The second extracellular loop of GPCRs determines subtype-selectivity and controls efficacy as evidenced by loop exchange study at A₂ adenosine receptors

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The second extracellular loop (EL2) of G protein-coupled receptors (GPCRs), which represent important drug targets, may be involved in ligand recognition and receptor activation. We studied the closely related adenosine receptor (AR) subtypes A₂A and A₂B by exchanging the complete EL2 of the human A₂B AR for the EL2 of the A₂A AR. Furthermore, single amino acid residues [Asp14845.27, Ser14945.28, Thr15145.30, Glu16445.43, Ser16545.44, and Val16945.48] in the EL2 of the A₂A AR were exchanged for alanine. The single mutations did not lead to any major effects, except for the T151A mutant, at which NECA showed considerably increased efficacy. The loop exchange entailed significant effects: The A₂A-selective agonist CGS21680, while being completely inactive at A₂A ARs, showed high affinity for the mutant A₂B(EL2-A₂A)AR, and was able to fully activate the receptor. Most strikingly, all agonists investigated (adenosine, NECA, BAY60-6583, CGS21680) showed strongly increased efficiencies at the mutant A₂B(EL2-A₂A) as compared to the wt AR. Thus, the EL2 of the A₂B AR appears to have multiple functions: besides its involvement in ligand binding and subtype selectivity it modulates agonist-bound receptor conformations thereby controlling signalling efficacy. This role of the EL2 is likely to extend to other members of the GPCR family, and the EL2 of GPCRs appears to be an attractive target structure for drugs.

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1. Introduction

G protein-coupled receptors (GPCRs) are a major class of targets for drugs on the market as well as for those presently in development. More than one third of current therapeutics are directed towards GPCRs [1]. GPCRs are divided into six different receptor classes (A to F) among which class A is the largest one comprising all rhodopsin-like GPCRs. In recent years many class A GPCRs have been co-crystallized with a variety of ligands ranging from inverse agonists/antagonists to full agonists [2–4]. Furthermore, the crystal structure of ternary complexes consisting of the agonist-bound β₂ adrenergic receptor and Gα protein, and of rhodopsin bound to a fragment of the Gα protein have been described revealing the conformational changes during receptor activation [5,6]. Each crystal structure provides a snapshot of a specific conformational stage along the activation process of the receptor. By comparing the X-ray structures of GPCRs from different families global patterns of conformational changes leading to receptor activation have emerged [2,3,7–10]. X-ray structures also provide insights into the respective binding pockets and allow the optimization of homology models that are highly useful for virtual screening [11–13]. This is particularly true for homology models of closely related receptor subtypes. One example is the A₂B adenosine receptor (A₂B AR) model [14,15], which has been constructed based on X-ray structures of the closely related A₂A AR [16,17].

Adenosine receptors (ARs) are members of class A GPCRs, subdivided into four distinct subtypes: A₁, A₂A, A₂B, and A₃. A₁ and A₂ ARs are Gα₁-coupled receptors whereas A₂A and A₂B ARs are coupled to Gα proteins thereby mediating an increase in intracellular CAMP levels. Furthermore, coupling of the A₁, A₂B, and A₃ ARs to Gα has been found in several cell types [18].

Due to their ubiquitous distribution throughout the body, ARs are involved in many physiological and pathophysiological processes. Ligands for ARs are therefore of considerable interest as novel drugs [18–20]. The A₂B AR subtype mediates antiinflammatory and immunosuppressive effects [21]. A₂A AR antagonists

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are currently in advanced clinical trials for Parkinson’s disease [22]; furthermore, they may be active in Alzheimer’s disease, and for the treatment of addiction and depression [18–20,23]. For the A2AAR subtype, antagonists are being developed and evaluated for the treatment of asthma and bronchial inflammation [24]. Further potential indications include cancer, diabetes, (neuro) inflammation, and Alzheimer’s disease [23,25,26].

The endogenous ligand adenosine (1, Fig. 1) and the vast majority of its derivatives, e.g. NECA (2) and CGS21680 (3), show considerably higher affinity for the human A2AAR than for the A2BAR. The reason for this and the amino acids involved are yet unknown. Sequence analysis of the human A2A and A2B ARs show an overall identity of 58% and a similarity of 73%, whereas the EL1 and especially the EL2 are less conserved exhibiting 44% and 34% identity, and 56% and 46% similarity, respectively [14,20].

In many GPCRs the EL2 is not well conserved regarding length, amino acid composition, and number of disulfide bonds. Nevertheless it participates in ligand recognition and binding as evidenced by crystal structures [27,28]. Meanwhile several X-ray structures of the human A2AAR are available, including inactive, antagonist-bound conformations (ZM241385, 3EMI [16]; XAC, 3REY and caffeine, 3RFM [29]), intermediate states, which are NECA (2YDV, [30]) and adenosine-bound (2YDO, [30]), respectively, and the fully activated UK-432097-bound conformation (3QAK, [17]). The resolutions of the crystal structures are in the same range for all structures with 2.6 Å for the inactive and NECA-bound structure, 2.7 Å for the fully active structure 3QAK, and 3.0 Å for the adenosine-bound structure, and 3.6 Å for the caffeine-bound structure 3RFM [16,17,29,30]. Only the most recent structure of the A2AAR in complex with ZM241385 (4EIVY), shows a significantly higher resolution of 1.8 Å [31]. In this structure several ordered water clusters, as well as lipids and cholesterol molecules are resolved. Except for the two transition-state structures 2YDV and 2YDO [30] and the inactive structure 4EIV [31], where the extracellular loops, especially EL2, are completely resolved, all other structures show very low loop resolution or incomplete loops. From the EL2 only the part close to TM5, containing the cysteine residues involved in disulfide bonds and the stabilizing β-sheet, which is also the most conserved part of the EL2, are resolved in all structures of the A2AAR, except for 3V9, where the β-sheet is missing [16,17,29–33].

Since each crystal structure only provides one single, fixed receptor conformation, and active conformations and loop structures are in most cases poorly resolved, mutagenesis studies are still the method of choice to investigate the role and the involvement of particular amino acids or whole loop structures. Furthermore, solving X-ray structures for all subtypes of a particular GPCR subfamily may be too costly and not practical [3], and for the A2B AR no X-ray structure is available.

In the present study we investigated the role of the EL2 of the A2B AR by exchanging the whole loop by the EL2 of the closely related A2AAR. Moreover, we investigated the role of single amino acids in the EL2 by exchanging them for alanine. Finally we performed homology modelling studies of the A2B AR based on recently published X-ray structural data of the A2AAR. Our main goal was to find explanations (i) for the relatively low affinity and potency of adenosine and its derivatives at A2B ARs in comparison to the closely related A2AAR subtype, and about subtype-selectivity for A2A versus A2B ARs, and (ii) to get more insight into the roles of the EL2 in GPCR activation by comparing two closely related receptors, which differ considerably in their EL2 length and sequence. By a complete loop exchange approach of closely related GPCRs we learned that the EL2 is not only involved in ligand recognition and receptor subtype selectivity, but beyond that, it plays an important role in receptor activation and ligand efficacy.

2. Methods

Chemicals were obtained from Roth (Karlsruhe, Germany) or AppliChem (Darmstadt, Germany) unless otherwise noted. All enzymes and competent bacteria were obtained from New England Biolabs (Frankfurt, Germany), primers, the vector pUC19, cell culture reagents and supplements, including antibiotics, were obtained from Life Technologies - Invitrogen (Darmstadt, Germany). Radioligands were obtained from Amer sham - GE Healthcare (Frankfurt, Germany).

2.1. Site-directed mutagenesis

The coding sequence for the human A2B AR was cloned into the plasmid vector pUC19. Point mutations leading to the desired amino acid exchanges were introduced by site-directed mutagenesis using the polymerase chain reaction (PCR). Complementary oligonucleotide primers were designed containing the corresponding mutations. Each mismatching base in this primer was flanked by 14–15 nucleotides at the 3’- and 5’-end. The PCR reaction mixture contained 20 ng of template DNA, 15 pmol of each, sense and antisense primer, 10 mM dNTPs, 1 × Thermopol reaction buffer and 1 U Vent polymerase (New England Biolabs, Frankfurt, Germany). PCR was performed as follows: 4 min at 94 °C, 20 cycles
consisting of 1 min at 94 °C, 1 min at 66 °C and 10 min at 72 °C followed by a final elongation step of 10 min at 72 °C. After digest with DpnI, the DNA was transformed into _Escherichia coli_ Top10 and isolated from individual clones. Sequencing was performed by GATC Biotech (Konstanz, Germany) [34]. Mutated receptor DNA was subsequently subcloned into the retroviral plasmid vector pLXS1 (Clontech, Heidelberg, Germany). Then transformation, isolation and sequencing of the newly constructed plasmids was performed.

### 2.2. Overlap extension mutagenesis

The complete second extracellular loop (EL2) of the human A<sub>2B</sub>AR was replaced by the EL2 of the A<sub>2A</sub>AR by overlap extension mutagenesis [35] PCR. The human A<sub>2A</sub>AR was cloned into the vector pLXS1. Seven primers with 20 or 21 bases each coding for a part of the EL2 of the human A<sub>2A</sub>AR, were designed. Half of the first primer was complementary to the plasmid and the other part represented an overhanging sequence, which coded for a part of the EL2 of the human A<sub>2A</sub>AR (EL2-A2A). In the following step a second primer hybridized to this overhanging part. PCR was performed from both sides of the loop in different reaction tubes. Both products had an overhanging part (16 bases) in the middle of the EL2. Subsequently overlap extension PCR was performed with a mixture containing 20 ng of template DNA of both products (see above), 10 mM dNTPs, 10 × KOD buffer, 1 U KOD Hot Start Polymerase and 25 mmol MgSO<sub>4</sub>; 4 min at 94 °C, 5 cycles consisting of 45 s at 94 °C, 45 s at 55 °C and 1 min at 72 °C followed by a final elongation step of 4 min at 72 °C. Then the primers for the amplification were added and the following PCR was performed: 4 min at 94 °C, 25 cycles consisting of 1 min at 94 °C, 1 min at 66 °C and 10 min at 72 °C followed by a final elongation step at 72 °C for 10 min. The DNA was transformed into _Escherichia coli_ Top10, isolated and sequenced.

### 2.3. Retroviral transfection of Chinese Hamster Ovary K1 cells

GP + envAM12 packaging cells [36] were plated into flasks 24 h before transfection using DMEM containing 10 % FCS, 100 μl penicillin G, 100 μg/ml streptomycin and 1 % ultraglutamine. The cells were then transfected using Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany). In order to increase infection efficiency, receptor DNA (63 %) was mixed with vesicular stomatitis virus G protein (VSV-G) DNA (37 %) [37] prior to transfection. After 15 h the medium was replaced by 3 ml of medium containing 5 mM sodium butyrate. The transfected GP + envAM12 cells were cultured at 32 °C and 5 % CO<sub>2</sub> for 48 h. The supernatant of the GP + envAm12 cells and 2 ml of the virus-containing solution was mixed with 4 μl of polybrene (4 mg/ml) and incubated for 2.5 h at 37 °C with CHO K1 cells. After 48 h these cells were selected for genetinic resistance in the presence of 800 μg/ml G418 [34].

### 3.1. Membrane preparations for radioligand binding assays

Membranes of CHO cells expressing the human A<sub>2B</sub>AR, human A<sub>2A</sub>AR mutants, or the human A<sub>2A</sub>AR, respectively, were prepared by adding ice-cold hypotonic buffer (5 mM Tris–HCl, 2 mM EDTA, pH 7.4) to the previously frozen cell culture dishes, and scratching the cells off. The resulting cell suspension was homogenized on ice for 20 s using an Ultra-Turrax® and subsequently spun down for 10 min (4 °C) at 1000 × g. The supernatant was then centrifuged for 60 min at 48,000 × g. The obtained membrane pellets were resuspended in 50 mM Tris–HCl buffer, pH 7.4, and again centrifuged under the same conditions. Then the membrane pellets were resuspended and homogenized in the required amount of 50 mM Tris–HCl buffer, pH 7.4, to obtain a protein concentration of 1–3 mg/ml. Aliquots of the membrane preparation were stored at -80 °C until used [38].

### 3.2. Radioligand binding assays

Competition experiments with [3H]PSB-603 were performed in a final volume of 500 μl containing 25 μl of test compound dissolved in 50 % DMSO/50 % Tris–HCl buffer (50 mM, pH 7.4), 275 μl buffer (50 mM Tris–HCl, pH 7.4), 100 μl of radioligand solution in the same buffer (final concentration 0.3 nM) and 100 μl of membrane preparation (30 μg protein per tube in buffer containing adenosine deaminase (2 U/ml, 20 min preincubation). Nonspecific binding was determined in the presence of 10 μM 8-cyclopentyl-1,3-dipropylxanthine (DPCPX). After an incubation time of 75 min at room temperature, the assay mixture was filtered through GF/B glass fibre filters using a Brandel harvester (Brandel, Gaithersburg, USA). Filters were washed four times (3–4 ml each) with ice-cold 50 mM Tris–HCl buffer, pH 7.4, containing 0.1 % bovine serum albumin (BSA). Then filters were transferred to minivials, incubated for 6 h with 2.5 ml of scintillation cocktail (LumaSafe plus, Perkin-Elmer, Rodgau, Germany) and counted in a liquid scintillation counter (TRICARB 2900TR, Packard/Perkin-Elmer, Rodgau, Germany) with a counting efficiency of 51 %. Three to six separate experiments were performed each in duplicates.

### 4. cAMP assays

CHO cells were seeded into a 24-well plate at a density of 200,000 cells/well 24 h before performing the cAMP assay. Cells were washed with Hank’s Balanced Salt Solution (HBSS; 20 mM HEPES, 135 mM NaCl, 5.5 mM glucose, 5.4 mM KCl, 4.2 mM NaHCO<sub>3</sub>, 1.25 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 0.8 mM MgSO<sub>4</sub>, 0.44 mM KH<sub>2</sub>P<sub>4</sub> and 0.34 mM Na<sub>2</sub>HPO<sub>4</sub>, pH adjusted to 7.3) with 1 U/ml of adenosine deaminase (ADA, Sigma–Aldrich, Taufkirchen, Germany). Then the cells were incubated in 300 μl HBSS at 37 °C and 5 % CO<sub>2</sub> for 2 h. ADA was omitted for experiments in which adenosine was used as an agonist. The phosphodiesterase inhibitor Ro20-1724 (Hoffmann La Roche, Grenzach, Germany; 100 μl, final concentration: 40 μM) was added to the CHO cells and they were incubated for 15 min at 37 °C and 5 % CO<sub>2</sub>. Then 100 μl of various dilutions of agonist (adenosine (Sigma–Aldrich, Taufkirchen, Germany), 5′-N-ethylcarboxamidoadenosine (NECA; Sigma–Aldrich, Taufkirchen, Germany), BAY60-8583 (Bayer HealthCare, Leverkusen, Germany); or CGS21680 (Tocris, Wiesbaden-Nordenstadt, Germany), respectively) in HBSS containing 5 % DMSO were added and the cells were incubated for 15 min at the same conditions as described above. cAMP formation was stopped by removing the supernatant from the cell suspension and subsequently lysing the cells by the addition of 500 μl of hot lysis buffer (90 °C; 4 mM EDTA, 0.01% Triton X-100, pH adjusted to 7.3). After one hour of incubation on ice, cAMP amounts of the lysates were determined by competitive radioligand binding
experiments. Competition experiments were performed with aliquots of 50 μl of cell lysates, 30 μl of $[^{3}H]$cAMP solution in lysis buffer (final concentration 3 nM) and 40 μl of cAMP binding protein [39] diluted in the same buffer (50 μg of protein per vial). For determining cAMP concentrations instead of cell lysate 50 μl of cAMP solutions containing various cAMP concentrations were added to obtain a standard calibration curve. Total binding was determined in the absence of cAMP and non-specific binding was measured in the absence of binding protein. The mixture was incubated for 60 min on ice and then filtered through GF/B glass fibre filters using a cell harvester (Brandel, Gaithersburg, USA). Filters were washed three times with 2–3 ml of ice-cold 50 mM Tris–HCl buffer, pH 7.4. Radioactivity on the filters was determined in a liquid scintillation counter (TRICARB 2900TR, Packard/Perkin-Elmer, Rodgau, Germany) after 6 h of preincubation with 2.5 ml of scintillation cocktail (LumaSafe plus, Perkin-Elmer, Rodgau, Germany). Three to five independent experiments were performed, each in duplicates. Amounts of cAMP were calculated by linear regression from a standard curve and normalized to the maximal effect induced by 100 μM of forskolin (set at 100%).

4.1. Homology modelling and docking

The original 3D homology model of the human A$_{2B}$AR was based on the inactive, inverse agonist–bound A$_{2A}$AR (3EML) [15,16]. After exchange of the EL2 (the A$_{2A}$ loop was exchanged for the A$_{2B}$ loop) the model was energy-minimized again and subjected to molecular dynamics simulation as previously described [14]. To investigate the binding mode of the A$_{2A}$-selective agonist CGS21680 in the mutant A$_{2B}$[EL2-A$_{2A}$]AR the model was refined based on the recently published X-ray structures, which represent the active receptor conformations, bound to the bulky synthetic agonist UK-432097 (3QAK), NECA (2YDV), and adenosine (2YDO), respectively [17,30]. The refined homology models were created using Modeller and then energy-minimized using GROMACS (3QAK and 2YDV-based models) or created using MOE and also energy-minimized (2YDO-based models). All models were used for molecular docking of CGS21680 using FlexX and AutoDock [40]. In addition CGS21680 was also docked into the X-ray structure 3QAK [17] of the A$_{2A}$ receptor using FlexX of the LeadIT package (BioSolveIT GmbH, St. Augustin, Germany).

Fig. 2. Topology model of the human wt A$_{2A}$AR and the human A$_{2B}$AR in which the second extracellular loop (EL2) is replaced by the EL2 of the human A$_{2A}$AR (abbreviated as A$_{2B}$[EL2-A$_{2A}$]). The larger loop (black) represents that of the A$_{2A}$AR, the smaller loop (blue) that of the A$_{2B}$AR. The following amino acid residues of the A$_{2B}$AR were exchanged for alanine by site directed mutagenesis and are highlighted: green D, Asp148<sup>46.2</sup>; blue: S, Ser165<sup>45.44</sup>; grey: T, Thr151<sup>45.39</sup>; red: E, Glu164<sup>45.45</sup>; pink: S, Ser165<sup>45.44</sup>, and purple: V, Val169<sup>45.46</sup>. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

![Fig. 2. Topology model of the human wt A$_{2A}$AR and the human A$_{2B}$AR in which the second extracellular loop (EL2) is replaced by the EL2 of the human A$_{2A}$AR (abbreviated as A$_{2B}$[EL2-A$_{2A}$]).](image)

Fig. 3. Alignment of the second extracellular loop of mammalian A$_{2A}$Rs. (h) human; (m) mouse; (r) rat; (*) identical amino acid residue; (;) conserved amino acid substitution; (,) semi-conserved amino acid substitution. The following amino acids are highlighted: framed: amino acid residues from adjacent transmembrane domains 4 and 5 (TM4, TM5); cyan highlighted: conserved cysteine residue (Cys45.50), involved in the essential disulfide bond with Cys3.25; bold: amino acid residues which were exchanged in the loop exchange A$_{2A}$ receptor mutant; underlined: amino acid residues forming β-sheets. The following amino acid residues were exchanged for alanine and single mutants were generated: green D, Asp148<sup>46.2</sup>; blue: S, Ser165<sup>45.44</sup>; grey: T, Thr151<sup>45.39</sup>; red: E, Glu164<sup>45.45</sup>; pink: S, Ser165<sup>45.44</sup>, and purple: V, Val169<sup>45.46</sup>. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

![Fig. 3. Alignment of the second extracellular loop of mammalian A$_{2A}$Rs.](image)
5. Data analysis

All data were analyzed by GraphPad Prism version 4.02 for Windows, GraphPad Software, San Diego California USA [38]. For curve fitting standard equations offered by Prism for one-site competition and two-site fits were used. The one-site fit was taken for the calculation, since the two-site fit did not lead to meaningful results. Levels of significance were determined using a two-tailed t-test.

6. Results

6.1. The second extracellular loop of the human A2 adenosine receptors

By comparing the EL2 of the human AR subtypes A2A and A2B, which show an overall similarity of 46% and an identity of 34% one can find high degrees of similarity at the region close to TM5, especially between the β-sheet and TM5 (see Figs. 2 and 3). The β-sheet is found in all A2AR X-ray structures [16,17,29,30], where it is in anti-parallel conformation with a second β-sheet in EL1. Both sheets are predicted to be present in the human A2B as well [14], being probably stabilized by the conserved disulfide bond between cysteine residues Cys45.50 in EL2 and Cys3.25 located in the extracellular part of TM3. The highly conserved cysteine residue in the EL2 gets the position number 45.50 in analogy to the Ballesteros-Weinstein nomenclature, where the most conserved residue of each helix is designated residue number 50, and amino acids are numbered in relation to these residues [27,41]. As shown in the alignment in Fig. 3 comparing the rat, mouse and human sequences, and in Fig. 4 comparing 14 mammalian A2A and A2B EL2 sequences, it becomes evident that mammalian A2A and A2B show high similarity between Cys45.50 and TM5. This region, which is highly conserved throughout evolution, is 86% identical and 100% similar between mammals. In contrast, as can be seen in the alignments (Figs. 3 and 4) even among the orthologs of individual subtypes, a large diversity in the EL2 region close to TM4 can be found. There is also some variation in length within and between the individual subtypes (see Figs. 3 and 4). The EL2 of the A2AR is already longer than that of other class A GPCRs [27]. The EL2 of the A2AR is even four amino acids longer than the EL2 of the A2B. It contains a particularly high number of cysteine residues, namely four. The A2B contains three cysteines in the EL2, all of which are involved in disulfide bond formation as demonstrated by the X-ray structures [16,17,29–33]. In the A2B only one of the cysteine residues, Cys45.50, is involved in an essential disulfide bond [14]. The only amino acid residues conserved in the variable part of the EL2 are the cysteine residues and the asparagine residues of the potential glycosylation sites.

The complete EL2 of the human A2BAR was exchanged for the corresponding loop in the human A2BAR subtype resulting in the loop mutant receptor A2B(RL2-A2A). Furthermore, five amino acids bearing functional groups (Asp148[45,27], Ser149[45,30], Thr151[45,30], Glu164[45,42], Ser165[45,42]), in addition to Val169[45,42], in the EL2 of the human A2BAR were exchanged for alanine in order to investigate their potential role in ligand binding and/or receptor activation (see Fig. 2). All receptors were stably expressed in Chinese hamster ovary cells. Four different agonists were used to characterize the effects of the loop exchange: the physiological agonist adenosine (1), its close, but metabolically more stable analog NECA (2), the A2A-selective agonist CGS21680 (3), and the A2A-selective agonist BAY50-6583 (4). Agonists 2 and 3 are nucleosides derived from adenosine (1), while 4 is a non-nucleoside agonist with an amidopyridine structure. The A2B-selective antagonist PSB-603 (5) served as a radioligand in
Table 1

Expression levels and $K_D$ values of wild type and mutant $A_{2B}$ receptors determined by homologous competition of PSB-603 vs. $[\text{3H}]$PSB-603. Data are mean ± SEM of three independent experiments unless otherwise noted.

<table>
<thead>
<tr>
<th></th>
<th>$B_{max}$ ± SEM (fmol/mg of protein)</th>
<th>$K_D$ ± SEM (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt $hA_{2B}$</td>
<td>271 ± 45$^{a}$</td>
<td>0.306 ± 0.117$^*$</td>
</tr>
<tr>
<td>wt $hA_{2A}$</td>
<td>390 ± 157$^{c,m}$</td>
<td>2.03 ± 1.05$^{m}$</td>
</tr>
<tr>
<td>$hA_{2B}$ (EL2-A2A)</td>
<td>65 ± 12$^{c,m}$</td>
<td>0.127 ± 0.043$^{m}$</td>
</tr>
<tr>
<td>D148A</td>
<td>458 ± 41$^{m}$</td>
<td>0.364 ± 0.088$^{m}$</td>
</tr>
<tr>
<td>S149A</td>
<td>1220 ± 259$^{c}$</td>
<td>0.488 ± 0.089$^{c}$</td>
</tr>
<tr>
<td>T151A</td>
<td>322 ± 11$^{m}$</td>
<td>0.467 ± 0.207$^{m}$</td>
</tr>
<tr>
<td>E164A</td>
<td>894 ± 59$^{m}$</td>
<td>0.470 ± 0.088$^{m}$</td>
</tr>
<tr>
<td>S165A</td>
<td>326 ± 27$^{m}$</td>
<td>0.282 ± 0.110$^{m}$</td>
</tr>
<tr>
<td>V169A</td>
<td>141 ± 19$^{d,ms}$</td>
<td>0.998 ± 0.231$^{d,ms}$</td>
</tr>
</tbody>
</table>

$a$ $n$ = 8.

$^{b}$ Determined using MSX-2 vs $[\text{3H}]$MSX-2.

$^{c}$ $n$ = 5.

$^{d}$ $n$ = 4.

$^{m}$ Not significantly different from wildtype $A_{2B}$AR (determined using the two-tailed t-test).

$^*$ $p < 0.05$ (determined using the two-tailed t-test).

$^*$ $p < 0.01$ (determined using the two-tailed t-test).

7. Radioligand binding

Radioligand binding assays were performed using membrane preparations of cells recombinantly expressing either the wt or the mutant $A_{2B}$ARs. Homologous competition assays using unlabeled PSB-603 versus $[\text{3H}]$PSB-603 as a radioligand allowed to estimate the expression levels of the wt and mutant receptors as well as the calculation of the $K_D$ values (see Table 1). The expression of the wt $A_{2A}$ and $A_{2B}$ARs was similar ($A_{2A}$, 390 ± 167 fmol/mg protein; $A_{2B}$, 271 ± 85 fmol/mg protein). The levels of most of the $A_{2B}$ mutant receptors did not show significant differences in expression levels from that of the wt receptor (see Table 1). Only the S149A (1220 ± 250 fmol/mg protein) and the E164A mutant (894 ± 59 fmol/mg protein) exhibited significantly higher expression as compared to the wt $A_{2B}$AR. The lowest expression level was observed for the loop exchange mutant $A_{2B}$ (EL2-A2A) (65 fmol/mg protein), which was 4-fold lower than that of the wt $A_{2B}$AR, the difference, however, did not reach statistical significance. The $K_D$ values determined for the mutant receptors were in the range of 0.1 to $\text{3H}$-labelled form (for structures of compounds see Fig. 1) and was also used in unlabeled form for homologous competition studies.

Fig. 5. Competition binding studies at the human wt $A_{2B}$AR and the $A_{2B}$ (EL2-A2A)AR mutant versus $[\text{3H}]$PSB-603 (0.3 nM). Membrane preparations from CHO cells stably expressing the receptors were used. Graph D shows for the wt $A_{2B}$AR a solid line (measured up to 180 μM CGS21680) and a dashed line (extrapolated); because of the limited solubility of CGS21680 higher concentrations could not be measured. Data points represent means ± SEM of at least three independent experiments performed in duplicates. $K_I$ values are listed in Table 2.
0.9 nM and not significantly different from the wt A2BAR (0.306 ± 0.117 nM, see Table 1). Since no agonist radioligand for A2BARs is available yet competition studies could only be performed versus the antagonist radioligand [3H]PSB-603 (Fig. 5 and Table 2). The antagonist PSB-603 itself showed similar affinity at the wt and the mutant A2BAR (EL2-A2A) receptor, exhibiting no significant difference in $K_i$ values (0.370 nM, and 0.115 nM, respectively, Fig. 5A and Table 2). The same was true for the adenosine derivative NECA (Fig. 5B), which showed an $K_i$ value of about 1 uM for both, wt and mutant A2BAR. The non-nucleosidic A2B-selective agonist BAY60-6583, however, displayed 12-fold higher affinity for the mutant as compared to the wt A2BAR. The same was true for the A2A-selective adenosine derivative CGS21680, which had virtually no affinity for the wt A2BAR (estimated $K_i$ value 98 μM), but showed >200-fold increased affinity for the mutant A2BAR (EL2-A2A) receptor. This difference was statistically highly significant (Table 2). Adenosine itself could not be investigated in binding studies since adenosine deaminase had to be present to remove endogenous adenosine present in the membrane preparations.

The A2B mutant receptors, in which selected single amino acids in the EL2 were exchanged for alanine, likewise did not show significantly different affinities for the antagonist PSB-603 (Table 2, for curves see Fig. 6). The nucleosidic agonist NECA, and the non-nucleosidic agonist BAY60-6583 also showed virtually identical affinity for each of the mutant as well as for the wt A2BAR (Table 2), with one minor exception. The S149A mutant appeared to exhibit slightly (3.3-fold) reduced affinity for NECA ($K_i$ 4.15 μM vs. 1.24 μM).

### 7.1. cAMP accumulation studies

In order to investigate the activation of the wt A2A and A2B receptor in comparison to that of the loop exchange mutant A2B(EL2-A2A)AR, cAMP accumulation studies induced by four different agonists (1–4) were performed (Fig. 7 and Table 3). As expected, adenosine was much more potent (55-fold) at the human wt A2A (EC50 83.3 nM) compared to the wt A2BAR (EC50 4550 nM) showing about the same degree of efficacy at both AR subtypes, as determined by a comparison with the effect of 100 μM forskolin (total amount of cAMP produced: 50–60 pmol). At the loop mutant receptor, its potency was similar as at the wt A2BAR (4-fold higher, but without reaching statistical significance). More conspicuously, its efficacy was considerably increased by 70% (from 34% to 58% related to the forskolin effect at 100 μM, set at 100%). Very similar effects were observed with the closely related adenosine derivative NECA, which appeared to be generally more potent and efficacious than adenosine. Like adenosine, NECA displayed almost identical potency at the wt A2B and the loop mutant A2B(EL2-A2A)AR (230 nM, and 199 nM, respectively). However, its efficacy was dramatically increased at the loop mutant by 50% (from 67% to 101%, normalized to the forskolin

### Table 2

Affinities of selected ligands for the wt human A2BAR compared to the mutant receptors determined in radioligand binding studies versus [3H]PSB-603 (0.3 nM). $K_i$ values were calculated based on the $R_C$ values obtained in homologous competition experiments (see Table 1). Data are mean ± SEM of three independent experiments unless otherwise noted.

<table>
<thead>
<tr>
<th>wt or mutant</th>
<th>$K_i$ ± SEM (nM)</th>
<th>PSB-603</th>
<th>NECA</th>
<th>BAY60-6583</th>
<th>CGS21680</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt A2BAR</td>
<td>0.370 ± 0.076$^a$</td>
<td>1240 ± 110$^a$</td>
<td>114 ± 36$^a$</td>
<td>98,200 ± 4600$^a$</td>
<td></td>
</tr>
<tr>
<td>hA2B(EL2-A2A)</td>
<td>0.115 ± 0.021$^{b,ns}$</td>
<td>854 ± 271$^{b,ns}$</td>
<td>8.93 ± 3.70$^{c,*}$</td>
<td>476 ± 268$^{c,**}$</td>
<td></td>
</tr>
<tr>
<td>D148A</td>
<td>0.319 ± 0.061$^{b,ns}$</td>
<td>1140 ± 74$^{b,ns}$</td>
<td>339 ± 106$^{ns}$</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>S149A</td>
<td>0.440 ± 0.035$^{b,ns}$</td>
<td>4150 ± 540$^{b,ns}$</td>
<td>286 ± 65$^{b,ns}$</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>T151A</td>
<td>0.431 ± 0.131$^{b,ns}$</td>
<td>2380 ± 1120$^{b,ns}$</td>
<td>257 ± 54$^{b,ns}$</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>E164A</td>
<td>0.425 ± 0.070$^{b,ns}$</td>
<td>1420 ± 98$^{b,ns}$</td>
<td>236 ± 46$^{b,ns}$</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>S165A</td>
<td>0.253 ± 0.055$^{b,ns}$</td>
<td>805 ± 12$^{b,ns}$</td>
<td>275 ± 23$^{b,ns}$</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>V169A</td>
<td>0.884 ± 0.129$^{b,ns}$</td>
<td>1190 ± 148$^{b,ns}$</td>
<td>n.d.</td>
<td>n.d.</td>
<td></td>
</tr>
</tbody>
</table>

n.d., not determined.

*: $p < 0.05$.

**: $p < 0.01$.

***: $p < 0.001$.

---

**Fig. 6.** Concentration-inhibition curves for ligands at the human wt A2BAR and A2BAR mutants obtained by site-directed mutagenesis versus [3H]PSB-603 (0.3 nM). Membrane preparations from CHO cells stably expressing the A2BAR or mutant receptors were used. Data points represent mean ± SEM of three independent experiments performed in duplicates. $K_i$ values are listed in Table 2.
Next, the A2B-selective nonnucleosidic agonist BAY60-6583 was tested: as expected, it was completely selective for the human wt A2B versus the A2AAR and about equipotent with NECA (Fig. 7). Like NECA, it displayed about the same potency at the wt and mutant A2BAR. As observed with the nucleosidic agonists BAY60-6583 exhibited a dramatically increased efficacy (by 90%) at the mutant A2B(EL2-A2A)AR compared to the wt A2BAR (from 49 to 94% normalized to the effect of 100 μM forskolin). The A2A-selective nucleosidic agonist CGS21680—a NECA derivative—was virtually inactive at the wt A2BAR. However, at the mutant A2B(EL2-A2A)AR, which contained the EL2 of the A2A AR subtype, a concentration-response curve could be determined and an EC50 value of 47 μM was calculated. Furthermore, the efficacy of CGS21680 at the loop mutant in comparison with the wt A2AAR was much higher (by 60%; Table 3 and Fig. 7). Thus, all four agonists showed significantly increased efficacies at the A2B(EL2-A2A) receptor mutant in comparison with both wt receptors (see Table 3).

The A2BARs with single mutations in the EL2 were also functionally studied. The two structurally diverse agonists NECA and BAY60-6583 were applied. The EC50 values of the mutants and the wt A2BAR for the agonists were all in the same range. The total amounts of cAMP produced amounted to 20–35 pmol and were therefore comparable for all constructs, except for T151A, which showed higher cAMP production (50 pmol) for NECA. Only in one case, at the E164A mutant, NECA appeared to be somewhat (about 4-fold) more potent than at the wt A2BAR. Efficacies of both agonists, NECA and BAY60-6583, were in general not significantly different at the mutant as compared to the wt A2BAR (see Table 4 and Fig. 8). There was, however, one exception: NECA appeared to be more efficacious at the T151A mutant than at the wt A2BAR (by 80%).
Table 3

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Human A2B</th>
<th>Human A2A</th>
<th>Human A2B-EL2-A2A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC50 ± SEM (nM)</td>
<td>Efficacy ± SEM (%)</td>
<td>EC50 ± SEM (nM)</td>
</tr>
<tr>
<td>Adenosine</td>
<td>4550 ± 2730</td>
<td>34 ± 6</td>
<td>83.3 ± 12.5</td>
</tr>
<tr>
<td>NECA</td>
<td>230 ± 45*</td>
<td>67 ± 7*</td>
<td>15.0 ± 2.2</td>
</tr>
<tr>
<td>BAY60-6583</td>
<td>165 ± 36*</td>
<td>49 ± 7*</td>
<td>&gt;100,000**d</td>
</tr>
<tr>
<td>CGS21680</td>
<td>&gt;1,000,000**d</td>
<td>n.d.</td>
<td>34.7 ± 7.5</td>
</tr>
</tbody>
</table>

n.d., not determined.

* n = 7.

† n = 6.

‡ n = 4.

§ No EC50 value could be determined, since the agonist is virtually inactive at the receptor.

‡‡ Not significantly different from wild type hA2B.

†† p < 0.05.

‡‡‡ p < 0.01.

Table 4

<table>
<thead>
<tr>
<th>Mutant</th>
<th>NECA</th>
<th>BAY60-6583</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC50 ± SEM (nM)</td>
<td>Efficacy ± SEM (%)</td>
</tr>
<tr>
<td>wt A2B</td>
<td>230 ± 45*</td>
<td>67 ± 7*</td>
</tr>
<tr>
<td>D148A</td>
<td>158 ± 30**</td>
<td>74 ± 5**</td>
</tr>
<tr>
<td>S149A</td>
<td>183 ± 40**</td>
<td>61 ± 2**</td>
</tr>
<tr>
<td>T151A</td>
<td>287 ± 49**</td>
<td>121 ± 13</td>
</tr>
<tr>
<td>E164A</td>
<td>62.5 ± 8.4*</td>
<td>97 ± 10**</td>
</tr>
<tr>
<td>S165A</td>
<td>316 ± 12**</td>
<td>95 ± 8**</td>
</tr>
<tr>
<td>V169A</td>
<td>90.1 ± 19.6**</td>
<td>65 ± 3**</td>
</tr>
</tbody>
</table>

a n = 7.

b n = 6.

* p < 0.05.

‡‡‡ Not significantly different from wild type.

7.2. Homology Modelling and Docking Studies

Homology models were constructed for the loop exchange mutant A2B(EL2-A2A) based on the crystal structure representing the active UK-432097 bound A2BAR (3QAK) conformation [17], and the NECA (2YDV) and adenosine (2YDO) bound A2BAR structures [30]. In all generated models of the mutant receptor two disulfide bonds were predicted, the conserved bond between Cys783.25 in TM3 and Cys1674.50 in EL2 and a second bond, between Cys723.19 (EL1) and Cys16035.42 (EL2) (see Fig. 9), which is also present in all A2BAR crystal structures [16,17,29–33]. In the EL2 of the wt A2BAR the second disulfide bond, which is seen in the A2BAR X-ray structures and in the model of the loop mutant, is not formed; its existence is unlikely because the two cysteine residues in EL2 are closer together and the formation of the second bond would destroy the β-sheet in EL2 (see Fig. 9) [14]. The homology model of the A2B(EL2-A2A)AR mutant also contains two β-sheets, one in the EL1 and the other one in the EL2, in antiparallel orientation as found in all A2BAR X-ray structures [16,17,29–33]. The β-sheets are probably held in place and stabilized by the two disulfide bonds.

Fig. 8. Concentration-response curves of cAMP accumulation studies in agonist-stimulated CHO cells stably expressing the human A2BAR or single mutants of the A2BAR using (A) NECA, or (B) BAY60-6583 as agonist. Data points represent mean ± SEM from three (mutants) or seven (wt) independent experiments performed in triplicates. Data are normalized to the effect of forskolin at 100 μM = 100%. Corresponding EC50 and efficacy values are summarized in Table 4.
which enhance the stability of the end of the loop close to TM5. In Fig. 9, the X-ray structure of the wt A2B AR [30], a homology model of the wt A2BAR [42], and a homology model of the A2B(EL2-A2A)AR mutant based on the X-ray structure of the active A2AAR conformation [17] are depicted.

The amino acid residues that were mutated to alanine are highlighted in the A2B AR model. Asp148[45,27], Ser149[45,28], and Thr151[45,30] are located close to TM4 in the part of the EL2, which is not present in the A2AAR. Glu164[45,43] and Ser165[45,44] are located in the helical part of the EL2 flanking the potential glycosylation site. Val169[45,48] is located close to TM5 and contributes part of the β-sheet, which is in the highly conserved part of the EL2.

The models of the mutant A2B(EL2-A2A)AR were used for molecular docking of the A2A-selective agonist CGS21680 (see Fig. 9), the A2B-selective agonist BAY60-6583, and the moderately selective agonist NECA. The ligand interactions were compared to those at the wt A2A and A2BARs. The adenine and ribose moieties CGS21680 in the model of the loop mutant A2B(EL2-A2A)AR were
found to interact with the same amino acids (Phe169, Asn250, Ser275, His276) as in the A2A AR. However, in the wt A2A AR model interactions between the ribose of CGS21680 and Ser7.42 and His7.43 appear not to be present. When further comparing the homology models of the wt A2A AR and the loop mutant A2A AR with CGS21680 docked in the ligand binding site an outward movement of helix 6 and a slight inward movement of helices 5 and 7 could only be observed for the model of the loop exchange mutant, but not for the wt A2A AR. Thus, only the A2A EL-A2A) mutant receptor behaved like the wt A2A AR in the presence of the agonist CGS21680 as found in the crystal structures representing an activated conformation of the A2A AR bound to the agonist UK-432097 [17] (see Fig. 10).

8. Discussion

G protein-coupled receptors are major targets in drug discovery. Information on their structure and function is therefore not only of interest in basic science but, in addition, has important practical implications in the development of better drugs. Besides the orthosteric binding site, which is occupied by the physiological agonist and by many drugs, evidence has recently accumulated that the extracellular region of the GPCR receptor proteins are directly involved in ligand recognition and receptor function. This is not only true for GPCRs activated by large proteins, but may in fact be the case for all GPCRs. The extracellular receptor portion represents the first point of contact with an externally approaching ligand molecule.

Closely related receptors usually share high similarity in the transmembrane helices, particularly in the parts that are close to the interior of the cell [3]. Most differences are typically found in the loop regions, especially in the extracellular loops (ELs), which are exposed to the external environment. Parts of the extracellular loop regions may exhibit a filter function for ligands: they may be involved in initial ligand recognition [28], or function as gatekeepers [43]. Especially the extracellular loop 2 (EL2) has been found to be directly involved in ligand binding and may contribute to subtype selectivity [43,44], while the extracellular loop 1 (EL1) may contribute to receptor activation as shown for the A2A AR [45].

Involvement of the EL2 in allosteric modulation of GPCRs has also been demonstrated [46–48]. It appears that the EL2 can have several different roles in many if not all GPCRs, and functions observed in one subclass may in many cases be generalized to others.

Most structural variety is observed within the EL2, while the other extracellular loops, EL1 and EL3, are less diverse. Several X-ray structures of the A2A AR, one of the four subtypes of ARs, have recently been published representing inactive and active conformations. We have now investigated the role of the EL2 of the most closely related AR subtype, the A2A AR, for which no X-ray structure is available. It is four amino acids longer than the EL2 of the A2A AR and contains an insertion of seven amino acids at the end of the EL2 which is connected to transmembrane helix 4. The side of the EL2 of the A2A AR, which is close to helix 4, was found to be relatively flexible, while the other half is conformationally fixed. The EL2 of the A2A AR appears to shape the binding pocket allowing access of ligands to the orthosteric binding site [3]. Previously, point mutations had been performed in the latter region, indicating that only one disulfide bond was essential for binding and function of the A2A AR (Cys87, Cys171) [14], and that amino acids near the cysteine cluster and close to helix 5, which probably form a β-strand structure, are involved in keeping the EL2 in a fixed and silent conformation [49]. We have mutated single amino acids that bear functional groups to alanine in more flexible half of the EL2 of the human A2A AR, which is close to helix 4: Asp146, S149, and T151 since we expected that such amino acid might be involved in ligand binding and/or receptor activation. In addition, we mutated three amino acids close to the cysteine cluster, Glu164, Ser165, and Val169, to alanine. Valine is part of the β-sheet and mutation might have an effect on the stability of the EL2. Mutation of Ser165 destroys the recognition sequence for potential glycosylation and may answer the question whether glycosylation is required at that position for receptor binding and function. Glu164 was selected because it is an amino acid bearing a functional, acidic group, which might play a role for initial ligand recognition and/or receptor activation.

However, none of the single mutants showed any major effect on ligand affinity or potency, neither in radioligand binding using the A2A AR-selective antagonist PSB-603, or the agonists NECA (adenosine derivative) and BAY6065-83 (non-nucleoside), respectively, nor in cAMP accumulation studies applying the two structurally diverse agonists. Very small effects were observed only with the mutants (minor reduction in affinity for NECA) and E164A (slightly increased potency for NECA in cAMP assays). The latter might be explained by a somewhat higher expression level of the E164A mutant compared to the wild-type receptor (3-fold, p < 0.01). With one exception, all mutants also behaved like the wt A2A AR with regard to efficacy. Only at the T151A mutant, located in the more flexible part of the EL2, NECA showed significantly increased efficacy (by 80% in relation to the effect of 100 μM forskolin set at 100%), indicating that this alteration facilitated a receptor conformation induced by NECA, but not by the nonnucleoside agonist BAY6065-83, that coupled more efficiently to the G protein than the NECA-activated wt A2A AR.

Our results could retrospectively be explained by homology models of the A2A AR based on X-ray structures of the active conformation of the A2A AR. Glu164 and Ser165 are located in the helical part of EL2, and since alanine is not prone to disrupt helical structures, the secondary structure may be preserved. Val169 is located in the more rigid part of EL2, and it can therefore be speculated that the shorter side-chain of alanine does not make a major difference and does not change the secondary structure much, except that the β-sheet is one amino acid residue shorter and the loop will become slightly more flexible by the mutation. The three other mutations of functional amino acids, D148A, S149A, and T151A are located closer to TM4 in the part which has no corresponding amino acid residues in the A2A AR (see Fig. 3). They do not seem to be involved in ligand binding. However, T151 appears to contribute to the control of the extent of G protein activation by the adenosine derivative NECA.

Next we pursued a new approach exchanging the complete EL2 of the human A2A AR for the EL2 of the related A2A AR resulting in the mutant A2A EL2-A2A AR. For all wt receptors and receptor mutants, we used efficient retroviral expression to obtain stably transfected CHO cells. Expression levels of the loop exchange mutant appeared to be about 4-fold lower than that of the wt A2A AR, but the differences were not statistically significant. The expression levels were in the same range as those reported for GPCRs including ARs in native tissues (wt A2A: 390, wt A2B: 271, loop exchange mutant: 65 fmol/mg protein) [50].

Due to the lack of an agonist radioligand for A2B ARs radioligand binding studies were performed with the A2B AR-selective antagonist radioligand [3H]PSB-603. Therefore, we could only label an inactive receptor conformation. The loop exchange had several major effects on ligand binding as well as on receptor function. While the affinity of the antagonist PSB-603 and the agonist NECA remained unaltered a small increase in affinity was observed for the A2B AR-selective agonist BAY60-6583 (12-fold). The most dramatic effect was seen for the A2B AR-selective agonist CGS21680, a NECA derivative bearing a large, substituted phenethyl residue that extends to the extracellular space (Fig. 9). While CGS21680 does not bind to the wt A2B AR even at high concentrations up to
100 μM, it showed high affinity for the A2B(EL2-A2A)AR mutant with an $K_i$ value of 476 nM, in the same range as the A2B standard agonist NECA ($K_i$ 854 nM) representing a more than 200-fold increase in affinity. These results show that the EL2 contributes to ligand selectivity in the A2BAR. Only agonists with large residues, extending to the cell surface appear to be affected (CGS21680 and BAY60-6583, but not NECA (compare [17]). Moreover, the EL2 governs receptor subtype selectivity, the A2A- selective agonist showing relatively high affinity for the A2BAR which contained the EL2 of the A2AR. The same effect was confirmed in functional cAMP accumulation studies. CGS21680, which did not activate the wt A2BAR, was able to fully activate the A2B(EL2-A2A)AR mutant, and an EC50 value could be determined. The smaller loop of the A2AR may be more open than the larger A2B loop and this could determine or at least contribute to receptor subtype selectivity.

However, the EL2 is not only involved in ligand binding contributing to ligand affinity and selectivity. We observed another, striking effect: The maximal effects of all agonists in stimulating receptor-mediated cAMP accumulation was dramatically increased in the loop exchange mutant as compared to the wt receptors. The reason for this cannot be due to increased expression levels of the A2B(EL2-A2A)AR mutant, since in fact, the expression of the EL2 was lower than that of the wt A2B and the A2AAR, although the differences were not statistically significant. The effect of increased efficacy was robust and strong, with 50–90% increase in efficacy for all four, structurally diverse agonists investigated, including the physiological agonist adenosine (see Fig. 7). Efficacy refers to the maximal effect achievable with a certain agonist or inverse agonist at a particular receptor. It reflects the stabilization of a ligand-specific state – a particular receptor conformation, which is ligand-specific [51]. Different agonists may show large differences in their efficacies, e.g. at the wt A2BAR NECA showed the highest efficacy while adenosine was less efficacious (Table 3). This means that NECA induces or stabilizes a receptor conformation that couples more efficiently to the Gi protein than adenosine. In the A2B(EL2-A2A)AR mutant even better coupling receptor conformations will be facilitated by the exchanged loop in the presence of agonists. At present it is, however, unclear, why the efficacy of agonists at the A2B(EL2-A2A)AR is not only higher in comparison with the wt A2B AR, but also when compared to the wt A2AAR.

When comparing the A2BAR homology models based on 2YDO, representing the transition state between inactive and fully active conformations with the models based on 3QAK representing the fully activated state of the receptor the common features thought to be involved in activation can all be observed, namely an inward movement of helices 3, 5, and 7 leading to a contraction of the lower side of the binding pocket and an outward movement of helix 6 which allows G proteins to bind [5,30]. In the G protein–coupled β2-adrenergic receptor it had been shown, that TM5 is extended by two helix turns when bound to Gi, [5]. In the case of the mutant A2B(EL2-A2A)AR one could speculate that a similar extension, which is observed in the agonist-bound homology models (complexed with CGS21680, NECA, or BAY60-6583) of the mutant receptor, promotes and/or stabilizes Gi binding, thereby increasing its signal transduction efficacy. One possibility is that the altered EL2 in the A2BAR might act like a covalently linked–positive allosteric modulator, or PAM [48]. In the absence of agonist, the receptor remained silent, however in the presence of an agonist, its maximal effect was strongly increased compared to the original EL2. Several noncovalent allosteric modulators have been found to interact with the EL2 of GPCRs [46,48,52]. PAMs at the A2AR subtype have recently been reported to interact with the EL2 of the receptor [53]. Allosteric modulators for the A2BAR are not known so far. But our results together with published data indicate that PAMs for A2BARs could likely be developed.

In conclusion, the replacement of the complete EL2 of the A2AR by the EL2 of the related A2BAR was found to be a powerful approach for investigating its role in ligand binding and receptor activation. The EL2 appears to have multiple roles, including (i) a filter or gate-keeper function for ligands, from initial recognition to ushering them to their destination, (ii) direct involvement in ligand binding, (iii) contribution to receptor subtype selectivity, (iii) keeping the receptor in a silent state, and (iv) inducing signalling selectivity for a certain signal transduction pathway as recently shown [46]. We have now found for the A2BAR that the EL2 is indeed responsible for ligand selectivity. Furthermore we have found that the EL2 can have a powerful impact on agonist efficacy. Our results may extend to other receptor subfamilies and represent a general role of the EL2 in GPCRs.

References


