Universiteit Leiden
The Netherlands

Activation of G protein-coupled receptors : the role of extracellular loops in adenosine receptors

Peeters, M.C.

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CHAPTER 6

THE SECOND EXTRACELLULAR LOOP OF THE ADENOSINE A_1 RECEPTOR PLAYS A ROLE IN BOTH RECEPTOR ACTIVATION AND ALLOSTERIC MODULATION

This chapter was based upon:

M.C. Peeters, L.E. Wisse, A. Dinaj, B. Vroling, G. Vriend, A.P. IJzerman.
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ABSTRACT

The adenosine A_1 receptor is a member of the large membrane protein family that signals through G proteins, the G protein-coupled receptors (GPCRs). GPCRs consist of seven transmembrane domains connected by thee intracellular and three extracellular loops. Their N-terminus is extracellular, the C-terminal tail is in the cytoplasm. The transmembrane domains in subfamilies that bind the same endogenous ligand, such as dopamine or adenosine, tend to be highly similar. In contrast, the loop regions can vary greatly, both in sequence and in length, and the role these loops have in the activation mechanism of the receptors remains unclear. Here, we investigated the activating role of the second and third extracellular loop of the human adenosine A_1 receptor. By means of an $(Ala)_3$ mutagenic scan in which consecutive sets of three amino acids were mutated into alanines and a classical alanine scan, we revealed a strong regulatory role for the second extracellular loop (EL2) of the human adenosine A_1 receptor. Besides many residues in the second and the third extracellular loops important for adenosine A_1 receptor activation, we also identified two residues in EL2, a tryptophan and a glutamate, that affect the influence of the allosteric modulator PD81,723. These results, combined with a comparison of the different receptor loop regions, provide insight in the activation mechanism of this typical class A GPCR and further emphasize the unique pharmacological profile the loops can provide to individual receptors, even within subfamilies of GPCRs.

INTRODUCTION

G protein-coupled receptors (GPCRs) constitute the largest family of membrane signaling proteins, able to bind and transmit signals of a wide variety of endogenous ligands ranging from proteins such as chemokines and gonadotropic hormones to small molecules such as adenosine [1]. The involvement in many physiological process as well as the ability to be targeted by synthetic ligands, make this family an attractive drug target. Over the last decade much progress has been made in understanding the activation mechanism of this large superfamily, greatly aided by the elucidation of several high resolution crystal structures [2,3,4]. These new insights combined with mutagenesis data have resulted in a paradigm shift in GPCR research. The limited view that ligand binding and G protein coupling only are important for signal transduction and receptor activation is broadening to include the distinct role of the extracellular domains of GPCRs [5]. The extracellular domains are the least conserved elements of GPCR structure, varying both in sequence and in length even within subfamilies. Also the structural divergence observed between the different crystal structures published so far, suggests that the role of the extracellular loops may be unique for each individual receptor. In that context mutagenesis studies may be informative in two aspects: they shed light on how the loops contribute to receptor activation and pinpoint to differences between family members.

In the current study, we examined the second and third extracellular loop (EL2 and EL3) of the adenosine A_1 receptor (A_1R), a typical class A GPCR. The A_1R is part of a small subfamily that recognizes the endogenous nucleoside adenosine. Four members of this family have been identified, the A_1R, $A_{2A}R$, $A_{2B}R$ and A_3R. The four subtypes have different affinities for the endogenous ligand; the A_1R is a high affinity receptor (Ki ≈ 100 nM) where the $A_{2B}R$ displays a very low affinity for adenosine (Ki ≈ 15,000 nM). Also their intracellular signaling pathways differ, with the A_1R and A_3R coupling to G_i proteins and subsequently decreasing cAMP levels, and the $A_{2A}R$ and $A_{2B}R$ coupling mainly to G_s proteins thereby increasing intracellular cAMP concentrations [6]. Already in the early nineties Olah and coworkers provided evidence that extracellular loops are involved in differences in ligand recognition between adenosine receptor subtypes [7]. The authors created chimeric receptors, substituting EL2 or a region encompassing transmembrane domains 6 and 7 (including EL3) of the A_1R into the A_3R resulting in enhanced affinities of both A_1R

selective agonists and antagonists compared to wild-type A_3R. A particularly important region responsible for the observed effects was shown to be the C-terminal part of EL2. The second extracellular loop of the adenosine A_1R might also contain a binding site for allosteric ligands as has recently been speculated by Narlawar et al. They studied the behavior of bivalent ligands that connect an orthosteric ligand with an allosteric modulator to probe the location of the allosteric site relative to the orthosteric site [8]. EL2 has also been suggested as the binding site for allosteric modulators at other GPCRs, such as the M_2 muscarinic acetylcholine receptor (M_2R) and the M_4 muscarinic acetylcholine receptor (M_4R) [9,10].

In contrast to EL2, the third extracellular loop (EL3) is very small in all adenosine receptor subtypes. Nonetheless, EL3 has been proposed to be important in signaling in various GPCR family members [11,12,13]. Furthermore, this loop is involved in shaping the ligand binding pocket of both the antagonist ZM241385 and the agonists UK-432097, NECA, and adenosine in the published crystal structures of the adenosine A_{2A} receptor [3,14,15]. In the UK-432097 bound active structure, EL3 appeared forced outwards to accommodate the large biphenylic substituent on the N^6 position of the adenine moiety [3]. This might indicate a role for EL3 too in activation of the receptor.

N-terminus

C-terminus

Figure 1. Snake-plot representation of the human adenosine A_1 receptor. The disulfide bridge conserved in many class A GPCRs links C80 and C169 (bold circles) and is indicated with the dotted line. The $(Ala)_3$ triplets in EL2 are shown with straight lines, also the section used for mutagenesis in EL3 is marked.

We performed a mutational analysis on both the second and third extracellular loop by using a classical alanine scan and investigated the effects on activation and ligand binding. Since EL2 is relatively large, we first scanned the loop by performing an $(Ala)_3$-scan in which triplets of amino acids were replaced by alanines. Interesting regions were then further characterized by single residue site-directed mutagenesis. To evaluate the mutant receptors, we made use of a robust yeast system, the MMY24 *S. cerevisiae* strain. This yeast system is an ideal background to monitor activation of a single GPCR, since its only endogenous GPCR has been removed from the system while still maintaining the complete GPCR-signaling machinery [16]. Several previous reports have proved this eukaryotic system to be predictive of the mammalian situation [17,18]. Besides investigating the effect of the alanine mutations on receptor activation and ligand binding, we also explored the ability of the allosteric modulator PD81,723 ((2-amino-4,5-dimethyl-3-thienyl)-[3-(trifluromethyl)-phenyl] methanone) to enhance the agonist-induced effect in the various mutant receptors. The results presented here, show a strong involvement of the second extracellular loop in receptor function by positively regulating A_1R activation. This is contradictory to the previously proposed role of EL2 as a negative regulator of the receptor [19,20]. Furthermore, we report a possible interaction of the allosteric modulator PD81,723 with a specific residue in EL2. Also EL3 is important in receptor activation; in particular two proline residues in this loop appear to be important in providing rigidity to this protein region.

MATERIALS AND METHODS

Mutagenesis

The *S. cerevisiae* expression vector containing the human adenosine A_1 receptor gene, pDT-PGK_hA_1R, that was used for all the mutagenesis procedures described in this paper was kindly provided by Dr. Simon Dowell from GSK (Stevenage, UK).

$(Ala)_3$-scan

For the initial screening of the second extracellular loop of the hA_1R, we replaced consecutive sets of three amino acids by an alanine; the $(Ala)_3$-scan. Where an alanine already existed, the residue was not mutated. Also the cysteine at position 169 was kept unchanged. The mutations were introduced using the QuikChange

Multi-Site Directed Mutagenesis system (Stratagene, Huizen, The Netherlands). The $(Ala)_3$-scan yielded ten mutant receptors.

Site-directed mutagenesis

The single alanine mutations introduced in the second extracellular loop as well as the single alanine scan of the third extracellular loop of the hA_1R were performed using the QuikChange II Site Directed Mutagenesis system (Stratagene, Huizen, The Netherlands). Twelve additional mutant receptors of EL2 and eight alanine mutant receptors of EL3 were created. All mutant receptor genes were verified by double-stranded sequencing (LGTC, Leiden, The Netherlands).

Transformation in MMY24 S. cerevisiae strain

pDT-PGK_hA_1R plasmids were transformed into an *S. cerevisiae* yeast strain according to the Lithium-Acetate procedure [21]. The strain is derived from the MMY11 strain [22] and was further adapted to communicate with mammalian GPCRs through the introduction of a chimeric G protein [16]. The genotype of the MMY24 strain is: *MAT**a**his3 leu2 trp1 ura3can1 gpa1_::G_i3 far1_::ura3 sst2_::ura3 Fus1::FUS1-HIS3LEU2::FUS1-lacZ ste2_::G418R.* To measure signaling of GPCRs, the pheromone signaling pathway of this strain was coupled via the FUS1 promotor to HIS3, a gene encoding the key enzyme in histidine production, imidazole glycerol-phosphate dehydrase. The degree of receptor activation was measured by the growth rate of the yeast on histidine-deficient medium.

Liquid yeast growth assay

To characterize the mutant receptors further, concentration-growth curves were generated in a liquid growth assay. Yeast colonies expressing wild type or mutant hA_1 receptor were inoculated in 2.5ml YNB-UL (Yeast Nitrogen Based medium lacking the markers uracil and leucine) and incubated overnight at 30°C. The cultures were diluted to an OD_{600} of 0.002 ($\approx 4 \cdot 10^4$ cells/ml) in medium without histidine (YNB-ULH medium), resulting in a final concentration of $1 \cdot 10^4$ cells/ml in the assay. Concentration-growth curves were performed in YNB-ULH medium with 7 mM 3AT, 0.8 IU/ml adenosine deaminase (ADA) (Roche Diagnostics, Almere, The Netherlands) and varying concentrations of CPA (N^6-cyclopentyladenosine) (Tocris Cookson Ltd, Avonmouth, United Kingdom) (10^{-7} – 10^{-11}M) in the presence or absence of 1 μM PD81,723 ((2-amino-4,5-dimethyl-3-thienyl)-[3-(trifluromethyl)-phenyl]methanone)

(synthesized in house). Growth represented by the absorbance at 595nm was measured over a period of 35 hrs in a Genios plate reader (Tecan, Durham, NC). Data was analyzed using nonlinear regression analysis software available in GraphPad Prism 5.0 (GraphPad Software, San Diego, CA).

Whole yeast cell radioligand binding experiments

Yeast cells expressing wild type or mutated A_1Rs were cultured overnight in rich YAPD (Yeast-extract Adenine Peptone Dextrose) medium. Cells were centrifuged for 5 minutes at 2000 xg, the pelleted cells were once washed with 0.9% NaCl. The cells were again centrifuged 5 minutes at 2000 xg and diluted in the assay buffer (50mM Tris-HCl pH7.4 + 1mM EDTA) to OD_{600}=40 (OD_{600} = 1 ≈ $2.5 \cdot 10^7$ cells/ml). Also, 1 U/ml ADA was added to the cells. Binding experiments were performed with 5 nM [^{3}H]DPCPX and a final cell concentration of $25 \cdot 10^7$ cells/ml in a total volume of 100 µl. Nonspecific binding was determined in the presence of 10 µM CPA. For whole competition binding curves a concentration range of 10^{-10}-10^{-5} M of the agonist CPA was used in the presence or absence of the allosteric modulator 10 µM PD81,723.
Samples were incubated for 1 hour at 25ºC while shaking vigorously to keep the yeast cells in suspension. Incubation was terminated by adding 1 ml ice-cold assay buffer. Bound from free radioligand was immediately separated by rapid filtration through Whatman GF/B filters pre-incubated with 0.1% polyethylenimine (PEI) using a Millipore manifold during which the filters were washed six times with ice-cold assay buffer. Filter-bound radioactivity was determined by scintillation spectrometry (Tri-Carb 2900TR; PerkinElmer Life and Analytical Sciences) after addition of 3.5 ml of PerkinElmer Emulsifier Safe.

Whole yeast cell extracts and immunoblotting

Whole protein cell extracts were made from the transformed yeast cells using trichloroacetic acid (TCA). From an overnight culture, $1.2 \cdot 10^8$ yeast cells were harvested in mid-log phase. The cells were washed twice with 20% TCA after which they were broken by vigorous vortexing in the presence of glass beads. The yeast cell extracts were separated on 12.5% SDS page gels and transferred to a PVDF transfer membrane (GE healthcare, Diegem, Belgium) using a semi-dry Western blotting set (Sigma–Aldrich, Zwijndrecht, The Netherlands). The antibody directed

against the C-terminal region of the adenosine A_1 receptor was used for immunodetection (Sigma-Aldrich, Zwijndrecht, The Netherlands). Densitometric analysis of the protein bands was performed using the volume analysis tool as present in the Quantity One imaging software from Bio-Rad (Hercules, CA). The aspecific band that is seen in all yeast extracts, including empty yeast cell extracts, was used as loading control. The ratio between specific A_1R protein bands and aspecific bands was determined and the wild-type receptor was set at 100%, the empty vector pDT-PGK at 0%.

Bioinformatic analysis

A multiple sequence alignment of the A_1R, $A_{2A}R$, $A_{2B}R$, and A_3R receptors was created with T-Coffee using the default settings, followed by small manual corrections based on structural considerations, sequence conservation and correlation patterns in EL2 and IL3 [23]. Start and end positions of the loops were selected based on manual inspection of the crystal structure of the human $A_{2A}R$ receptor (PDB: 3EML). The Phylip package was used to calculate distances between the individual receptors (both the complete receptor sequences as well as the loop segments), and to generate the distance trees [24]. The scoring tables are shown in Chapter 7, Figure 5).

RESULTS

(Ala)$_3$-scan of the second extracellular loop (EL2)

To gain insight in the role of the second extracellular loop of the human adenosine A_1 receptor (hA_1R) in receptor activation, we first set out to identify specific regions in the loop that influence activity. For this purpose, we designed an (Ala)$_3$-scan, where consecutive sets of three amino acids were mutated into alanine residues **(Figure 1)**. Where an alanine residue already existed, the corresponding codon was kept unchanged. Also, the cysteine at position 169 was not mutated, since this residue has been proven in the past to be essential for receptor function, as it is part of the highly conserved disulfide bridge with C80 in TM3 [25].

The (Ala)$_3$-scan rendered 10 different mutant receptors that were tested for their activation profile in a liquid yeast growth assay. This assay is based on a gene

reporter system incorporated in the *S. cerevisiae* MMY24 strain. When the expressed human receptor is activated by an agonist, the yeast pheromone signaling pathway is activated through a chimeric yeast-mammalian G protein leading to subsequent transcription of the HIS3 reporter gene. As a result, the yeast cells produce the essential amino acid histidine that allows the cells to grow on histidine-deficient medium in a dose dependent manner. Concentration-growth curves of all 10 $(Ala)_3$ mutant receptors are shown in **Figure 2.**

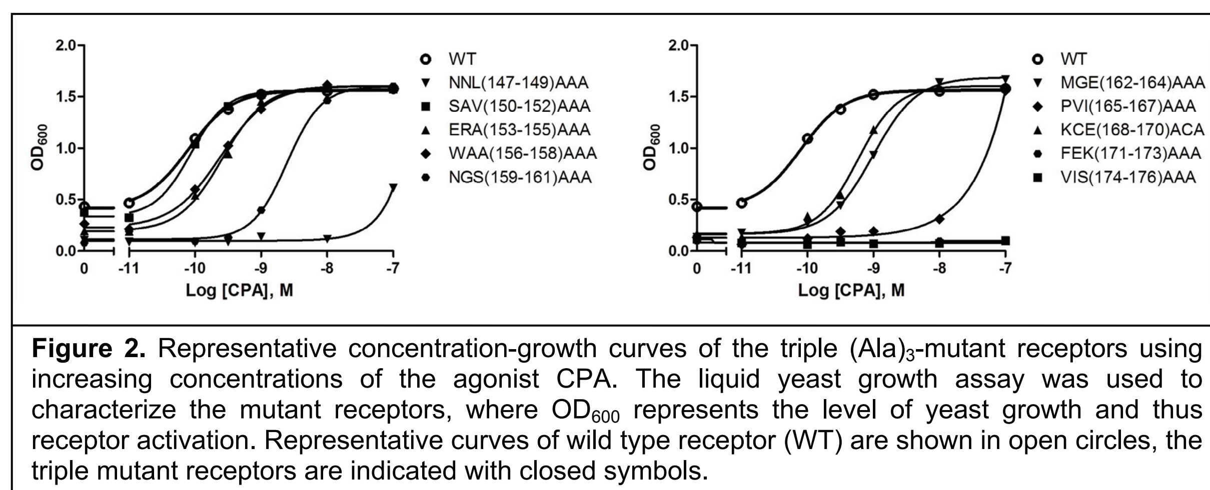


Figure 2. Representative concentration-growth curves of the triple $(Ala)_3$-mutant receptors using increasing concentrations of the agonist CPA. The liquid yeast growth assay was used to characterize the mutant receptors, where OD_{600} represents the level of yeast growth and thus receptor activation. Representative curves of wild type receptor (WT) are shown in open circles, the triple mutant receptors are indicated with closed symbols.

The level of constitutive activity the wild-type A_1R displays is relatively high, approx. 25% of the maximal response under the assay conditions used. Except for mutant SAV(150-152)AAA, all triple mutant receptors show a decrease in potency for the selective A_1R agonist N^6-cyclopentyladenosine (CPA). SAV(150-152)AAA is also the only mutant receptor that did not display a significant decrease in constitutive activity (see also **Table 1**). Especially the start and end of the loop sequence, represented by triplets NLL(147-149), FEK(171-173), and VIS(174-176) are highly sensitive to the alanine mutations, showing no or barely any response to the agonist. Three other triple mutant receptors that show a more than one log unit decrease in potency were NGS (159-161)AAA, MGE(162-164)AAA, and PVI(165-167)AAA (**Figure 2, Table 1**). A Western blot analysis showed that all receptors were expressed in the yeast cells, even the mutant receptors that were greatly affected by the alanine mutations. A number of receptors appeared to be present in larger quantities compared to the wild type A_1R, most notably for the SAV(150-152)AAA construct **(Figure 3)**.

Table 1. Characterization of the adenosine A_1R mutant receptors of the second extracellular loop, both triple $(Ala)_3$-mutants and single alanine mutants, using the liquid yeast growth assay. The level of constitutive activity (CA) of wild type receptor (WT) and mutants is represented by the OD_{600} +/ SEM measured in the absence of CPA. EC_{50} values of the agonist CPA (nM) and percentage maximal activity (Emax) are shown as means ± SEM of three independent experiments, each performed in duplicate. Mean values derived from the concentration-growth curves were used for calculation of the fold EC_{50} value, the shift of EC_{50} in the presence of the allosteric modulator PD81,723 (PD81).

Mutant	CA (OD_{600})	EC_{50} (nM) CPA	% Emax	EC_{50} (nM) + PD81	Shift EC_{50} + PD81
WT	0.43 +/- 0.04	0.10 +/- 0.01	100 +/- 1	0.06 +/- 0.01	1.6
NNL(147-149)AAA	0.10 +/- 0.03	> 100	39 +/- 3		
SAV(150-152)AAA	0.38 +/- 0.03	0.10 +/- 0.04	100 +/- 1	0.06 +/- 0.03	1.6
ERA(153-155)AAA	0.19 +/- 0.03	0.28 +/- 0.01	100 +/- 1	0.19 +/- 0.05	1.5
E153A	0.10 +/- 0.01	0.43 +/- 0.02	101 +/- 1		
R154A	0.50 +/- 0.03	0.12 +/- 0.02	103 +/- 1		
WAA(156-158)AAA	0.26 +/- 0.04	0.25 +/- 0.08	100 +/- 1	0.22 +/- 0.09	1.1
NGS (159-161)AAA	0.08 +/- 0.01	2.4 +/- 0.2	102 +/- 2	1.7 +/- 0.3	1.4
N159A	0.25 +/- 0.03	0.35 +/- 0.01	100 +/- 1		
G160A	0.09 +/- 0.03	0.71 +/- 0.01	101 +/- 2		
S161A	0.12 +/- 0.02	0.41 +/- 0.07	94 +/- 1		
MGE(162-164)AAA	0.13 +/- 0.03	1.0 +/- 0.06	106 +/- 1	0.44 +/- 0.05	2.2
M162A	0.47 +/- 0.16	0.12 +/- 0.02	102 +/- 1		
G163S	0.03 +/- 0.01	2.1 +/- 0.09	96 +/- 1		
E164A	0.46 +/- 0.15	0.15 +/- 0.002	102 +/- 1		
PVI(165-167)AAA	0.13 +/- 0.05	> 100	99 +/- 1		
P165A	0.15 +/- 0.05	0.61 +/- 0.05	102 +/- 1		
V166A	0.08 +/- 0.01	0.64 +/- 0.04	101 +/- 1		
I167A	0.06 +/- 0.003	4.5 +/- 0.2	103 +/- 1		
KCE(168-170)ACA	0.17 +/- 0.03	0.56 +/- 0.02	101 +/- 1	0.33 +/- 0.06	1.7
E170A	0.10 +/- 0.01	0.40 +/- 0.06	102 +/- 1		
FEK(171-173)AAA	0.10 +/- 0.01	No response			
VIS(174-176)AAA	0.11 +/- 0.002	No response			

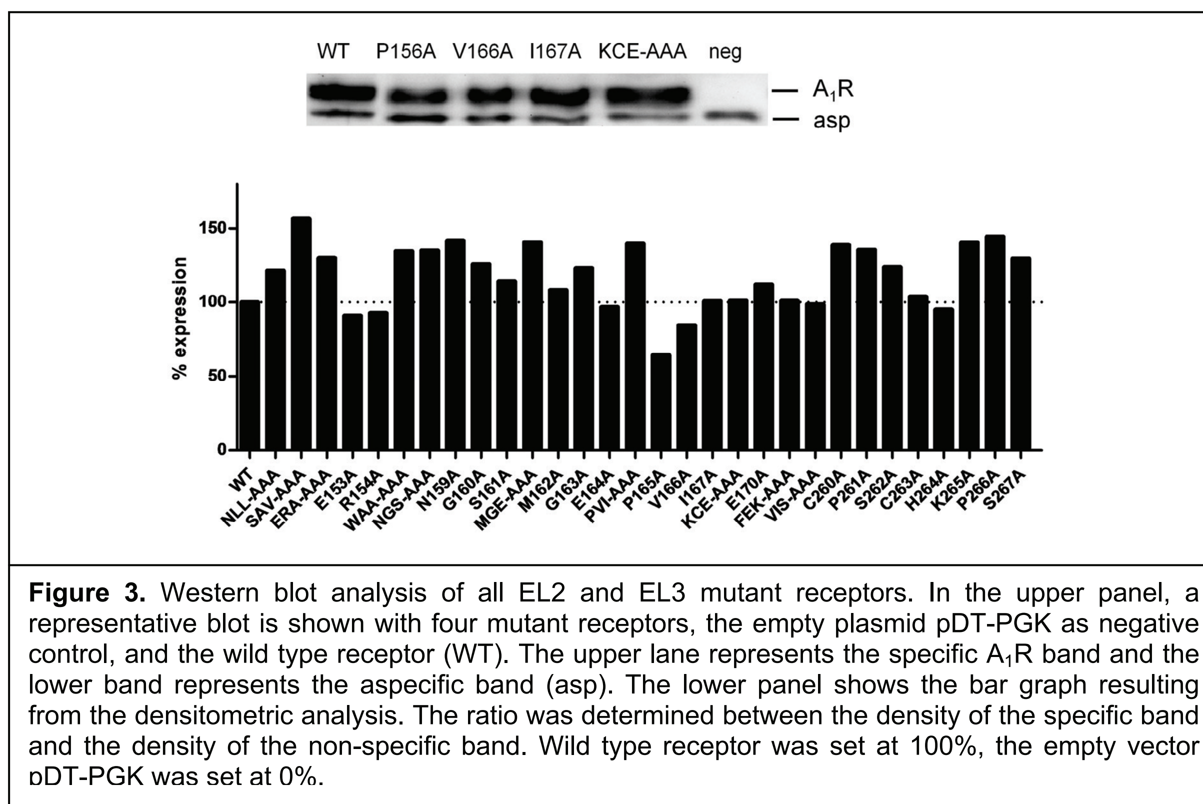


Figure 3. Western blot analysis of all EL2 and EL3 mutant receptors. In the upper panel, a representative blot is shown with four mutant receptors, the empty plasmid pDT-PGK as negative control, and the wild type receptor (WT). The upper lane represents the specific A_1R band and the lower band represents the aspecific band (asp). The lower panel shows the bar graph resulting from the densitometric analysis. The ratio was determined between the density of the specific band and the density of the non-specific band. Wild type receptor was set at 100%, the empty vector pDT-PGK was set at 0%.

Single alanine mutations EL2

To further investigate specific residues that influence adenosine A_1 receptor activation, we created single alanine mutations of several positions where we mainly focused on the central part of the loop. The $(Ala)_3$ mutant receptor WAA(156-158)AAA only contained a mutation of the tryptophan at position 156 and already represented a single mutant receptor. The 12 new single mutant receptors were analyzed in the functional liquid yeast growth assay (**Table 1**). A selection of the resulting concentration-growth curves is shown in **Figure 4**.

Similar to the $(Ala)_3$ mutant selection, most single alanine mutations compromised receptor activation both in response to the agonist CPA and independently of a ligand (constitutive activity). Even though several mutant receptors displayed a large potency decrease, they were all able to reach (near) maximal activation levels compared to wild-type receptor in response to CPA. Looking at the three most affected $(Ala)_3$ mutants, we observed an additive effect of the individual alanine mutant receptors within the NGS(159-161) triplet with position G160 having the largest, 7-fold potency decrease as well as a 4.8-fold decrease in constitutive activity. The 10-fold decrease in CPA potency of the $(Ala)_3$ mutant MGE(162-164)AAA appeared solely due to the single mutant G163A that even shows a potency

decrease of 21-fold compared to wild type receptor as well as a 14-fold decrease in basal activity. Mutating the other 2 residues in this triplet, M162 and E164 resulted in a negligible effect on activation when investigated separately. These two residues appeared to rescue activation to some extent when combined with G163A. In the triplet PVI(165-167), again an additive effect was seen of which the main effect is caused with isoleucine 167 mutated to an alanine, resulting in a 45-fold decrease of agonist potency. This mutant receptor also showed the largest impact on constitutive activity of the three single alanine mutants, showing a 7.2-fold decrease compared to wild-type receptor. Both mutant receptors P165A and V166A showed a 7-fold decrease in CPA's potency. These receptors were the only mutants that showed a somewhat decreased expression level compared to the wild-type receptor (**Figure 3**).

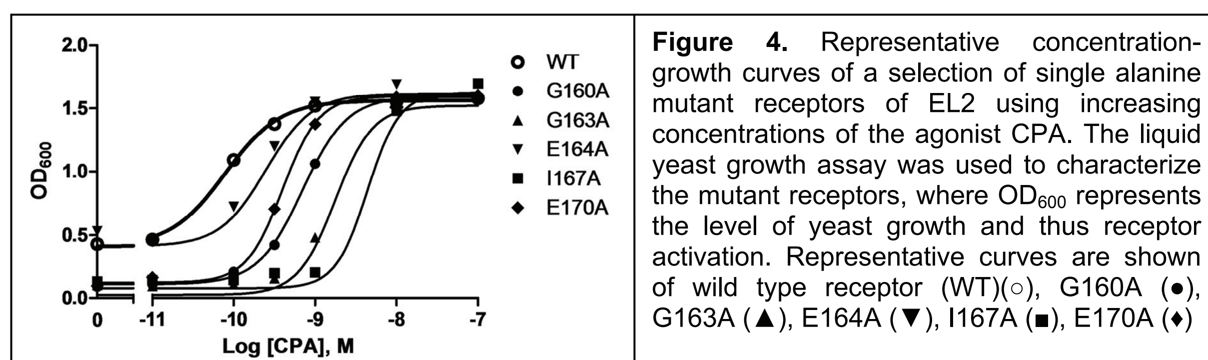


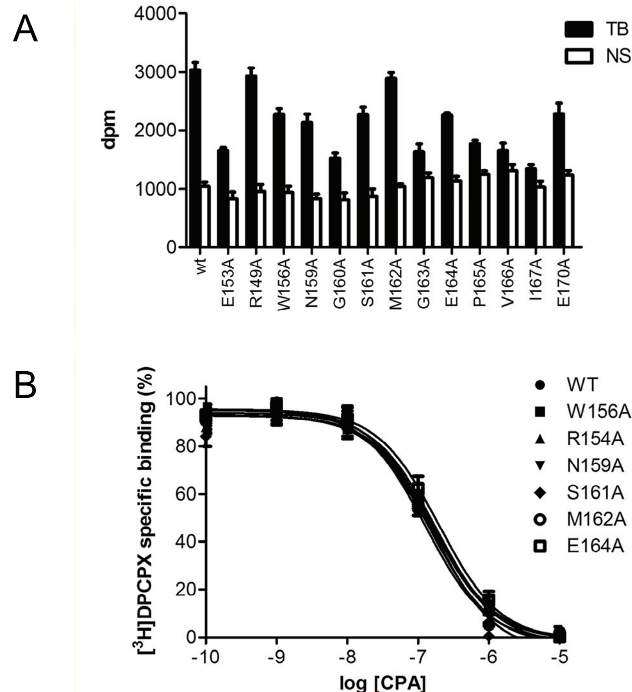
Figure 4. Representative concentration-growth curves of a selection of single alanine mutant receptors of EL2 using increasing concentrations of the agonist CPA. The liquid yeast growth assay was used to characterize the mutant receptors, where OD_{600} represents the level of yeast growth and thus receptor activation. Representative curves are shown of wild type receptor (WT)(○), G160A (●), G163A (▲), E164A (▼), I167A (■), E170A (♦)

Next, we performed radioligand binding experiments using the single mutant collection, including the W156A mutant receptor. We firstly determined whether a large enough window could be obtained to perform whole competition binding curves. For the binding experiments a concentration of 5 nM of the antagonist $[^3H]$DPCPX was used, a concentration that is more than 3 times the Kd value of the wild type receptor (Kd_{wt} on yeast: ≈ 1.5 nM, data not shown). Even with this increased amount of radioligand and an excess of unlabeled CPA, for a number of the mutant receptors, only a low level of specific binding was observed **(Figure 5A, Table 2).** The mutant receptors for which we observed specific binding greater than 60% compared to wild type receptor were used to perform full competition binding curves (**Figure 5B, Table 2**). From the competition binding curves, no decrease in affinity for CPA was observed, even though some of the mutants, like S161A and N159A, did show a decrease in potency for CPA in the yeast growth assays.

Table 2. Radioligand binding experiments of the single alanine mutations in the second extracellular loop, using the selective antagonist [^{3}H]DPCPX. Single point measurements and competition binding curves were performed using the unlabeled agonist CPA. Percent specific binding and IC_{50} values are shown as means ± SEM of three independent experiments, each performed in duplicate. Mean values derived from the competition binding curves were used for calculation of the shift in IC_{50} in the presence of the allosteric modulator PD81,723 (PD81)

Mutant	% specific binding	IC_{50} (nM)	IC_{50} (nM) + PD81	Shift IC_{50} + PD81
WT	100	167 +/- 25	69 +/- 8	2.4
E153A	41 +/- 5			
R154A	103 +/- 8	166 +/- 38	72 +/- 17	2.3
W156A	74 +/- 11	161 +/- 6	141 +/- 10	1.1
N159A	71 +/- 6	170 +/- 10	64 +/ 3	2.6
G160A	34 +/- 5			
S161A	77 +/- 4	169 +/- 11	75 +/- 18	2.3
M162A	89 +/- 4	130 +/- 5	55 +/- 5	2.4
G163A	12 +/- 1			
E164A	60 +/- 2	192 +/- 7	58 +/- 4	3.3
P165A	18 +/- 3			
V166A	14 +/- 3			
I167A	8 +/- 2			
E170A	43 +/- 5			

Figure 5.
Radioligand binding experiments of single alanine mutant receptors of EL2.
(*A*) Single point competition binding assay with [^{3}H]DPCPX.
Binding experiments were performed at a radioligand concentration of 5 nM. Non-specific binding was determined using 10 µM CPA.
(*B*) Competition binding curves with [^{3}H]DPCPX using increasing concentrations of unlabeled CPA as displacer of wild type receptor (●) and the single mutants W156A (■), R154A (▲), N159A (▼), S161A (♦), M162A (○), and E164A (□). Total binding was set at 100%, non-specific binding at 0%.



Allosteric modulation

To investigate whether our mutants were sensitive to allosteric modulation, we performed concentration-growth curves with CPA on the $(Ala)_3$ mutants in the presence of 1 μM PD81,723, a selective allosteric modulator of the A_1R. Where we normally use 10 μM of the allosteric modulator in binding studies, we were not able to exceed a concentration of 1 μM in the functional experiments due to the intrinsic agonistic effect of PD81,723 that concealed the modulating effect on CPA activation. The increase in agonist potency in the presence of PD81,723 observed for the wild type receptor was 1.6 fold under these conditions. A significant decrease was noticed for mutant receptor W156A and an increase in response to PD81,723 was observed for mutant receptor MGE(162-164)AAA **(Figure 6A, Table 1)**.

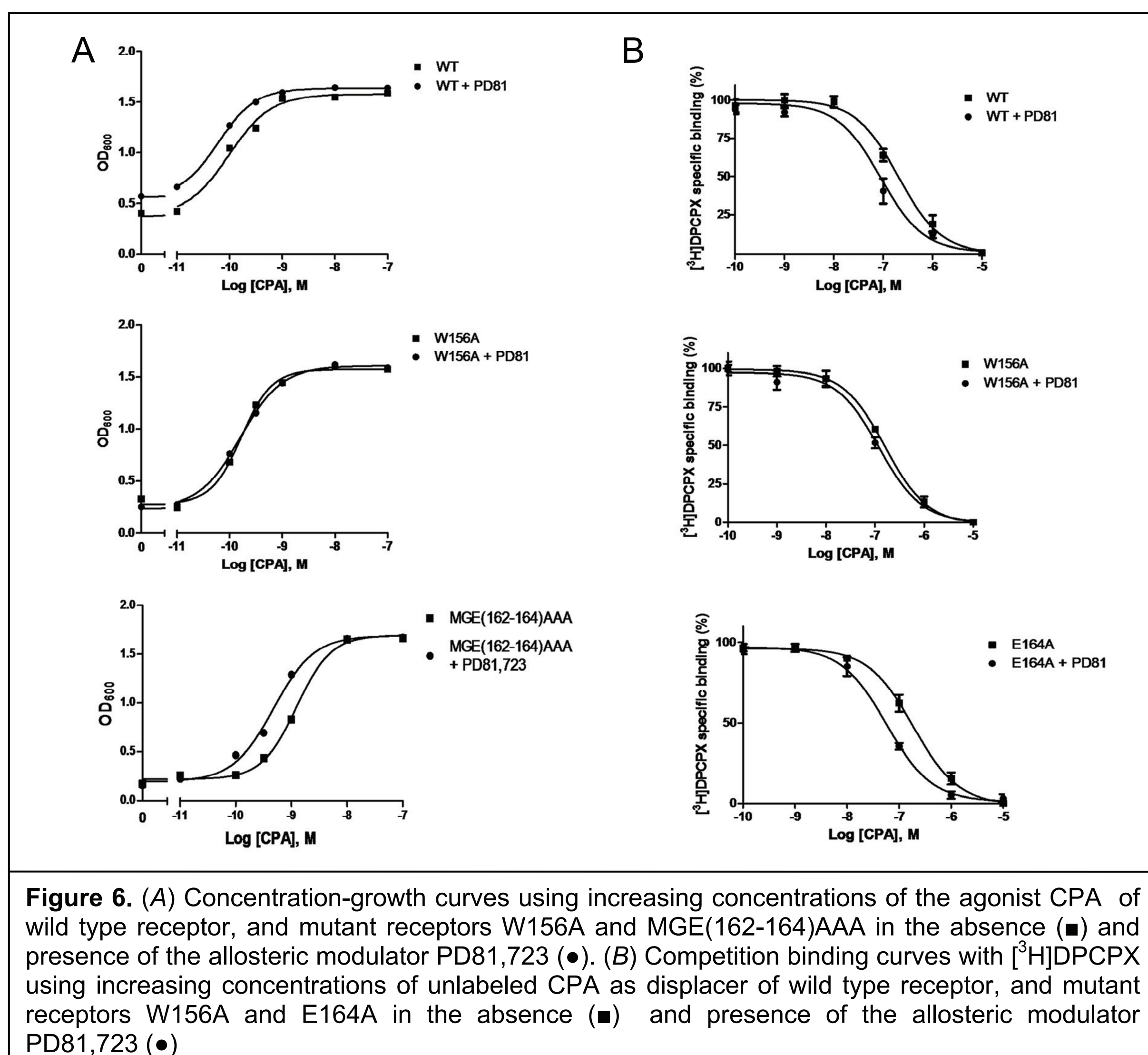


Figure 6. (*A*) Concentration-growth curves using increasing concentrations of the agonist CPA of wild type receptor, and mutant receptors W156A and MGE(162-164)AAA in the absence (■) and presence of the allosteric modulator PD81,723 (●). (*B*) Competition binding curves with [^{3}H]DPCPX using increasing concentrations of unlabeled CPA as displacer of wild type receptor, and mutant receptors W156A and E164A in the absence (■) and presence of the allosteric modulator PD81,723 (●)

When subjecting the single mutant receptors W156A and E164A (part of the MGE triplet) to radioligand competition binding experiments in the presence of 10 µM PD81,723, we noticed similar effects **(Figure 6B)**. The affinity of CPA for the wild type receptor was increased 2.4 fold when competition curves were performed in the presence of the allosteric enhancer PD81,723. In contrast, mutant receptor W156A showed no significant change in CPA affinity in the presence of PD81,723 and mutant receptor E164A showed an increase in affinity that was even greater than observed for wild type, namely 3.3 fold **(Table 2)**.

Alanine scan of the third extracellular loop

The third extracellular loop was also subjected to an alanine scan. The loop is relatively small, therefore, we performed a single alanine scan in which all eight residues were mutated **(Figure 1)**. The results of the functional liquid yeast growth assays are listed in **Table 3**.

Table 3. Characterization of the adenosine A_1R mutant receptors of the third extracellular loop, using the liquid yeast growth assay. The level of constitutive activity (CA) of wild type receptor (WT) and mutants is represented by the OD_{600} +/ SEM measured in the absence of CPA. EC_{50} values of the agonist CPA (nM) and percentage maximal activity (Emax) are shown as means ± SEM of three independent experiments, each performed in duplicate.

Mutant	CA (OD_{600})	EC_{50} (nM)	Fold EC_{50}	% Emax
WT	0.43 +/- 0.03	0.10 +/- 0.01	1.0	100 +/- 1
C260A	0.26 +/- 0.02	0.14 +/- 0.01	1.5	100 +/- 1
P261A	0.27 +/- 0.09	0.25 +/- 0.07	2.5	96 +/- 3
S262A	0.27 +/- 0.06	0.19 +/- 0.02	1.9	103 +/- 1
C263A	0.31 +/- 0.6	0.10 +/- 0.02	1.0	102 +/- 1
H264A	0.38 +/- 0.13	0.16 +/- 0.05	1.6	102 +/- 1
K265A	0.16 +/- 0.08	0.18 +/- 0.03	1.8	102 +/- 1
P266A	0.21 +/- 0.09	0.36 +/- 0.06	3.6	101 +/- 0.5
S267A	0.16 +/- 0.07	0.22 +/- 0.01	2.2	101 +/- 1

Compared to the mutagenesis results observed for EL2, the effects on activation by the mutations in EL3 were relatively small. The largest effect was seen when the two

prolines were mutated, P261A and P264A, where CPA's potency was decreased by respectively 2.5 and 3.6 fold, respectively. Of the eight EL3 mutant receptors, only H264A showed similar levels of constitutive activity. Basal activity was decreased in all other mutants, with K265A and S267A displaying the largest decrease of 2.7-fold. All mutant receptors were again able to reach maximal activation levels in response to CPA. A Western blot analysis showed that the mutant receptors were expressed at similar levels compared to WT or even somewhat higher **(Figure 3).**

Bioinformatics analysis - loop similarity

The adenosine A_1 receptor is a member of a small subfamily together with three other adenosine receptors, $A_{2A}R$, $A_{2B}R$, and A_3R. In transmembrane sequence, the A_1R is most related to the A_3R, and these two receptors also share the same signaling pathway, coupling both to G_i proteins. The question remains though, whether the loops and their function are just as comparable. Also, we were wondering to what extent we can compare the structural information of the extracellular loops that is available from the crystal structure of one subtype, the $A_{2A}R$, to other adenosine receptor subtypes. To investigate this, we performed a sequence similarity analysis in which we compared all loop sequences of the receptors with each other. Multiple sequence alignments were created with the T-Coffee alignment method using the complete sequence of all four adenosine receptors as well as the three intracellular loops (ILs) and the three extracellular loops (EL2) separately [23]. Based on these multiple sequence alignments we calculated the distance of the loop and receptor sequences using the Phylip package and created distance trees that represent the relative similarity of the fragments [24]. The results of the analysis are shown in **Figure 7.** The distance tree placed left of the snake plot represents the similarity of the full receptor sequences. This tree shows that within the adenosine subfamily, the $A_{2A}R$ and $A_{2B}R$ are most similar to each other and that also the A_1R and A_3R are grouped together. A similar tree is observed for IL3, the loop that is thought to be the most influential in G protein coupling [26]. In contrast, the other intra- and extracellular loops show different results. The EL2 is most similar in the $A_{2A}R$ and $A_{2B}R$, however, the A_1R shows the largest distance from the A_3R and is also quite different from the $A_{2A}R$. In EL3, the A_1R resembles the $A_{2A}R$ most, closely followed by the $A_{2B}R$. Also here, the loop of the A_3R resembles the A_1R least.

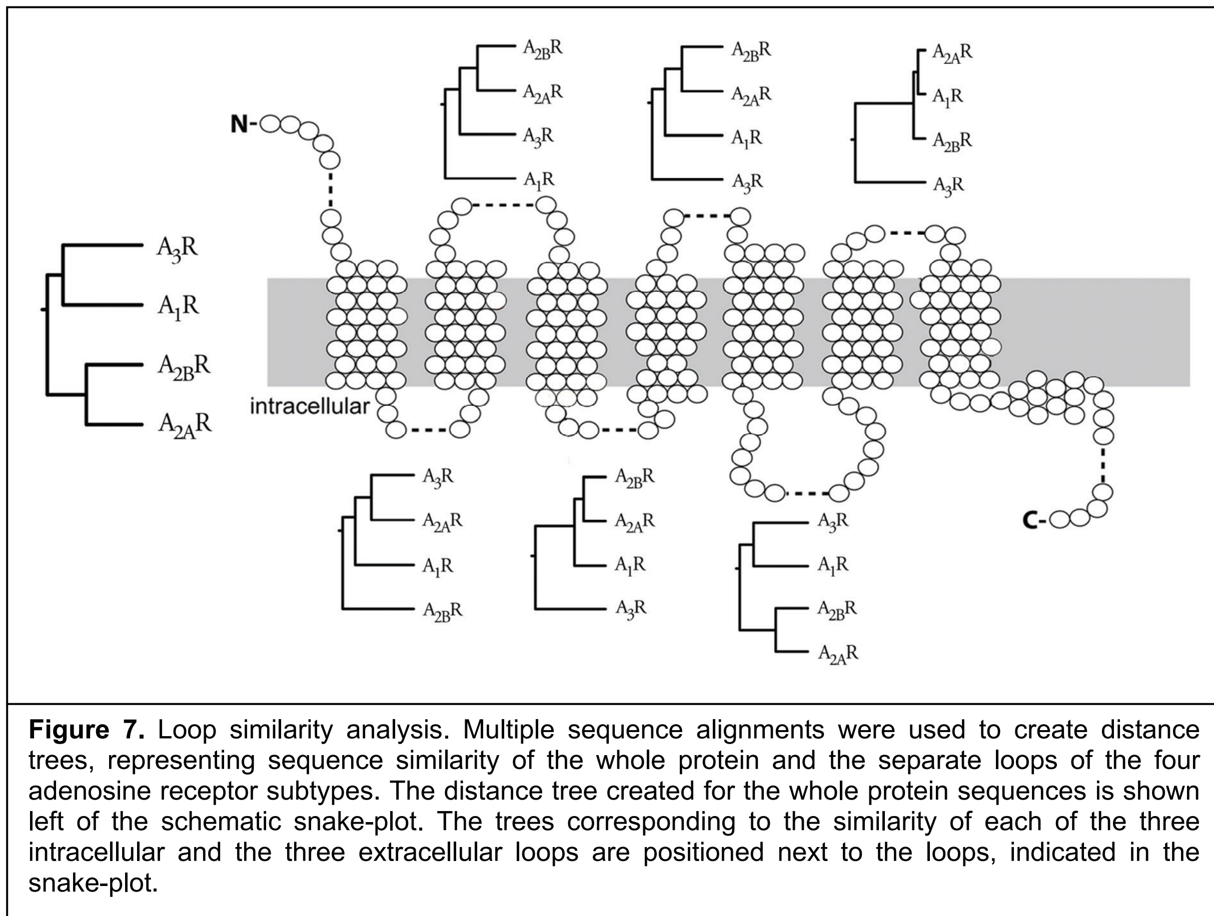


Figure 7. Loop similarity analysis. Multiple sequence alignments were used to create distance trees, representing sequence similarity of the whole protein and the separate loops of the four adenosine receptor subtypes. The distance tree created for the whole protein sequences is shown left of the schematic snake-plot. The trees corresponding to the similarity of each of the three intracellular and the three extracellular loops are positioned next to the loops, indicated in the snake-plot.

DISCUSSION

Over the last decade, the extracellular domains of GPCRs have gained significant interest [5]. Both experimental data and the published crystal structures clearly demonstrate that the extracellular loops are not just involved in cell surface expression and anchoring in the cell membrane, but are active participants in the activation mechanism. Of the adenosine receptors subfamily, we now have access to both inactive and active structures of the $A_{2A}R$ subtype that have provided us with new insights in how the receptor transitions between the two conformational states [3,14,15]. They show that the second and third extracellular loops interact with the bound ligands and are shaping the ligand binding site, indicating a role in binding and recognition of the ligand, but also in initiating receptor activation. However, the extracellular region is highly divergent even within the adenosine receptor subfamily and it is so far unclear to what extent we can extrapolate information obtained from

one receptor to another. We therefore decided to explore both the second and third extracellular loop in another adenosine receptor subtype, the adenosine A_1 receptor.

A positive regulating role of EL2 in adenosine A_1 receptor activation

To investigate the role of the second extracellular loop of the human adenosine A_1 receptor, we made use of an $(Ala)_3$-scan as well as a classical single alanine point mutation method. Many residues were identified to be important for adenosine A_1 receptor activation. Except for a low number of residues that could be mutated without consequences for receptor activation (SAV(150-152), R154, and M162), all alanine mutations caused the receptor to display a decreased activation profile (**Table 1)**. The mutation E170K is the only naturally occurring variant in EL2 that has been described in the Natural Variant (NaVa) database [27], and has been associated with colorectal cancer [28]. Mutating the glutamic acid into the much smaller alanine in our investigation resulted in a 4-fold decrease in agonist potency. The low level of specific binding obtained in the radioligand binding experiments indicates that also antagonist binding was compromised (**Figures 4 and 5**). The mutant receptors that displayed the largest decrease in receptor activity had mutated glycines and a mutated isoleucine, G160A, G163A, and I167A , that showed a 7-fold, 21-fold, and 45-fold decrease in CPA potency, respectively, as well as a large decrease in constitutive activity (**Table 1, Figure 4**). All three mutant receptors also showed low specific binding of $[^3H]$DPCPX in radioligand binding experiments (**Table 2**). Of all amino acids, glycine residues provide the largest flexibility to a protein structure, while alanine provides more constraints to the protein backbone. It therefore appears that EL2 of the A_1R is greatly dependent on the level of flexibility induced by the glycine residues. Also, since none of the mutated residues showed any increased constitutive activity or agonist potency, we conclude from these results that EL2 in the wild-type A_1R acts as a positive regulator of the receptor activation mechanism. It is not only involved in activation of the receptor in response to a ligand, but also in maintaining the level of basal activity that is relatively high for the human A_1R (**Figure 4**). Interestingly, the decrease in constitutive activity and potency did not involve a vast decrease in maximal activation levels (Emax). The mutant receptors are thus still fully functional, but appear to require a higher energy level to change conformational states.

One unique EL2 for each individual receptor?

There has been much speculation of how the second extracellular loop influences GPCR activation. Several reports have described an important role of EL2 in both positive and negative regulation of the activation mechanism [5]. A saturation mutagenesis study on the complement factor 5a receptor (C5aR) revealed many constitutively active receptors. These results led to the conclusion that EL2 might play an unexpected role as a negative regulator of receptor activation [19,20]. Other mutagenesis studies corroborated this suggested role of EL2, like mutagenesis studies of the of the M_1 and M_2 muscarinic acetylcholine receptor (M_1R and M_2R) and a site-directed mutagenesis study on the thrombin receptor that also resulted in several EL2 mutants with increased constitutive activity [9,29,30]. Contrary to these findings, several other EL2 mutagenesis studies did not yield constitutively active mutants, suggesting another role for EL2 [31,32]. Also, a random mutagenesis screen on another subtype of muscarinic acetylcholine receptors, the M_3R, led to the identification of about twenty mutant receptors containing single amino acid changes in EL2 that were inactive in yeast and proved to be important for efficient agonist-induced M_3R activation but not for agonist binding [33]. The apparent contradictory results observed in the muscarinic acetylcholine receptors, suggest that even within closely related subfamilies, the role of EL2 can differ.

Recently, our laboratory conducted a low frequency random mutagenesis screen on the adenosine A_{2B} receptor, a family member of the A_1R. This study revealed a particular "hotspot" of residues in a cysteine-rich region of EL2 that resulted in constitutive activity of the receptor [34]. This implies that also within the small adenosine receptor subfamily, EL2 can adopt a different role in activating the receptor. Where EL2 of the $A_{2B}R$ seems to contain a motif that negatively regulates activation, EL2 of the A_1R appears to act as a positive regulator. This raises questions on how to interpret the recently published crystal structures of the adenosine $A_{2A}R$ in view of the other members of the subfamily and how reliable homology models can be concerning the extracellular loops. EL2 varies greatly in both amino acid sequence and length among the adenosine receptors and also the number of extracellular cysteines is different. The $A_{2A}R$ contains three extracellular cysteines in the loop that are all able to form disulfide bridges that determine the extracellular structure [3,14,15]. The A_1R contains only a single cysteine residue in EL2 that is part of the conserved cysteine bridge with TM3. A loop similarity analysis

we conducted further underlines the individual character of the extracellular loops. Phylogenetic analysis classically groups the $A_{2A}R$ and $A_{2B}R$ subtypes and the A_1R and A_3R subtypes together [35], this is also shown in the distance tree we created for the full receptor sequences in **Figure 7**. A similar similarity tree is observed when comparing the third intracellular loop (IL3) of all four subtypes. This loop is thought to be the key determinant in G protein coupling [26]. The other intra- and extracellular loops however, show a different classification. In EL2 the $A_{2A}R$ and $A_{2B}R$ are still most similar, however, the A_1R is most distant from the A_3R. Of all adenosine receptor subtypes, the A_1R is most similar to the $A_{2B}R$. However, this distance seems to be large enough for the loop to act differently in receptor activation and suggests that structurally the loops are hard to compare.

Involvement of residues W156 and E164 in allosteric modulation

Besides investigating the role of EL2 in activation alone, we also studied its involvement in the enhancing effect of the A_1R allosteric modulator PD81,723. Many GPCRs, including the adenosine A_1 receptor, have been shown to be allosterically modulated by both small molecule ligands and ions [36,37]. In the family of muscarinic acetylcholine receptors (MRs) several residues have been identified that participate in the binding of allosteric ligands [38]. In the M_2R an EDGE motif centrally located in EL2 was shown to be involved in the binding of prototypical MR modulators [9,39]. Also, a phenylalanine in EL2 of the M_4R has been demonstrated to interact with the allosteric agonist LY2033298, whereas it did not influence binding of orthosteric agonists [10].

Of all four adenosine receptor subtypes, the A_1R receptor is the most studied receptor on this subject and several allosteric modulators have been described for this receptor [6,40]. Recently, Narlawar et al. suggested that the allosteric modulator binding site in the A_1R might reside close to or within the second extracellular loop by an approach linking the orthosteric and allosteric site with bivalent ligands and docking studies [8]. Allosteric binding sites can originate by chance during evolution and are therefore likely to be found in a less conserved region. EL2 might indeed be a probable site of allosterism considering its high sequence variability even within subfamilies.

To investigate if one of our EL2 alanine mutant receptors changed the effect on allosteric modulation, we tested the effect of PD81,723, a selective allosteric enhancer of agonist binding and function on the A_1R, on the receptor's potency for CPA. A first functional test on the $(Ala)_3$ mutant receptors revealed that PD81,723 lost its ability to increase CPA potency with mutant WAA(156-158)AAA (W156A), but seemed to increase this effect on mutant receptor MGE(162-164)AAA (**Table 1, Figure 6A**). Subsequent radioligand binding experiments showed that also the increase in CPA affinity by PD81,723 on mutant W156A was lost. In contrast, the single mutant E164A, part of the triple mutant MGE(162-164)AAA, showed an increased effect of PD81,723 on CPA affinity compared to wild type receptor (**Table 2, Figure 6B**). The corresponding position in the $A_{2A}R$, E161, showed an increase in affinity for the nonxanthine adenosine antagonist CGS 15943 (9-chloro-2-(furyl)[1, 2 , 4]triazolo[1, 5-c]quinazolin-5-amine) (6 fold) when mutated to an alanine but not for other ligands [41]. Mutant E164A in the A_1R showed a decreased specific binding with $[^3H]$DPCPX (60%) and no significant change in CPA affinity (**Table 2**).
The other mutant receptors tested showed responses to PD81,723 comparable to wild-type A_1 receptor. These results imply a role of EL2 in binding and modulation of the allosteric enhancer PD81,723 and confirm the hypothesis that an allosteric binding site might be present in EL2 of the A_1R.

EL3 in A_1R activation

Similar to the mutagenesis study on the EL2 of the A_1R, an alanine scan performed on all 8 amino acids of the third extracellular loop did not yield any mutants that increased the activation profile. All mutant receptors, except H264A, showed a decrease in constitutive activity. Two prolines in EL3, P261 and P266, were found to be most sensitive to the alanine conversion showing a decrease in CPA potency of 2.5- and 3.6-fold compared to wild type respectively (**Table 3)**. Mutant receptor P261Q of the hA_1R has been described in the GPCR Natural Variant (NaVa) database as a naturally occurring polymorphism and arose from the NIH full-length cDNA project [27,42]. No functional data is available for the P261Q mutant receptor and the mutation has so far not been linked to any disease state. However, our results indicate that this proline residue is important for normal function of the A_1R. Proline residues in general are quite rigid amino acids, often introducing a kink in the

backbone. The effect of the proline mutations in our study implies that this rigidity in EL3 is an important feature in receptor activation. Similar to the EL2 mutant receptors, all EL3 alanine mutations were still able to reach the maximal level of activation in response to CPA. Even though the proline mutations affect basal activation levels and agonist potency, they are fully functional in transmitting the activation signal.

High resolution structures of the adenosine A_{2A} receptor subtype were published both in an inactive state with the antagonist ZM241385 and in an active conformation with the agonists UK- 432097, NECA, and adenosine bound to the receptor [3,14,15]. This receptor subtype contains many extracellular cysteine residues that are all capable of forming disulfide linkages. Also in EL3, a disulfide bridge is seen in the structures. The A_1R is the only other adenosine receptor subtype that would be able to form such a disulfide bridge. It is mainly due to this resemblance that in our loop similarity study, the A_1R is closest related to the $A_{2A}R$ (**Figure 7**). The EL3 of the A_3R is far distant in similarity to the other subtypes and most distant from the A_1R, which can be explained by the low number of amino acids present in the A_3R. Scholl et al. performed a study on all nine native cysteines present in the human adenosine A_1 receptor, two of which are located in the third extracellular loop. Neither cysteine residues were shown to influence agonist and antagonist binding. Our study shows that also for A_1R activation, C260 and C263 are not essential and that mutation to an alanine also does not decrease expression levels (**Table 3 and Figure 3**). This suggests that if the A_1R is also able to connect the two cysteines in a disulfide bridge, this is not an essential structural feature for normal function of this receptor. Further research will show whether the possible disulfide bridge is involved in other processes, such as ligand selectivity or ligand directed signaling.

In conclusion, by applying site-directed scanning mutagenesis on the second and third loop of the adenosine A_1 receptor, we identified a number of residues important for receptor activation. Contrary to a putative role for the second extracellular role, as previously described, to act as a dimmer switch for activation, the loop appears to be an activator of the adenosine A_1 receptor. Also, we provided evidence that EL2 accommodates at least part of the allosteric binding site.

The results presented here, provide new insights in the role extracellular loops play in the activation mechanism of class A GPCRs and further emphasize that this role can very well vary between individual receptors, even within subfamilies.

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