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The role of the second and third extracellular loops of the adenosine A1 receptor in activation and allosteric modulation

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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 three intracellular and three extracellular loops. Their N-terminus is extracellular, the C-terminal tail is in the cytoplasm. The transmembrane domains in receptor 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 alanine residues in EL2 and a classical alanine scan in EL3, 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.

1. 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 [2–4]. 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 et al. 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

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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. [8]. 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. 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 [11–13]. 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.

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 long, 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 *Saccharomyces 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-(trifluoromethyl)-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.

Table A.1
Primers used for the $(Ala)_3$-scan.

Forward primer	Amino acid change
5′-ctgacccctatgtttggctgggccgctgcgagtgcggtggagcgggcctg-3′	NNL → AAA
5′-ggctggaacaatctggctgcggcggagcgggcct-3′	SAV → AAA
5′-ctgagtgcggtggcggcggcctgggcagcc-3′	ERA → AAA
5′-gtggagcgggccgcggcagccaacgg-3′	WAA → AAA
5′-gggcctgggcagccgccgccgccatgggggagcccg-3′	NGS → AAA
5′-gcagccaacggcagcgcggcggcgcccgtgatcaagtg-3′	MGE → AAA
5′-ggcagcatgggggaggccgcggccaagtgcgagttcga-3′	PVI → AAA
5′-ggggagcccgtgatcgcgtgcgcgttcgagaaggtcat-3′	KCE → ACA
5′-gagcccgtgatcaagtgcgaggccgcggcggtcatcagcatggagtacat-3′	FEK → AAA
5′-gatcaagtgcgagttcgagaaggccgccgccatggagtacatggtctacttca-3′	VIS → AAA

2. Materials and methods

2.1. 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).

2.1.1. $(Ala)_3$-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 QuickChange Multi-Site Directed Mutagenesis system (Stratagene, Huizen, The Netherlands). Primers used for this procedure can be found in Appendix A (Table A.1). The $(Ala)_3$-scan yielded ten mutant receptors.

2.1.2. 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 QuickChange II Site Directed Mutagenesis system (Stratagene, Huizen, The Netherlands). Primers used for this procedure can be found in Appendix A (Tables A.2 and A.3). 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).

2.2. 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

Table A.2
Primers used for site-directed mutagenesis EL2.

Forward primer	Reverse primer	Amino acid change
5′-gagtgcggtggcgcgggcctggg-3′	5′-cccaggcccgcgccaccgcactc-3′	E153A
5′-agtgcggtggaggcggcctgggcagc-3′	5′-gctgcccaggccgcctccaccgcact-3′	R154A
5′-ggcctgggcagccgccggcagcatgggg-3′	5′-ccccatgctgccggcggctgcccaggcc-3′	N159A
5′-ggcagccaacgccagcatggggg-3′	5′-cccccatgctggcgttggctgcc-3′	G160A
5′-ggcagccaacggcgccatgggggagccc-3′	5′-gggctcccccatggcgccgttggctgcc-3′	S161A
5′-agccaacggcagcgcgggggagcccgtg-3′	5′-cacgggctcccccgcgctgccgttggct-3′	M162A
5′-cggcagcatggcggagcccgtga-3′	5′-tcacgggctccgccatgctgccg-3′	G163A
5′-ggcagcatgggggcgcccgtgatcaag-3′	5′-cttgatcacgggcgcccccatgctgcc-3′	E164A
5′-cagcatgggggaggccgtgatcaagtg-3′	5′-cacttgatcacggcctccccatgctg-3′	P165A
5′-atgggggagcccgcgatcaagtgcgag-3′	5′-ctcgcacttgatcgcgggctcccccat-3′	V166A
5′-tgggggagcccgtggccaagtgcgagttcg-3′	5′-cgaactcgcacttggccacgggctccccca-3′	I167A
5′-ggggagcccgtgatcgcgtgcgagttcgagaa-3′	5′-ttctcgaactcgcacgcgatcacgggctcccc-3′	K168A
5′-cccgtgatcaagtgcgcgttcgagaaggtcatc-3′	5′-gatgaccttctcgaacgcgcacttgatcacggg-3′	E170A