested from analysis of the monoclinic crystal form of CGS21680-bound A_{2A}R. CGS21680 was bound in a slightly different conformation compared with that observed in the orthorhombic crystal form, similar to that observed in NECA-bound A_{2A}R, and the density for the extracellular region was very weak, suggesting that this region was poorly ordered, presumably due to its high flexibility. This is consistent with the weak reduction in affinity for CGS21680 binding when amino acid residues making contacts with the (2-carboxyethyl)phenylethylamino group were mutated to alanine. That the extracellular region can adopt multiple conformations, and that this can have an impact on ligand binding is observed upon comparison of the two structures of A_{2A}R bound to ZM241385 (PDB IDs 3EML and 3PWH; Jaakola et al., 2008; Dore et al., 2011). ZM241385 contains a phenylethylamino extension from the triazolotriazine core of the ligand, analogous to the (2-carboxyethyl)phenylethylamino extension on CGS21680, which adopts different conformations in the two A_{2A}R crystal structures (Fig. 7). This may be a result of the potential pH sensitivity of the salt bridge between Glu169^{EL2} and His264^{EL3} that is present in the A_{2A}R structure determined at pH 5.5–6.5 (3EML) but is broken in the structure determined at pH 8–8.75 (3PWH). It is known that agonist binding to the A_{2A}R is pH sensitive (Askalan and Richardson, 1994; Hiley et al., 1995), with higher affinities observed at a lower pH, and with the pH-sensitive amino acid residue proposed to be a histidine based on the implied pK value of 7.0 (Askalan and Richardson, 1994). Further work will be required to establish whether His264^{EL3} is indeed mediating the pH effect observed in A_{2A}R. Consideration of the previous factors thus suggests that it is conceivable that the crystal structure of CGS21680-bound A_{2A}R represents only one of a number of different poses of the

(2-carboxyethyl)phenylethylamino moiety, which may go some way to explain the negligible effect of the alanine substitutions of Ser67^{2.65} and Glu169^{EL2} upon the binding of CGS21680. Another confounding factor that precludes a full understanding of the reasons for the specificity of CGS21680 is that the high sequence divergence of the extracellular regions of the A₁, A_{2A}, A_{2B}, and A₃ receptors suggests that the structures of these regions may be different from the extracellular region of A_{2A}R. Thus, further insights will only be gained once the structures of the A₁, A_{2B}, and A₃ receptors have been determined.

Analysis of the A_{2A}R mutants in the extracellular region of the receptor resulted in the surprising observation that the basal activity of the receptor could be abolished by single amino acid changes. Specifically, the mutants S67A^{2.65}, E169A^{EL2}, and H264A^{EL3} all showed virtually no basal activity even when expression levels of the receptor were highly elevated. A lesser effect was observed for the mutant L267A^{7.32}. This is consistent with the concept of allosteric modulators of receptors binding to analogous regions at the top of the orthosteric binding site in other receptors, such as the adenosine A₁ receptor (Peeters et al., 2012) and the muscarinic M₂ receptor (Kruse et al., 2013). It is difficult to rationalize why these mutations significantly reduce basal activity, but it has

Fig. 7. Comparison of the binding mode of ZM241385 in two different A_{2A}R structures. Structures of ZM241385-bound A_{2A}R crystallized at either pH 5.5–6.5 (PDB ID 3EML; receptor in gray and ZM241385 in pink) or pH 8–8.75 (PDB ID 3PWH; receptor in rainbow coloration and ZM241385 in yellow) were compared in PyMOL and displayed in the illustrated representation. The different conformations of the ligands and the residues involved in salt bridge formation are highlighted by double-headed arrows.

been consistently observed that the binding of small agonists (adenosine and NECA) results in a contraction of the ligand binding pocket (Lebon et al., 2011b), as observed in the β -adrenergic receptors (Rasmussen et al., 2011a; Warne et al., 2011), and the closure of the extracellular loops over the entrance of the binding pocket. This may be facilitated by the interaction between Glu169^{EL2} and His264^{EL3} that form a hydrogen bond upon agonist binding, which would clearly be absent upon mutating either residue to Ala. Orthologous techniques for crystallography such as NMR, spin labeling, and molecular dynamics simulations will be needed to further study the dynamics of the extracellular region for additional insights into this interesting phenomenon.

Coordinates and structure factors have been deposited with the Protein Data Bank, PDB ID [4UG2](#) for the orthorhombic crystal form and [4UHR](#) for the monoclinic crystal form.

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Authorship Contributions

Participated in research design: Lebon, Tate.

Conducted experiments: Lebon, Edwards.

Performed data analysis: Lebon, Leslie.

Wrote or contributed to the writing of the manuscript: Lebon, Leslie, Tate.

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