The activation mechanisms of G protein-coupled receptors : the case of the adenosine A2B and HCA2/3 receptors

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Summary

G protein-coupled receptors (GPCRs) are one of the largest families of membrane proteins, with approximately 800 different GPCRs in the human genome. Signalling via GPCRs regulates many biological processes, such as cell proliferation, differentiation, and development. Moreover, many hormones and neurotransmitters are ligands for these receptors, and hence it is not surprising that many drugs, either mimicking or blocking the action of the bodily substances, have been developed. It is estimated that 30-40% of current drugs on the market target GPCRs. Further identifying and elucidating the functions of GPCRs will provide opportunities for novel drug discovery. The budding yeast *Saccharomyces cerevisiae (S. cerevisiae)* is a very important and useful platform in this respect. There are many advantages of using a yeast assay system, as it is cheap, safe and stable; it is also convenient for rapid feasibility and optimization studies. Moreover, it offers "null" background when studying human GPCRs. New developments regarding human GPCRs expressed in a yeast platform are providing insights into GPCR activation and signalling, and facilitate agonist and antagonist identification.

In **Chapter 1**, a general description of GPCRs, GPCR activation, G protein and G protein selectivity is presented. In this thesis, we focused our attention on the human adenosine A_{2B} receptor ($hA_{2B}R$) and the family of hydroxycarboxylic acid HCA_2 and HCA_3 receptors. Applications of the *S. cerevisiae* system in GPCR research are introduced as well.

In **Chapter 2**, we summarized the latest findings regarding human GPCRs in studies using *S. cerevisiae*, ever since the year 2005 when we last published a review on this topic. We described 11 families of GPCRs in detail, while including the principles and developments of each yeast system applied to these different GPCRs and highlight and generalize the experimental findings of GPCR function in these systems.

The expression of human GPCRs in *S. cerevisiae* containing chimeric yeast/mammalian G_{α} subunits provides a useful tool for the study of GPCR

activation. In **Chapter** 3, we used a one-GPCR-one-G protein yeast screening method in combination with molecular modeling and mutagenesis studies to decipher the interaction between GPCRs and the C-terminus of different α-subunits of G proteins. We chose the $hA_{2B}R$ as a paradigm, a typical class A GPCR that shows promiscuous behavior in G protein coupling in this yeast system. The wild-type $hA_{2B}R$ and five mutant receptors were expressed in 8 yeast strains with different humanized G proteins, covering the four major classes: $G_{\alpha i}$, $G_{\alpha s}$, $G_{\alpha q}$ and $G_{\alpha 12}$. Our experiments showed that a tyrosine residue (Y) at the C-terminus of the G_{α} subunit plays an important role in controlling the activation of GPCRs. Receptor residues $R103^{3.50}$ and $I107^{3.54}$ are vital too in G protein coupling and the activation of the $hA_{2B}R$, whereas $L213^{IL3}$ is more important in G protein inactivation. Substitution of $S235^{6.36}$ to alanine provided the most divergent G protein coupling profile. Finally, $L236^{6.37}$ substitution decreased receptor activation in all G protein pathways, although to a different extent. In conclusion, our findings shed light on the selectivity of receptor-G protein coupling, which may help in further understanding GPCR signaling.

In **Chapter 4**, we continued to investigate the $hA_{2B}R$. We used a single-GPCR-one-G protein yeast screening method in combination with mutagenesis studies, molecular modeling and bio-informatics to investigate the importance of the different amino acid residues of the $NPxxY(x)_6F$ motif and helix 8 in $hA_{2B}R$ activation. A scanning mutagenesis protocol was employed, yielding 11 single mutations and one double mutation of the $NPxxY(x)_6F$ motif and 16 single mutations of helix 8. The amino acid residues $P287^{7.50}$, $Y290^{7.53}$, $R293^{7.56}$ and $I304^{8.57}$ were found to be essential, since mutation of these amino acid residues to alanine led to a complete loss of function. Western blot analysis showed that mutant receptor $R293^{7.56}A$ was not expressed, whereas the other proteins were. Amino acid residues that are also important in receptor activation are: $N286^{7.49}$, $V289^{7.52}$, $Y292^{7.55}$, $N294^{8.47}$, $F297^{8.50}$, $R298^{8.51}$, $H302^{8.55}$ and $R307^{8.60}$. The mutation $Y290^{7.53}F$ lost 50% of efficacy, while $F297^{8.50}A$ behaved similar to the wild-type receptor. The double mutation, $Y290^{7.53}F/F297^{8.50}Y$, lost around 70% of efficacy and displayed a lower potency for the reference agonist 5'-(*N*-ethylcarboxamido) adenosine (NECA). This study provides new insight into the molecular interplay

and impact of TM7 and helix 8 for hA_{2B} receptor activation, which may be extrapolated to other adenosine receptors and possibly to other GPCRs.

In **Chapter 5**, we focused our attention on the family of HCA receptors, a GPCR family of three members, of which the HCA_2 and HCA_3 receptors share 95% sequence identity but differ considerably in C-terminus length with HCA_3 having the longest tail. The two receptors were expressed and analysed for their activation profile in *S. cerevisiae* MMY yeast strains that have different G protein G_α subunits. The $hHCA_2$ receptor was promiscuous in its G protein coupling preference. In the presence of nicotinic acid the $hHCA_2$ receptor activated almost all G protein pathways except $G_{\alpha q}$ (MMY14). However, the G_α protein coupling profile of the $hHCA_3$ receptor was less promiscuous, as the receptor only activated $G_{\alpha i1}$ (MMY23) and $G_{\alpha i3}$ (MMY24) pathways.

We then constructed two mutant receptors by 'swapping' the short (HCA_2) and long (HCA_3) C-terminus. The differences in HCA_2 and HCA_3 receptor activation and G protein selectivity were not controlled, however, by their C-terminal tails, as we observed only minor differences between mutant and corresponding wild-type receptor. This study provides new insights into the G protein coupling profiles of the HCA receptors and the function of the receptor's C terminus, which may be extended to other GPCRs.

In **Chapter 6**, we focused on the design and synthesis of HCA_2 agonists with the additional quality of having a long residence time on the receptor, as the latter parameter may be linked to *in vivo* efficacy. Structure-affinity relationship (SAR) and structure-kinetics relationship (SKR) studies were combined to investigate a series of biphenyl anthranilic acid agonists for the HCA_2 receptor. In total, 27 compounds were synthesized and twelve of them showed higher affinity than nicotinic acid. Two compounds, **6g (**IC_{50} **=** 75 nM) and **6z** (IC_{50} = 108 nM) showed a longer residence time profile compared to nicotinic acid, exemplified by their kinetic rate index (KRI) values of 1.31 and 1.23, respectively. The SAR study resulted in the novel 2-F, 4-OH derivative (**6x**) with an IC_{50} value of 23 nM as the highest affinity HCA_2 agonist of the biphenyl series, although it showed a similar residence time as nicotinic acid. The SAR and SKR data suggest that an early compound selection based on binding kinetics is a promising addition to

the lead optimization process.

In **Chapter 7**, we summarized our findings in the present thesis and described the advantages of the *S. cerevisiae* system and the importance of residues in conserved sequence motifs, such as DRY and NPxxY. Future perspectives for drug discovery based on our findings with respect to receptor activation and G protein coupling are discussed. All in all, the variety of methods described in this thesis provided us a detailed understanding of receptor function, suggesting that novel avenues for further drug discovery on these established targets is entirely feasible.