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

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SUMMARY

The quest for the exact activation mechanism of GPCRs is still very much ongoing. With each new discovery in the GPCR research field, it becomes more evident how very complex these proteins work. Even though the number of elucidated structures are increasing fast and many questions about what a receptor looks like and how it functions can be answered more confidently, even more new questions have arisen. The research in this thesis has aimed to contribute an important part of the puzzle and answer a few of these new questions.

In **Chapter 1**, subjects that are being discussed in this thesis were introduced. Main themes, like mutagenesis, constitutive activity and the S. cerevisae system received most attention in this chapter. The research described in this thesis has focused most on the extracellular loops of GPCRs and their role in the activation mechanism. We therefore devoted **Chapter 2** to an elaborate description of what we have learned so far about the role of the extracellular loops using recent literature as well as the crystal structures that were available at that time.

In **Chapter 3**, the first extracellular loop (EL1) of the adenosine A_{2B} receptor ($A_{2B}R$) was investigated. From a random mutagenesis screen in a S. cerevisiae yeast expression model using the first part of the receptor, we discovered a mutant receptor with two amino acid changes in EL1: F71L and D74G. Thorough mutational and pharmacological analysis of these two positions taught us that this part of the receptor was essential in activation of the $A_{2B}R$. At position 71 the property of hydrophobicity played an important role, while at position 74 hydrophilicity was key. The elucidation of the crystal structure of the adenosine A_{2A} receptor greatly helped us in explaining these results. The two positions appear to reside at the edges of a β-strand in EL1 that can form a sheet with the second extracellular loop (EL2). The properties of residues 71 and 74 are essential in maintaining this very important three-dimensional protein structure.

In **Chapter 4**, we applied a similar random mutagenesis screen for an increased activation profile as was described in Chapter 3, now using the second part of the $A_{2B}R$ encompassing transmembrane domain 4 (TM4), the second extracellular loop (EL2), and transmembrane domain 5 (TM5). The results revealed three “hotspots” important for constitutive activity as well as agonist potency. The clusters of amino

acids appear responsible for maintaining the subtle equilibrium that exist between the active conformation R^* and the inactive conformation R of the receptor. These residues are not necessarily directly involved in either ligand binding or G protein coupling.

After screening for increased active mutant receptors, we also wanted to investigate mutations that result in deactivation of the receptor in **Chapter 5**. For this purpose, we developed a new screening method using the same S. cerevisiae system, the MMY24 strain, that allowed us to specifically select for this inactive phenotype. In order to expand on the knowledge obtained in Chapter 4, we again used the mutated library of the $A_{2B}R$ in which random mutations were introduced in the fragment encompassing TM4, EL2, and TM5. The mutated receptors that were identified from the screen all showed a decrease in both constitutive activity as well as agonist potency. A particular important region located at the extracellular half of TM5 was discovered with $C190^{5.46}$ as a key player that is important in facilitating the conformational changes in the process of receptor activation. A comparison with the data obtained in Chapter 4 showed that a cysteine-rich cluster in EL2 acts as a negative regulator of activation, keeping the receptor silent, whereas the cluster at the top of TM5, only several residues downstream in the receptor, acts in an opposite way.

In **Chapter 6**, we investigated another subtype of the adenosine receptor subfamily; the adenosine A_1 receptor (A_1R). The role in activation of the second and third extracellular loop (EL2 and EL3) was examined by means of a triple alanine scan (Ala_3-scan) and a singular alanine scan (Ala-scan). Many residues in both loops proved important for A_1R function, all influencing receptor activation negatively when mutated. Especially EL2 appears to act as a positive regulator in receptor activation. This is contradictory to the role of the motif identified in EL2 of the $A_{2B}R$ that appears to have an opposite role. Furthermore, we identified two residues in EL2, a tryptophan and a glutamate, that affect the influence of the allosteric modulator PD81,723.

The research described in the previous chapters was brought together in a general discussion in **Chapter 7**. By combining all the results and conclusions of the individual chapters, we obtained a more complete view of how adenosine receptors can be activated. Also, this chapter provides future perspectives based on these final conclusions.