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Chapter 7

Conclusions and future perspectives

The main theme of this thesis, allosteric modulation effectuated through the sodium ion site of GPCRs, is inspired by the important role that this site appears to play in GPCR signaling. As sodium ions are abundant under physiological conditions they may affect GPCR signaling considerably. Receptor activation causes a substantial rearrangement of the sodium ion site, suggesting that it has an important role in this process.¹

Chapter 2 reviews the current knowledge on allosteric modulation of amiloride and its derivatives binding to the sodium ion site of Class A GPCRs. Chapters 3 to 5 follow-up on the recent crystal structure of the adenosine A_{2A} receptor with a sodium ion bound.² Chapters 3 and 4 complement the crystal structure with additional results from combined biochemistry, biophysical, molecular dynamics, and mutational studies. Chapter 5 describes the synthesis of novel amiloride derivatives that bind in the sodium ion site but also protrude into the orthosteric binding site. In Chapters 3 to 5, radio-labeled ligands were used to quantify ligand binding to the receptor, and Chapter 6 describes an alternative approach towards ligand binding assays. Instead of using a radio-label, mass spectrometry was used to quantify the binding of an unlabeled ligand to the adenosine A₁ and A_{2A} receptors.

Conclusions

A versatile allosteric site and tool compounds to probe its properties

Even though the sodium ion site is a well conserved allosteric site among Class A GPCRs, it is versatile in the ligands that it can bind and the resulting allosteric effects. Not only does it bind the sodium ion, but also the small molecule amiloride and its derivatives. Chapter 2 of this thesis reviews the variety of amiloride derivatives and the different allosteric effects that they can exert on Class A GPCRs. A general trend is the higher affinity for GPCRs of amilorides with lipophilic substituents over the parent amiloride. Amilorides have been found to allosterically modulate adenosine, adrenergic, dopamine, chemokine, muscarinic, serotonin and gonadotropin-releasing hormone receptors. Of these, the adenosine, α -adrenergic, and dopamine receptors experience the highest degree of modulation by amiloride and analogues. Due to the fact that the sodium ion site is well conserved it is to be expected that amilorides can also bind and modulate other GPCRs not yet investigated for their sensitivity.

The allosteric effects triggered by amilorides binding to GPCRs can be divided in positive and negative allosteric modulation, and competitive and noncompetitive displacement of the orthosteric ligand. The type of allosteric effect depends on whether the orthosteric ligand is an agonist or an antagonist and on the specific GPCR type. Even for closely related sub-types of GPCRs the effects can be quite different. For most receptors amilorides act as negative allosteric modulators of both agonist and antagonist binding. However, for some receptors amilorides display probe dependency as they act as positive allosteric modulators of agonist binding but not of antagonist binding. Examples of this amiloride probe dependency have been found for the adenosine A_3 and the α_{2A} -adrenergic receptors. In the case of the α_{2B} -adrenergic receptor differently substituted amiloride derivatives can even have either negative or positive allosteric effects on antagonist binding. The flexibility of such a conserved site to exert different allosteric effects by the binding of very similar molecules is intriguing and suggests a role for amino acids not directly involved in sodium ion binding.

A physiological role for allosteric modulation

Allosteric modulation as a general concept has been found on many different targets, also beyond the GPCR superfamily. As a definition, allosteric sites are distinct from orthosteric sites where endogenous ligands bind. This implies that no (known) endogenous ligands bind to allosteric sites, and hence allosteric ligands are usually found in high-throughput screens of large collections of non-endogenous ligands.³ This does not exclude that allosteric sites have no endogenous ligands binding to them, but in general these have not been found.⁴ Without the need to accommodate the binding of endogenous ligands, allosteric sites have supposedly less evolutionary pressure to stay the same. This is an advantage for drug design for sub-types of GPCRs. The orthosteric site of receptor sub-types (for instance the adenosine A₁, A_{2A}, A_{2B}, and A₃ receptors) endure evolutionary pressure to keep similar features, as they bind the same endogenous ligands, and this is a challenge when designing ligands selective for one sub-type. Allosteric sites which do not feel this evolutionary pressure can have more diverse features, making it easier to develop ligands that are sub-type selective.^{4,5}

The allosteric sodium ion binding site is an exception to this rule. First of all, the sodium ion binds in this site, which may be defined as an endogenous ligand, for its abundant presence in the body. The sodium ion site is remarkably well conserved amongst Class A GPCRs, suggesting major evolutionary pressure to maintain its features.¹ Indeed, one of the conclusions of Chapter 3 of this thesis is that the physiological concentration of sodium ions is high enough to occupy the sodium ion site of 75 % of adenosine A_{2A} receptors present in the body, substantially reducing their sensitivity to activation by endogenous adenosine. This points to a role of sodium ions as suppressors of in-vivo adenosine A_{2A} receptor activation. Again, the high conservation of the sodium ion site amongst Class A GPCRs suggests that this is an important general mechanism for organisms to control GPCR activity. Indeed, the inhibitory effect of sodium ions at physiologically relevant concentrations has been found for many GPCRs. This also implies the importance to control sodium ion concentrations for in vitro ligand binding assays.

Sodium ions stabilize the adenosine A_{2A} receptor in the inactive state

Even without access to structural information, a few decades of studying the effects of sodium ions and amiloride on GPCRs established that they bind to an allosteric site, instead of the orthosteric site. The recent elucidation of GPCR crystal structures with a bound sodium ion revealed the molecular details of its binding in the allosteric sodium ion site. Previous crystal structures of GPCRs in the antagonist-bound state likely had a sodium ion bound in the sodium ion site, as usually a large concentration of sodium ions is added to stabilize the inactive state of the receptor, but the relatively small ion could not be detected due to the limited resolution of these structures.¹ The first crystal structure with sufficiently high resolution to reveal a bound sodium ion was of the adenosine A_{2A} receptor.² The sodium ion was held by a ionic interaction with Asp52^{2,50}, confirming previous results tying this residue to sodium ion effects,⁶ and a hydrogen-bonding network of waters and amino acids forming the sodium ion site. One of the most eye-catching differences between the inactive sodium ion bound structure and the active structure was the occlusion of the sodium ion site in the active structure, which suggested that binding of a sodium ion to an agonist-bound receptor is impossible.

Chapter 3 follows up on this crystal structure studying in more detail the differences in allosteric effects exerted by sodium ions and amilorides binding in the sodium ion site. The allosteric effects of sodium ions, amiloride, and its analogue HMA on orthosteric ligand binding to the adenosine A_{2A} receptor were evaluated by a combination of molecular dynamics, radioligand binding, and thermostability studies. It was concluded that an antagonist ([³H]ZM-241,385) and a sodium ion can bind to the receptor simultaneously, while the binding of an agonist ([³H]NECA) and a sodium ion excludes each other. The results indicated that binding of a sodium ion to the sodium ion site stabilizes the receptor in its inactive conformation, thereby prohibiting agonist binding, but facilitating antagonist binding. The stabilization of Trp246^{6,48} by the sodium ion appeared to play an important role in maintaining the inactive conformation. This fits well with the previous identification of Trp246^{6,48} as a “toggle switch” for receptor activation.⁷

In contrast to sodium ions, amiloride and HMA displaced both the agonist and antagonist, but they still displayed distinct allosteric effects between the two. Similar to sodium ions, the amilorides displaced agonist [³H]NECA competitively, while the amilorides and antagonist [³H]ZM-241,385 could bind simultaneously. Unlike sodium ions

however, the amilorides displaced the antagonist in a noncompetitive manner, likely by pushing Trp246^{6.48} into a different rotameric position. This suggests that amiloride binding results in a delicate balance between improved stability of the inactive receptor conformation and an indirect, noncompetitive, interference with antagonist binding.

Mutations to further investigate the sodium ion site

The presence or absence of a sodium ion in its binding site seems to dictate the conformation of the receptor in either its inactive or active states, respectively. Hence, Chapter 4 of this thesis focuses on the amino acids forming the sodium ion site and their purpose in receptor activation. Site-directed single point mutations into alanine of Asp52^{2.50}, Ser91^{3.39}, Trp246^{6.48}, Asn280^{7.45}, and Asn284^{7.49} were evaluated for their effect on orthosteric ligand binding, allosteric modulation by sodium ions and amilorides, and receptor activation. Except for Asp52^{2.50}, the mutation of these sodium ion site amino acids lowered the affinity of agonist [³H]NECA binding substantially, but did not affect antagonist [³H]ZM-241,385 binding as much. As Trp246^{6.48} is the only residue interacting directly with an agonist binding in the orthosteric site, the residues Ser91^{3.39}, Asn280^{7.45}, and Asn284^{7.49} must be important for maintaining a conformation of the receptor that is indirectly suitable for agonist binding.

All these sodium ion site mutations either abrogated or reduced the negative allosteric effect of sodium ions on agonist binding, but had mixed effects on amiloride and its derivative HMA. D52A^{2.50} reduced their potency, confirming the docking pose of the amilorides in which their positively charged guanidinium moiety engages in an ionic bond with the negatively charged Asp52^{2.50}, similar to the sodium ion. In contrast, W246A^{6.48}, N280A^{7.45}, and N284A^{7.49} increased the potency of the amilorides, with a remarkably large effect of the Trp246^{6.48} mutation. This indicates that these residues hinder the binding of amiloride and HMA into the sodium ion site.

Finally, mutation of the amino acids forming the sodium ion site substantially affected receptor activation. Mutations D52A^{2.50} and N284A^{7.49} completely abolished the receptor's ability to be activated, while mutations S91A^{3.39} and N280A^{7.45} induced constitutive activity of the receptor, and mutations S91A^{3.39}, W246A^{6.48}, and N280A^{7.45} decreased the agonist activation response of the receptor. From this it can be concluded that besides their effect on sodium ion, amiloride, and orthosteric ligand binding, the amino acids forming the sodium ion site are involved in the activation mechanism of the adenosine A_{2A} receptor.

In molecular dynamics simulations, D52A^{2.50} caused the sodium ion to dissociate promptly towards a vestibule pocket, formed by Glu13^{1.39} and His278^{7.43}, suggesting a pathway for sodium ion entering the sodium ion site. Indeed these residues had been found to be important for allosteric modulation by sodium ions in a previous mutation study.⁸ His278^{7.43} is also important for agonist binding, as shown in the same study and in the agonist-bound crystal structure of the adenosine A_{2A} receptor, suggesting a second site next to the sodium ion site where sodium ions can antagonize agonist binding.

Larger amiloride derivatives reach into the orthosteric binding site

The abundance of different 5'-substituted amiloride analogues that can allosterically modulate the adenosine A_{2A} receptor but also GPCRs in general, encourages further investigation of the possibilities for different moieties on this position. In Chapter 5, different phenethyl substitutions on the 5' position of amiloride were evaluated for their allosteric effect on binding of the antagonist [³H]ZM-241,385 to the wild-type and W246A^{6.48} mutated adenosine A_{2A} receptor. On the wild-type receptor, the 4-ethoxyphenethyl substituted amiloride yielded a higher potency than reference amiloride HMA, while methoxy and dioxolylphenethyl derivatives had potencies similar to HMA. Docking of these derivatives of amiloride in the sodium ion site suggested that their phenethyl moieties entered a hydrophobic pocket close to the sodium ion site, explaining the preference for lipophilic moieties on this position.

Just as for HMA, the novel phenethyl amiloride derivatives had a higher potency for the W246A^{6.48} mutated receptor, confirming their binding in the sodium ion site. Indeed, docking of these amiloride phenethyl-derivatives revealed a steric clash with residue Trp246^{6.48}, pushing it towards another rotameric position, just as observed for amiloride and HMA in Chapter 3. The bulkiest dioxolyl and ethoxyphenethyl derivatives benefited the most in their potency from the absence of the tryptophan, suggesting that bulkier 5' substituents increase the steric clash with Trp246^{6.48}.

The docking modes of the amiloride derivatives predicted that their elongated phenethyl substituents protrude into the orthosteric site pocket and can engage in a direct competition with orthosteric ligands. This was supported by the observed effects of the amilorides on the dissociation kinetics of antagonist [³H]ZM-241,385 on the wild-type receptor. Whereas HMA accelerated antagonist dissociation the most, signifying a noncompetitive interaction, the phenethyl derivatives had less effect on antagonist

dissociation. The dioxolylphenethyl derivative did not affect the dissociation of the antagonist at all, signifying a completely competitive displacement of the antagonist.

An unlabeled ligand binding assay

In Chapters 3 to 5 of this thesis radioligand binding assays were applied to quantify ligand binding to the adenosine A_{2A} receptor. Although radioligands are recognized as robust and reliable tools in the measurement of ligand binding, they have their drawbacks in terms of safety, production costs, and waste disposal. Alternative ligand binding assays are therefore of interest. Such an alternative approach was sought in the quantification of the binding of unlabeled ligands to their targets by mass spectrometry, or MS binding. The ongoing development of mass spectrometers increases their sensitivity and has opened the possibility to accurately quantify the small amounts of ligands that are found in ligand binding assays, usually in the pM range. The group of Wanner has pioneered the MS binding assay for several targets, among which GPCRs.⁹ In Chapter 6 of this thesis, we developed and validated the MS binding assays for additional GPCR targets, the adenosine A₁ and A_{2A} receptors.

As unlabeled marker ligands for the MS binding assay DCPX and ZM-241,385 were chosen, ligands with a high selectivity and affinity for the adenosine A₁ and A_{2A} receptors, respectively. The application of marker ligands in MS binding is analogous to radioligands in radioligand binding, and the availability of radio-labeled versions of these ligands makes for a straightforward validation of the MS binding assay by radioligand binding assays. Although the ligand that is binding to the receptor itself is unlabeled, it is good practice in mass spectrometry to add a fixed concentration of internal standard to each injected sample to increase the accuracy of MS quantification. Preferably this is a deuterated version of the marker ligand, which has the same column retention time due to similar chemical properties, but can be discerned in MS by its different molecular weight. For this purpose, deuterium-labeled DCPX and ZM-241,385 were synthesized. Subsequently, an LC-MS method to quantify pM concentrations of the marker ligands was developed, with their deuterium-labeled counterparts as internal standards.

With the MS quantification method developed, saturation, association, and dissociation MS binding assays were performed and validated against radioligand binding assays. Furthermore, displacement assays were performed and validated in which the

affinity of other ligands can be indirectly measured through their competition with the marker ligand. Finally, the MS binding assay was for the first time successfully applied to the competition association assay, in which the marker ligand competes with another ligand for association to the receptor. This allows for the determination of association and dissociation rates of the other ligand indirectly through fitting the association data of the marker ligand to the model of Motulsky and Mahan.¹⁰

The necessity to use an internal standard in the mass spectrometry quantification step was scrutinized. The internal standard is used to compensate for sources of signal distortion in MS quantification. Ligand binding assays typically use multiple measuring points to determine affinity and kinetic properties of ligands, and this already leads to a certain degree of compensation for deviations of individual points. This aspect of ligand binding assays could mean that compensation of MS quantification results by an internal standard does not add significantly to their accuracy. Indeed, similar K_i , k_{on} , and k_{off} values were obtained from data that was either compensated by internal standard quantification and from data without this compensation, indicating that an internal standard is not strictly necessary in MS binding assays. Thus, the MS binding assay allows to measure affinity and kinetic ligand properties without the need to synthesize any labeled ligand.

Future perspectives

Protein structure elucidation and GPCRs

The field of GPCR structural biology has been greatly advanced by the elucidation of the X-ray crystal structures of 30 different types of GPCRs. As to the sodium ion site, the elucidation of sodium ion-bound GPCR structures has revealed the molecular details of sodium ion binding, adding importantly to the already existing body of pharmacological evidence of modulation of GPCRs by sodium ions. The availability of structural information inspires new pharmacological studies of the sodium ion site, as exemplified by Chapters 3 to 5 of this thesis.

Many GPCRs remain to be crystallized though, as the GPCR family consists of more than 800 different receptors. As GPCRs are membrane proteins, they are hydrophobic in nature and this is one of the challenges in growing crystals that are viable for X-ray diffraction. This has mainly to do with their stability when taken out of their natural membrane environment. Except for rhodopsin, GPCR crystal structures could only be solved with the help of different protein engineering methods, such as thermostabilizing point mutations, insertion of hydrophilic regions obtained from other proteins, and deletion of flexible loop and terminus regions.¹¹ Additionally, the resulting engineered GPCRs should reach sufficiently high expression levels to produce the large quantities of pure protein needed for the crystallization process. One approach to facilitate the elucidation of crystal structures of the remaining human GPCRs, is to systematically screen all of them for suitable engineered fusion constructs with sufficiently high expression levels.¹²

A limitation of X-ray crystallography is that it only catches a snapshot of the protein structure that is frozen in time. However, GPCRs are dynamic proteins with many different possible conformations. The comparison of antagonist (inactive) and agonist (active) bound structures may reveal the global movements required for receptor activation, but less stable intermediate conformations will be likely missed as the actual activation process is not followed. Techniques that allow following protein dynamics in time are in full development for GPCRs, for example solid- and solution-state nuclear magnetic resonance (NMR) and serial femtosecond crystallography (SFX).

NMR allows detecting the dynamics of isotopically labeled amino acids. The study of GPCRs by NMR has been primarily focused on GPCR regions due to protein size restrictions of this technique.¹³ However, through isotope labeling of a limited number of amino acids spread over the receptor, the global dynamics of the receptor's backbone can be followed, as recently applied to the β_1 -adrenergic receptor¹⁴ and the adenosine A_{2A} receptor.¹⁵ SFX enables to gather data from protein crystals by X-ray free electron laser (XFEL) without damaging the proteins, in contrast to X-ray crystallography.¹⁶ This allows structural data to be obtained at room temperature, thus physiologically more relevant than X-ray crystallography, and from smaller crystals, simplifying the crystal growth step. Until now SFX has been used to obtain structural GPCR snapshots, for example for the serotonin 5-HT_{2B} receptor,¹⁷ but it holds the promise of time-resolved structural studies of GPCR activation, for example on the conformational changes of rhodopsin induced by light.¹⁸

Advances in molecular dynamics

The availability of structural information opened the possibility to apply molecular dynamics to computationally simulate GPCR dynamics.¹⁹ With the captured snapshot from a crystal structure as a starting point, molecular dynamics simulates 'in silico' the subsequent events on an atomic and femtosecond scale. This allows to follow various processes, such as structural rearrangements upon receptor activation,²⁰ ligand association,²¹ and ligand dissociation.²² As techniques to follow structural receptor dynamics in real-time are still nascent, molecular dynamics may provide a good alternative. Even if molecular dynamics is a powerful tool to understand receptor dynamics on a molecular scale, it remains a simulation depending on force-fields that only approximate actual atomic interactions. This makes it desirable to support conclusions drawn from 'in silico' molecular dynamics studies by 'wet lab' biochemical experiments, as demonstrated in Chapters 3 and 4 of this thesis.

One of the drawbacks of molecular dynamics is the vast amount of computational power needed to simulate complex systems such as GPCRs on an atomic scale.¹⁹ Calculations to complete microsecond simulations typically take weeks or months, while conformational rearrangements upon GPCR activation may take milliseconds to complete.^{23,24} Certain 'tricks' can be applied to increase calculation speeds and simulation lengths, such as reducing energy barriers and temperature accelerated molecular dynamics,²⁵ but these also reduce the accuracy of the simulation. However, the availability

of computing power keeps expanding exponentially, which continuously enables molecular dynamics to tackle more complex systems while decreasing calculation times and increasing simulation accuracy.

Bitopic ligands and their implications

Next to crystal structures with a sodium ion bound, a few crystal structures with small molecule allosteric modulators bound have been elucidated to date, namely of the M2 muscarinic receptor and the metabotropic glutamate 1 and 5 receptors.²⁶⁻²⁸ Most of GPCR crystal structures have been co-crystallized with high affinity orthosteric ligands, as these reinforce protein stability substantially.²⁹ The generally low affinity of allosteric modulators for GPCRs is a challenge for acquiring high resolution crystal structures with these co-crystallized. Amiloride and HMA are no exception with their affinities in the μM range. A crystal structure of the adenosine $\text{A}_{2\text{A}}$ receptor with an amiloride derivative co-crystallized would add much information to the findings of Chapters 3 to 5 of this thesis, for instance the exact mechanism behind the observed probe dependency and the differences in competitive interaction with orthosteric ligands. The results of Chapter 5 imply the possibility to synthesize a bitopic ligand, which binds in both the allosteric sodium ion site and the orthosteric site of the adenosine $\text{A}_{2\text{A}}$ receptor. The increase in possible interactions of such a bitopic ligand upon binding to the receptor would likely result in a higher affinity than of an 'ordinary' amiloride derivative, making it a viable option for co-crystallization with the adenosine $\text{A}_{2\text{A}}$ receptor.

Another drawback of amiloride and HMA is their nonselectivity, as they do not only bind to many GPCRs but also to other proteins. This makes their application in cell-based assays complicated, as it would be hard to pin-point their effects to one target. However, cell-based assays are necessary to study the effects of amiloride binding on the receptor activation. The recent finding that HMA induces the same conformation of the adenosine $\text{A}_{2\text{A}}$ receptor as a partial agonist emphasizes the value of such a study.¹⁵ A bitopic ligand could offer a solution, as it may be more selective through interaction with less conserved residues in the orthosteric site. The same interactions with the orthosteric site will probably mean that they do not behave as 'classic' allosteric modulators, but it would still be interesting to study their effects on GPCR signaling in a more physiologically relevant system.

Mass spectrometry binding without internal standard

The application of MS binding without the need for a deuterium-labeled internal standard in Chapter 6 of this thesis has interesting implications for its further development. However, the lack of an internal standard would require a careful control of factors that distort mass spectrometry read-outs, i.e. evaporation rates during the elution step, and drift and ion suppression during the quantification step. Evaporation rates may be reduced by improvements in the elution protocol, for example by incorporating an evaporation step and subsequent resuspension of the residue in fixed amounts of elution buffer. Improved drift suppression might be reached by ongoing improvements in mass spectrometry equipment. Regarding ion suppression, we observed in our work that variable ion suppression was mainly caused by the presence of changing concentrations of 'cold' ligand in the elution buffer. For displacement and competition association assays this situation is a given, but in saturation, association, and dissociation assays the 'cold' ligand is inherently absent, thereby removing a substantial source of ion suppression. Without the need to synthesize a deuterium labeled internal standard, the development of LC-MS quantification protocols for marker ligands would be relatively fast, and this would allow to directly determine the K_D value and k_{on} and k_{off} rate constants of a series of ligands.

Other mass spectrometry prospects in membrane protein research

Mass spectrometry is also applied in other ways in membrane protein research. One example is endogenous or native mass spectrometry (nMS) which identifies different membrane protein-lipid or ligand complexes by their different sizes.³⁰ Differently to the MS binding assay, the protein-ligand complex is not denatured before the mass spectrometry detection step, so that the intact native membrane protein assembly can be probed. To keep the protein intact during the nMS analysis asks for a more complex approach than with MS binding, but has the possibility to yield valuable information about protein interactions, as endogenous protein ensembles with lipids, peptides, and drugs can be detected and identified by their differences in mass. This technique may therefore yield great opportunities to find new (endogenous) allosteric modulators of membrane proteins and GPCRs in particular.

Final note

This thesis provides a detailed insight in the value of the allosteric sodium ion site for GPCR functioning. Inspired by a high resolution crystal structure of the adenosine A_{2A} receptor with a sodium ion bound, we explored different aspects of the sodium ion site. This resulted in insights into the probe dependency of sodium ions and amilorides, evidence for the crucial role of the amino acids of the sodium ion site in receptor signaling, and opportunities to design novel bitopic amiloride derivatives that bind in both the sodium ion site and the orthosteric site. Furthermore, an unlabeled ligand binding assay was developed for the adenosine A₁ and A_{2A} receptors by means of mass spectrometry. This MS binding assay proves to be an excellent alternative for the conventional radioligand binding assay, without the need to synthesize any labeled ligand.

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