Non-ribose ligands for the human adenosine A1 receptor

Klaasse, E.C.

CHAPTER 8

General Discussion,
Conclusions and Perspectives

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General Conclusions

The research described in this thesis has provided novel data and promoted new insights in structure activity relationships of the adenosine A_1 receptor with respect to traditional agonists such as CPA and *R*-PIA as well as new, non-adenosine like agonists. Ligand binding, second messenger activation and internalization of the adenosine A_1 receptor were studied, and the effect of allosteric modulators such as PD81,723, sodium ions and GTP on these processes was investigated. Fusion proteins were used as tools to study inverse agonism, constitutive activity and internalization of the receptors.

Nine different fusion proteins of human adenosine A_1 receptors fused to ^{351}Cys mutated $G_{i\alpha}$-subunits were studied. From [^{35}S]GTPγS binding experiments, it appeared that all the A_1-$G_i\alpha$ fusion proteins tested had a higher basal receptor activity than the unfused adenosine A_1 receptor, thereby providing improved conditions to observe inverse agonism. Compared to unfused adenosine A_1 receptors, the affinity of CPA at wild-type A_1-$G_i\alpha$ fusion proteins (^{351}Cys) increased more than eightfold, while the affinity of DPCPX did not change significantly. Furthermore, we showed that the allosteric enhancer of agonist binding, PD81,723, elicited similar effects on ligand binding; i.e. CPA binding to the A_1-$G_i\alpha$ fusion proteins was enhanced, whereas the affinity of DPCPX was hardly affected. Moreover, sodium ions were unable to decrease agonist binding to the majority of the A_1-$G_i\alpha$ fusion proteins. Sodium ions act through a highly conserved aspartate residue in TM helix II, presumably inducing a change in receptor conformation towards the inactive R state. It may be that the physical connection between receptor and $G_i\alpha$-subunit causes a conformational change, which, in turn, prevents the sodium ions from binding to the adenosine A_1 receptor. Alternatively, sodium ions may promote the formation of an inactive state (R) of the receptor, which cannot or only partly be achieved in receptors already fused with a G protein.

Concerning internalization, PD81,723 decreased the threshold of the CPA concentration necessary to induce internalization of the adenosine A_1YFP receptor, rather than accelerating the slow internalization process. CPA by itself was able to internalize 25% and 40% of the receptors at a concentration of 400 nM or 4 µM, respectively. In the presence of 10 µM PD81,723, however, a low level of internalization was obtained already at 40 nM of CPA, and at 400 nM CPA 59% of the receptors internalized, an increase of 34%. The allosteric inhibitor SCH-202676 (10 µM) on the other hand effectively prevented CPA-induced internalization of the receptor. It should however be noted that recent studies revealed that SCH-202676 is not a true allosteric modulator. It acts as an agent which reversibly modifies sulfhydryl groups of cysteine residues in cell membrane preparations and thus in

receptors. Addition of either PD81,723 or SCH-202676 alone had no effect on internalization.

Recently, selective agonists for the human adenosine A_1 receptor with an unusual structure lacking the ribose moiety of traditional adenosine-like agonists were synthesized. The affinity of these 2-amino-4-(3 and/or 4-disubstituted phenyl)-6-(substituted)sulfanyl-pyridine-3,5-dicarbonitriles (Figure. 8.1) is determined by the substitution pattern of the phenyl ring whereas the efficacy is determined by the substituents on both the phenyl ring and the sulfanyl chain. Di-methoxyphenyl substituted compounds had very little affinity. The monosubstituted compounds recognized one binding state/site with higher nanomolar affinity. cAMP studies revealed an activity profile ranging from partial agonism to partial inverse agonism. 3,4-Methylenedioxyphenyl-substituted compounds recognized two binding states/sites with a picomolar affinity for the high affinity site. These compounds were full agonists that largely superseded the prototypic agonist CPA in affinity and potency. This is a remarkable new class of agonists for the A_1R, since the ribose group of the adenosine analogues was always thought to be crucial for activation of the receptor.

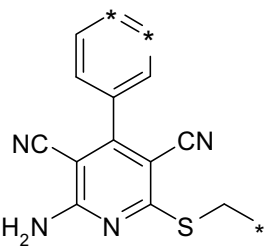

Figure 8.1. Core structure of 2-amino-4-(3 and/or 4-disubstituted phenyl)-6-(substituted)sulfanyl-pyridine-3,5-dicarbonitriles. The * represent the substitution positions.

One of these non-adenosine compounds, LUF6037, showed a very high affinity for the human adenosine A_1 receptor that extended far beyond that of the reference full agonist CPA (100-fold higher affinity). Remarkably, the allosteric modulator PD81,723 was not able to enhance the affinity of LUF6037 for the human adenosine A_1 receptor. Whereas the thermodynamic interaction of LUF6037 with the receptor was characteristic of a full agonist and cAMP production could indeed be inhibited as much as with CPA, LUF6037 was unable to induce internalization of the human adenosine A_1YFP receptor. This finding, together with the inefficacy of PD81,723, suggests that LUF6037 has an at least partially different interaction with the receptor binding pocket compared to the known adenosine-like agonists. This behaviour of LUF6037 towards the adenosine A_1R is a novel example of collateral efficacy, which may be therapeutically advantageous in drug resistance due to receptor internalization, as seen with traditional full agonists.

Finally, LUF5834, one of the non-adenosine agonists was tritiated and [^{3}H]LUF5834 was evaluated as a radioligand for the adenosine A_1 receptor. It recognized two high-affinity binding sites with a mere 10-fold difference in K_d values, occupying all the receptor binding sites present on the cell membrane. Displacement experiments revealed that LUF5834 is not sensitive to PD81,723 or GTP addition. Furthermore, an ‘extra’ third site was revealed in displacement experiments with the inverse

agonist DPCPX. This extra third site most probably reflects another representative of the conformational space of the R state of the receptor. Based on these results, an extended receptor equilibrium was proposed for the different receptor states.

Perspectives

Allosteric Modulators

In light of the above described findings, it would be very interesting to find out what mechanism underlies the enhancing effect of PD81,723 on the activity of the adenosine A_1 receptor. PD81,723 is known to act as an allosteric enhancer specifically enhancing agonist binding to the adenosine A_1 receptor[1]. Batthacharya et al, recently developed six chimaeras between the A_1R and $A_{2A}R$, dually coupled to the $G_i\alpha$ and $G_s\alpha$ subunit. Amongst others, the third intracellular loop (3ICL) of the A_1R and $A_{2A}R$ was exchanged, which is responsible for G protein coupling. It appeared that PD81,723 cannot enhance agonist binding to an $A_{2A}R$ equipped with the 3ICL of the A_1R. In contrast, PD81,723 was able to increase the potency of CPA for the chimaera between the A_1R and the 3ICL of the $A_{2A}R$. These results indicate that the recognition site of PD81,723 may likely reside within the transmembrane domain although an interaction with extracellular loops or intracellular loops other than ICL3 can not be fully excluded. PD81,723 acts in a way that directly stabilizes the receptor to a conformational state, capable of coupling with $G_i\alpha$ and $G_s\alpha$[2]. Data from Figler et al. also suggest that simultaneous binding of orthosteric ligands and allosteric enhancers convert the A_1AR from partly to fully coupled to G proteins and prevents rapid uncoupling upon binding of $GTP\gamma S$[3]. The fact that $G_i\alpha$ coupling is necessary to observe the effect of the allosteric enhancer PD81,723 on agonist binding is also supported by Kournounakis et al. They found that a mutation of Thr277 to Ala in the A_1R not only decreased agonist affinity but also inhibited the enhancing effect of PD81,723. Insensitivity of the A_1R mutant T277A to PD81,723 may be linked to the fact that this mutant appears to be uncoupled from G proteins[4]. In support of this theory that coupling to G_i is essential to see the enhancing effects of PD81,723, I report in this thesis that PD81,723 increased ligand binding to A_1-G_i fusion proteins to the same extent as to wildtype A_1R. Thus, the enhancing effect of PD81,723 on ligand binding to the A_1R is probably not due to facilitating interaction with the G protein, but rather by stabilizing a pro-active state of the receptor. In addition, I report in this thesis that PD81,723 exerts its action in cooperation with an adenosine-like agonist, but not with one of the new, nonadenosine-like agonists, lacking a ribose group. This is in line with the conclusion from this thesis that the new, nonadenosine-like agonists interact with the receptor binding pocket in a partially different way from the traditional agonists. Probably, the already very high affinity of the nonadenosine-like agonists, compared to that of the traditional adenosine derived analogues,

cannot be significantly further enhanced by the addition of PD81,723. Alternatively, the interaction of non-ribose compounds with the A_1 receptor may be insensitive to the addition of PD81,723.

Collateral efficacy

The efficacy of a drug is generally determined by the drug's ability to produce a biological response. In the classical receptor-occupancy theory, the efficacy is considered an intrinsic property of the ligand/receptor pair, and it is often assumed to be the same for all the responses evoked by this pair. However, very recently it has become clear from several studies in the GPCR field that receptor activation is not a linear sequential process whereby a single receptor activation state triggers all possible relevant downstream signaling pathways within the cell. Instead, a view of 'collateral efficacy', in which ligands can produce various combinations of downstream signaling effects is presented. In literature, the concept of collateral efficacy is also known as 'ligand-directed trafficking of receptor signalling' (LDTRS), 'functional selectivity', 'biased agonism', 'ligand-biased efficacy', or 'pluridimensional efficacy'[6-8]. The findings in this thesis concerning the new, nonadenosine-like agonists provide an additional example of collateral efficacy. In this case, both adenosine and nonadenosine-like agonists are able to bind to the receptor and to inhibit cAMP production, but a difference occurs in the ability to promote internalization.

Like the adenosine analogues, some of the nonadenosine-like agonists are full agonists, displaying even higher affinities than CPA for the A_1R and inhibiting the cAMP production to the same extent as CPA. However, where CPA was able to induce internalization of the A_1YFP receptor, the nonadenosine-like agonists were completely inactive in this respect. An important next step in this line of research would be to investigate where in the internalization cascade the effects of the nonadenosine-like agonists deviate from the adenosine analogues. For example, upon agonist binding, the extent and sites of phosphorylation of the receptors in desensitization as a first step to internalization may be examined with help of western-blotting, pull-down assays and mass spectroscopy. Furthermore, cotransfection of cells with for instance fluorescently labeled β-arrestin would teach whether β-arrestin will be recruited upon agonist stimulation.

Identification of new chemical entities

More than 10 selective agonists are currently in clinical trials to treat adenosine receptor subtype related pathologies, namely cardiac arrhythmias and neuropathic pain (A_1R agonists), myocardial perfusion imaging and anti-inflammatory agents (A_{2A}R agonists), cardiac ischemia (A_{2B}R agonists), rheumatoid arthritis and colorectal

cancer (A_3R agonists). Until now, only adenosine itself has been approved[9]. Therefore, another promising line of research would be to develop selective nonadenosine-like agonists for all adenosine subtypes, taking into consideration the concepts of collateral efficacy. The same approach as for the new adenosine A_1 ligands can be followed, namely using modeling techniques to retrieve electronic and spatial properties of the existing ligands for the adenosine receptor subtypes, and clustering them into a new model. This knowledge-based drug design approach has proven to be a good starting point for the development of new series of compounds. In this way, hopefully, a new generation of drugs with unique therapeutic profiles and a minimum of side effects can be developed to treat diseases in which adenosine receptors are involved.

Internalization

Especially for the A_3R, there is a need to develop new agonists. The A_3R is known to internalize in a matter of minutes[10], which is a distinct disadvantage in agonist drug treatment protocols with the currently available agonist drugs. However, it should be taken into account that the adenosine A_3R may contain a nuclear localization signal (NLS) which may be responsible for the fast 'internalization'. New agonists unable to exploit this property will be very welcome.

In order to facilitate internalization studies of the other adenosine receptor subtypes, preparation of fluorescently labeled adenosine A_{2A}-, A_{2B}- and A_3- receptors will be useful. Niebauer et al. already equipped the C-terminus of the adenosine A_{2A} receptor with a GFP tag, resulting in an A_{2A}GFP-receptor that should already be suitable for such internalization studies[11,12]. Alternatively, cell surface biotinylation can also be used to measure receptor expression on the plasma membrane[13].

Another way to study aspects of the internalization cascade would be to develop fluorescent agonists for the adenosine A_1R, $A_{2A}R$, $A_{2B}R$ and A_3R. To achieve this, one has to couple fluorescent probes to agonists while retaining a high affinity, high efficacy and good selectivity for the above mentioned receptor subtypes. It is recommended to develop a series of fluorescent agonists for each receptor subtype, in order to pick the one with the best properties. Another option is to vary the spacer length between the ligand and the fluorescent tag. However, it has to be taken into account that at a certain stage of internalization the ligand will dissociate from the receptor and that only the ligand can be traced. Briddon et al. already developed a fluorescent antagonist for the adenosine A_1R, XAC-BY630, used to quantify ligand-receptor binding at a single cell level[14]. With help of Fluorescence Correlation Spectroscopy (FCS), they were able to quantify the XAC-A_1R interactions at the cell membrane in an area as small as 0.1–0.2 μm^2, allowing single ligand-receptor complexes to be studied. Furthermore, since the detection volume of the FCS can be

directed to specific areas of the cell membrane, such as caveolae, insight into differences in ligand-receptor interactions within different membrane domains of the same cell can be determined.

Receptor activation states

A ground-breaking line of investigation would be to explore the conformational space of the adenosine A_1R population. Saturation- and binding studies with the new, nonadenosine-like radioligand [^{3}H]LUF5834, using the inverse agonist DPCPX as displacing ligand, revealed that the A_1R population can adopt at least three states. The inactive state seems to be represented by at least two conformations, R^1 and R^2. A third conformation represents a G protein-coupled receptor, R* state. Since there is only a 10-fold difference in the two K_d-values of [^{3}H]LUF5834, and the K_d-values are in the nM range, this agonist radioligand is able to label the whole adenosine A_1R population, which is an uncommon feature for an agonist radioligand. Therefore, follow-up studies exploiting the special properties of this new agonist radioligand may provide urgently needed deeper insight in the conformational space of a GPCR. For example, it would be very interesting to study if this feature can be observed using other inverse agonists or antagonists, or if this is exclusive for DPCPX. To further explore the composition of the A_1R population and the behaviour of [^{3}H]LUF5834, radioligand binding studies with a range of reference ligands representing full agonists, partial agonists, neutral antagonists in the presence or absence of different allosteric modulators (e.g. Na^+-ions, amiloride analogues) need to be performed.

Taking recent literature into account, it becomes clear that receptors do not have a single active state, but can adopt a population of different active states that induce a variety of responses called ‘collateral efficacy’, depending on the nature of agonist or allosteric modulator that is bound. The results obtained in this thesis extend these concepts and suggest that the allosteric enhancer PD81,723 ‘assists’ in promoting the active conformation of the adenosine A_1R, induced by the binding of adenosine derived agonists. In addition, the new, non-ribose agonists may promote an active conformation of the A_1R, that is subtly different in that it is not susceptible to the action of PD81,723 and does not trigger an internalization pathway. Remarkably, this active conformation of the A_1R stabilized by these non-ribose agonists seems to much more subtly modulate adenylyl cyclase activity, in order to explain the wide variety of inverse agonists, partial agonists and full agonists in a series of 12 compounds with relatively small differences in R-groups. This more ‘active’ conformation may also explain the high affinities observed in this series of compounds.

In the future, the development of new compounds for and the mechanism involved in ´receptor-research´ will never be linear again, but will have to consider the concepts of collateral efficacy.

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