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Chapter 1

General introduction

From ancient times on mankind has searched for ways to cure diseases. The first written records originate from the Mesopotamians and Egyptians who already had a keen interest in the causes and the healing of diseases.^{1,2} In these times healing was strongly tied to religion and superstition, and diseases were mainly ascribed to supernatural powers. Still, the basics of objective and empirical medicine started to emerge, including the description and use of natural products to cure diseases.³ This mixture of superstition and rationalism in the approach to medicine remained more or less the same until the advent of scientific experimental methods in the 16th and 17th centuries. The field of pharmacology emerged as late as the 19th century, when the concept of chemical structures and their relationship with their pharmacological action became a subject of study.⁴ In the following period impressive advancements were made with the formulation of receptor theory and the discovery of numerous receptor types and their endogenous ligands.^{5, 6} Today, pharmacology is a very diverse field with elements of molecular biology, genetics, chemistry, and bio-informatics, to name but a few disciplines. Together, these disciplines help to understand why a drug works and give exciting opportunities to develop new drugs.

G protein-coupled receptors

Of the many drug targets currently identified the G protein-coupled receptors (GPCRs) constitute a large fraction. The family of GPCRs encompasses more than 800 different cell membrane-bound receptors,⁷ which regulate many different aspects of human physiology, such as vision, homeostasis, and the immune system. This makes it no surprise that they are targeted by 30% to 40% of all drugs on the market.⁸ The general role of GPCRs is to relay a diverse collection of extracellular signals, such as photons, hormones, odorants, and neurotransmitters, to the intracellular environment. At the intracellular side GPCRs interface with G proteins that can further transduce the signal. GPCRs share a common structure of seven transmembrane alpha-helices connected by extra- and intra-cellular loops (Figure 1). According to their structural architecture, GPCRs can be subdivided in five different families: rhodopsin-like (class A), secretin-like (class B), glutamate-like (class C), adhesion-like, and frizzled/taste2-like receptors.⁷ Of these, the class A rhodopsin-like receptors constitute by far the largest group.

Allosteric modulation and the sodium ion site

The ligands that can activate GPCRs know a wide variation in both size and chemical structure, from large proteins and peptides to small molecules and ions. The binding sites on GPCRs can be categorized as orthosteric sites, where endogenous ligands bind and activate the receptor, and allosteric sites, where ligand binding can modulate receptor activation (Figure 1). Most drugs target the orthosteric site as antagonists thereby blocking endogenous ligand binding and subsequent activation of the receptor, or as agonists that activate the receptor. Drugs that target the allosteric site may have a less ‘blunt’ effect, as they enhance or dampen the activation by endogenous ligands.⁹⁻¹¹ This has the advantage that the action is more localized in both time and space, as these allosteric modulators in the most stringent definition only have an effect when the endogenous ligand is present at the orthosteric site.

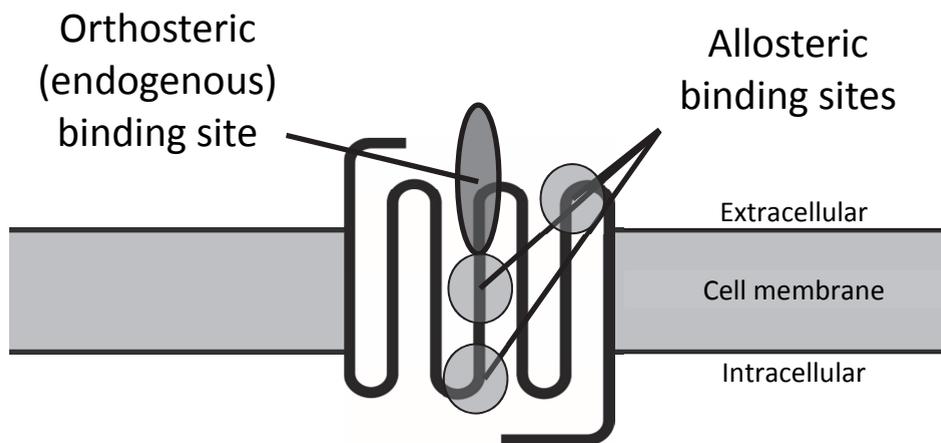


Figure 1. Schematic representation of a GPCR embedded in the cell membrane. Seven transmembrane alpha-helices cross the cell membrane, with an extracellular N-terminus, an intracellular C-terminus, and six intra- and extra-cellular loops that connect the transmembrane regions. The orthosteric binding site is represented by an oval and the allosteric binding sites by circles. The middle circle approximates the location of the sodium ion site, in the central region of the receptor just below the orthosteric site. Note that the situation may be different for each GPCR considering the location and the number of binding sites.

The concept of allosteric modulation is a promising one for the discovery of new drugs, and quite a few allosteric modulators have been found for GPCRs.¹² However, the molecular basis of allosteric activity is still largely unknown. The increasing availability of GPCR crystal structures sheds more and more light on the molecular aspects of ligand

binding and GPCR activation.¹³⁻¹⁵ Recently, crystal structures have been solved with sufficiently high resolution to be able to spot a sodium ion in an allosteric site of several GPCRs, bound by the highly conserved amino acid Asp^{2.50} (Numbering according to Ballesteros and Weinstein¹⁶).¹⁷⁻²¹ Next to these solved crystal structures, sequence alignment indicates that this sodium ion site is very conserved and present in almost all class A GPCRs.²² This affirmed the results of earlier studies in which sodium ions were described as allosteric modulators of GPCRs.²³

The small molecule amiloride has been found to be an allosteric modulator for GPCRs as well. Amiloride is used therapeutically as a potassium sparing diuretic by blocking renal epithelial sodium channels (ENaCs).²⁴ For GPCRs, amilorides have been found to compete with sodium ions for the same allosteric site.²⁵ The feature of the sodium ion binding site to bind both small ions and small molecules is intriguing. Moreover, sodium ions and amilorides exhibit different allosteric modulation patterns, relative to each other but also between the different GPCRs, as discussed for sodium ions by Katritch et al.²² and for amilorides in Chapter 2 of this thesis. This makes the sodium ion site an excellent candidate of research to further understand the mechanisms behind allosteric modulation.

Adenosine receptors

The first crystal structure in which a sodium ion binding site was identified was of the adenosine A_{2A} receptor.¹⁷ Adenosine receptors belong to the class A rhodopsin-like GPCRs, and as the name suggests adenosine is the endogenous ligand. Caffeine and theophylline are antagonists and have a wide-spread use as a stimulant due to their ability to block adenosine receptors. The group of adenosine receptors encompasses four different subtypes with different structural features and physiological roles, named the adenosine A₁, A_{2A}, A_{2B}, and A₃ receptors. These receptors are well-documented by a few decades of research yielding a good understanding of their physiological role and a considerable number of selective agonists and antagonists is available.^{26, 27} Adenosine A_{2A} receptors couple to G_s and G_{olf} proteins and activation stimulates intracellular cAMP production.²⁸ Adenosine A_{2A} receptors are found in many different tissues, such as the brain striatum, leukocytes, the lung, and the heart. For their wide presence in the body adenosine A_{2A} receptors regulate many different physiological processes, amongst which psychiatric

behavior, the sleep-wake cycle, the immune system, myocardial oxygen consumption, and angiogenesis. With these diverse roles the adenosine A_{2A} receptor attracts substantial clinical interest for the development of drugs. Selective antagonists have been or are currently in clinical trials for treatment of Parkinson's disease and cocaine addiction, and the same is true for selective agonists to replace adenosine as a coronary vasodilator in myocardial perfusion imaging and treatment of sickle cell anemia.²⁸

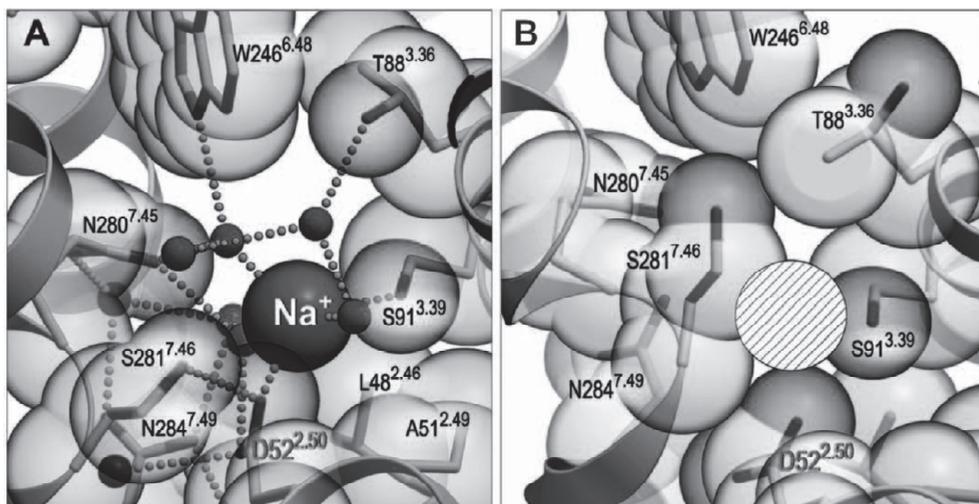


Figure 2. The sodium ion site in the crystal structures of the inactive (**A**) and the active state (**B**) of the adenosine A_{2A} receptor. In **A** the sodium ion is shown as a dark grey sphere, coordinated by D52^{2.50} through a salt bridge (dotted lines) and by waters (small solid spheres) and residue S91^{3.39} through hydrogen bonds (dotted lines). In **B** the pocket is collapsed leaving no room for the sodium ion to bind (hatched sphere). The receptor backbone is shown as ribbons, residues lining the sodium ion site are shown as sticks, and carbon and oxygen atom spheres are transparent. The crystal structure **A** was co-crystallized with antagonist ZM-241,385 and **B** with agonist UK-432,097.²⁹ The binding site of these orthosteric ligands is located above residue W246^{6.48} and is clipped from this detailed view. From Liu et al.¹⁷ Reprinted with permission from AAAS.

For the adenosine A_{2A} receptor several crystal structures bound to agonists and antagonists have been solved.^{30, 31} The recently solved crystal structure with a bound sodium ion added substantially to our understanding of the molecular mechanism of adenosine A_{2A} activation.¹⁷ This crystal structure was co-crystallized with the selective antagonist ZM-241,385 and was hence a snapshot of the 'inactive state' of the receptor. Comparison with the 'active state' of the receptor co-crystallized with an agonist made clear that the sodium ion site undergoes substantial changes between the active and the

inactive states of the receptor (Figure 2). The apparent central role of the allosteric sodium ion site in the activation of the adenosine A_{2A} receptor urged us to further investigate it in Chapters 3-5 of this thesis.

Ligand binding assays

To further explain the information derived from crystal structures the 'wet lab' is still indispensable. The field of pharmacology provides a multitude of different experimental setups to probe receptor and ligand characteristics. Most of these methods are dependent on labeled probes, for example radioligands and fluorescent ligands. Radioligand binding is a robust and well-tried method to probe pharmacological properties of ligands,³² and this method served us well in this thesis. However, the use of radiolabeled ligands is expensive and requires careful handling regarding safety and waste disposal. Therefore new methods to assess ligand binding are being developed, such as fluorescent labeling.³³ Labeling with bulky fluorescent moieties brings however its own risk of changing the pharmacological properties of a ligand. A label-free alternative for ligand binding assays is offered by mass spectrometry (MS).

Advancements in mass spectrometry have made it possible to accurately measure the small quantities of ligand usually found in ligand binding assays. The use of mass spectrometry in ligand binding assays was coined 'mass spectrometry binding' (MS binding) by the group of Wanner. They developed it for several targets, amongst which the dopamine D₂ receptor,³⁴ the GABA transporter mGAT1,³⁵ and the serotonin transporter.^{36,37} MS binding essentially follows a similar protocol as radioligand binding up to the point of the quantification of bound ligand, except for the use of an unlabeled ligand instead of a radioligand.

In both radioligand and MS binding assays the ligand is allowed to bind to a crude membrane extract of cells expressing the target receptor. Then the reaction mix consisting of the ligand and receptor is filtered over a glass fiber filter, which only retains the membrane with the ligands bound to the receptor, while the unbound ligand is separated. After the filtration step radioligand and MS binding assays diverge. In the radioligand binding assay a scintillation fluid is applied to the filter after which the amount of radioligand can be quantified by scintillation counting. In the MS binding assay the bound ligand is first eluted from the filter by an organic solvent after which the ligand amount in

the solvent is quantified by a mass spectrometer. Before the detection by the mass spectrometer the ligand and remaining membrane content is separated by liquid chromatography (LC). Both the LC method and the MS detection method have to be developed for every ligand specifically, as each ligand has a different molecular mass and a different retention time on an LC column. A ligand for which the LC-MS method is developed is also called a marker ligand, and can be used in the same manner as a radioligand in radioligand binding assays to determine pharmacological binding properties of other, unlabeled ligands.

The types of radioligand binding assays of which equivalents have been applied to MS binding by the group of Wanner are ligand saturation, displacement, association, and dissociation assays. Another type of radioligand binding assay is the competition association assay, in which the kinetic properties of a ligand can be determined without measuring binding of that ligand directly. Instead the ligand of interest competes for binding to the target with a radioligand of which binding can be followed directly. Fitting the resulting association data of the marker ligand to a model describing competition association derives the k_{on} and k_{off} for the competing ligand.³⁸ In this thesis, we describe the development of an MS binding assay for the adenosine A₁ and A_{2A} receptors and its validation for the different types of ligand binding assays, including for the first time the competition association assay.

This thesis

In **Chapter 2** the current knowledge of allosteric modulation of class A GPCRs by amilorides is reviewed. We describe how the allosteric effects of amiloride and its analogues can be very different, depending on the GPCR and the orthosteric ligand in question. In **Chapter 3** we focus on the mechanism of allosteric modulation by sodium ions in the adenosine A_{2A} receptor by integrating the results of molecular dynamics, radioligand binding, and thermostability experiments. This approach allowed us to further explore the difference in allosteric modulation by sodium ions and amilorides between agonists and antagonists. In **Chapter 4** we combine molecular dynamics, radioligand binding, and functional assays in a mutagenesis study to investigate the role of the amino acids that form the sodium ion site of the adenosine A_{2A} receptor in ligand affinity and receptor signaling. We found that the sodium ion site was not only important for sodium ion and amiloride binding, but also for the overall receptor conformation and signal transduction. In **Chapter 5** we describe the synthesis and biological evaluation of a number of amiloride analogues for the adenosine A_{2A} receptor. We found novel potent amiloride analogues that all displace orthosteric ligands, but by different allosteric mechanisms.

In these studies radioligands have been indispensable. Yet, the use of radioactively labeled ligands in the lab has its drawbacks, and hence **Chapter 6** is dedicated to the development of a non-radioactive ligand binding protocol based on mass spectrometry (MS binding) for the adenosine A_{2A} receptor and its close homolog the adenosine A₁ receptor. We validated that the MS binding assay works for different types of radioligand binding assays, and in addition we combined it for the first time with the competition association method to determine ligand binding kinetics.

To conclude, we discuss in **Chapter 7** the results of the research presented in this thesis in general together with the further implications for the field of GPCR pharmacology.

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