Allosteric modulation by sodium ions and amilorides of G protein-coupled receptors : a closer look at the sodium ion site of the adenosine A2a receptor and development of a mass spectrometry ligand binding assay for adenosine A1 and A2a receptors

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Summary

The study of allosteric modulation offers new insights in the activation mechanisms of G protein-coupled receptors (GPCRs). The sodium ion site is an allosteric site that is well conserved among Class A GPCRs. Even though it is well conserved, the allosteric sodium ion site is versatile in its allosteric effects and the size of ligands that can bind, from sodium ions to 5'-amino and 2-guanidino substituted amilorides **(Chapter 2)**. The adenosine A_{2A} receptor was the first GPCR to be crystallized with a sufficiently high resolution to reveal a sodium ion bound in the sodium ion site. In this thesis I applied the adenosine A_{2A} receptor as a model GPCR for the study of allosteric modulation effected by the sodium ion site **(Chapters 3 – 5)**, and together with the adenosine A_1 receptor for the development of a ligand binding assay based on mass spectrometry **(Chapter 6)**.

The sodium ion site and the orthosteric site of the adenosine A_{2A} receptor can be occupied simultaneously when an antagonist is bound in the orthosteric site, but not when an agonist is bound **(Chapter 3)**. Sodium ions seem to stabilize the inactive conformation, thereby promoting antagonist binding, but excluding agonist binding at a physiologically relevant sodium ion concentration. By binding into the sodium ion site, amiloride analogues also exhibit distinct patterns of agonist and antagonist modulation.

Next to its effect on orthosteric ligand binding, the allosteric sodium ion site facilitates receptor signaling **(Chapter 4)**. Mutation of the amino acids that form the sodium ion site influenced the affinity of ligands binding to it and to the orthosteric site, but also changed the capacity of the receptor as a whole to be activated by agonists. Mutation of the polar residues in the sodium ion pocket was shown to either abrogate (D52A$^{2.50}$ and N284A$^{7.49}$) or reduce (S91A$^{3.39}$, W246A$^{6.48}$, and N280A$^{7.45}$) the negative allosteric effect of sodium ions on agonist binding. Mutations D52A$^{2.50}$ and N284A$^{7.49}$ completely abolished receptor signaling, while mutations S91A$^{3.39}$ and N280A$^{7.45}$ elevated basal activity and mutations S91A$^{3.39}$, W246A$^{6.48}$, and N280A$^{7.45}$ decreased agonist-stimulated receptor signaling. In molecular dynamics simulations D52A$^{2.50}$ directly affected the mobility of sodium ions, which readily migrated to another pocket formed by Glu13$^{1.39}$ and His278$^{7.43}$. The D52A$^{2.50}$ mutation also decreased the potency of amiloride with respect to orthosteric ligand

displacement, but did not change orthosteric ligand affinity. In contrast, W246A$^{6.48}$ increased some of the allosteric effects of sodium ions and amiloride, while orthosteric ligand binding was decreased.

Amiloride can be extended with substituents at the 5' position to produce amiloride derivatives with different allosteric properties and similar or even higher potencies than HMA at the wild-type adenosine A_{2A} receptor **(Chapter 5)**. The potency of a series of 5'-substituted amiloride derivatives was assessed by their ability to displace orthosteric radioligand [^{3}H]ZM-241,385 from both the wild-type and sodium ion site W246A$^{6.48}$ mutant adenosine A_{2A} receptors. Of this series, 4-ethoxyphenethyl-substituted amiloride 12l was found to be more potent than HMA. Similar to amiloride and HMA, the novel amiloride derivatives showed significantly higher potencies at the W246A$^{6.48}$ mutant adenosine A_{2A} receptor, implying that Trp246$^{6.48}$ hinders the binding of these amiloride derivatives. HMA showed the largest allosteric effect on [^{3}H]ZM-241,385 dissociation, whereas the most potent novel amilorides 12h, i, k, and l showed reduced or no effects on this dissociation process. This indicates that these novel amilorides engage in a more direct competition with the orthosteric ligand, and it can be hypothesized that they do so by intrusion into the orthosteric pocket. The striking differences in effect on the dissociation of [^{3}H]ZM-241,385 between the wild-type and W246A$^{6.48}$ adenosine A_{2A} receptors imply that Trp246$^{6.48}$ influences the nature of the allosteric interaction by amilorides.

Mass spectrometry is a valid alternative to radioligand detection for the quantification of ligand binding to the adenosine A_1 and A_{2A} receptors **(Chapter 6)**. Despite the robustness of radioligand binding assays, they carry inherent disadvantages in terms of safety precautions, expensive synthesis, special laboratory requirements, and waste disposal. Mass spectrometry is a method that can selectively detect ligands without the need of a label. The sensitivity of mass spectrometry equipment increases progressively, and currently it is possible to detect the low ligand quantities that are usually found in ligand binding assays. We developed a label-free mass spectrometry ligand binding (MS binding) assay for the adenosine A_1 and A_{2A} receptors. Radioligand binding assays for both receptors are well established, and ample data is available to compare and evaluate the performance of an MS binding assay. To prove the feasibility of MS binding on the adenosine A_1 and A_{2A} receptors we first developed a mass spectrometry detection method

for unlabeled DPCPX and ZM-241,385, which are ligands with high selectivity and affinity for the respective receptors. To serve as internal standards, both compounds were also deuterium-labeled. Subsequently, we investigated whether the two unlabeled compounds could substitute for their radiolabeled counterparts as marker ligands in binding experiments, including saturation, displacement, dissociation, and competition association assays. Furthermore, we investigated the accuracy of these assays if the use of internal standards was excluded. The results demonstrate the feasibility of the MS binding assay, even in the absence of a deuterium-labeled internal standard, and provide great promise for the further development of label-free assays based on mass spectrometry for other GPCRs.

Chapter 7 concludes this thesis by reflecting on its contents and their place in the current state of GPCR research, and on the future perspectives that this field of research has to offer.