Non-ribose ligands for the human adenosine A1 receptor

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Affinities, activities, allosteric modulation & internalization

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'Het leven is wat je gebeurt, terwijl je andere plannen maakt'
Acda & de Munnik

Maak je dus geen zorgen voor de dag van morgen, want de dag van morgen zorgt wel voor zichzelf. Elke dag heeft genoeg aan zijn eigen last.
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Contents

CHAPTER 1

General introduction

General Introduction

Within the mammalian body, communication is essential for the regulation of all physiological functions. Signaling from the extracellular environment to the cell's interior is conducted in most cases by cell surface receptors. The largest class of membrane bound receptors consists of the Guanylyl-nucleotide-binding Protein-Coupled Receptors, also known as the G protein-coupled receptors or GPCRs. These GPCRs can be activated by a wide variety of ligands, such as ions, peptides, hormones[1], neurotransmitters, chemokines or odorants[2]. Not surprisingly, GPCRs are currently the largest group of drug targets[3]. Membrane bound receptors consist of a single polypeptide, arranged in 7 transmembrane (7TM) helices that are oriented perpendicular to the membrane (Figure 1.1). Upon activation of the receptor by agonist binding, the G protein binds to the receptor and induces an intracellular signal via a second messenger, e.g. phospholipase A or C, or adenylyl cyclase. G proteins consist of an α, β and γ subunit. Currently, 16 α, 5 β and 14 γ isoforms are known, implicating a great variety in G proteins[4]. In the inactive state, the G_α subunit contains guanosine-5'-diphosphate (GDP) in its binding site. Upon activation of the receptor, GDP is exchanged for guanosine-5'-triphosphate (GTP), leading to activation or inhibition of a second messenger effector protein.

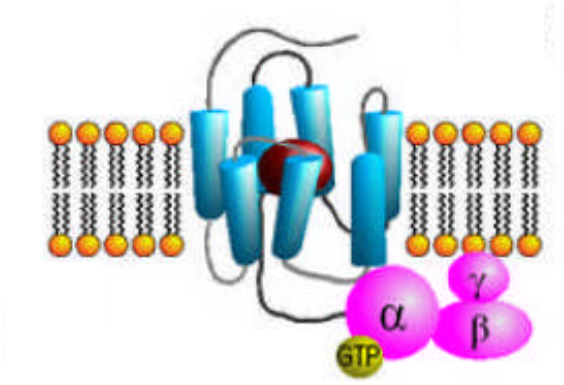

Figure 1.1. Structure GPCR

The GPCR superfamily can be divided into different sub-classes, based on similar structural features. Class A receptors or rhodopsin-like receptors form the largest sub-family, and share a series of conserved amino acid residues. Amongst others, adenosine receptors are members of this Class A receptors[5].

Adenosine receptors and their subtypes

Adenosine receptors are named after their endogenous ligand, adenosine, which is widespread in the body with a basal concentration in the nM-μM range (Figure 1.2)[6]. Besides being a neuromodulator, adenosine is also acting as a local hormone. It has a very short half-life (seconds) and is produced instantaneously when and where it is needed[7]. Extracellularly, adenosine is formed by the breakdown of the abundantly present ATP by 5'-ectonucleotidase.

In 1929, Drury and Szent-Györgi were the first to describe the actions of adenosine on the heartbeat and arterial pressure[8]. Almost 50 years later Burnstock proposed two types of receptors, P_1 and P_2, which could be distinguished by their preference

for either adenosine or adenine nucleotides, respectively[9]. The P_1 receptor was subsequently divided into A_1 (or R_i) and A_2 (or R_s) adenosine receptors according to their ability to either inhibit or stimulate the cAMP production, respectively[10,11]. In 1983, it was found that the A_2 receptor showed high and low affinity binding sites in the brain[12]. Accordingly, the adenosine A_2 receptor was subdivided into A_{2A} receptors with a high affinity binding site (striatum) and A_{2B} receptors with a low affinity binding site (throughout the brain)[13]. The adenosine A_1, A_{2A}, and A_{2B} receptors were discovered with help of ´classical´ ligand pharmacology. In contrast, the existence of the adenosine A_3 receptor subtype was discovered with help of molecular biology studies, and later confirmed with pharmacological experiments[14,15].

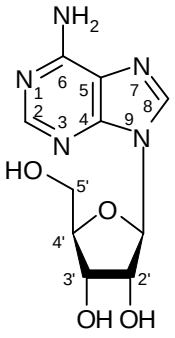

Figure 1.2. Chemical structure of adenosine

Cloning

As molecular biological techniques evolved in the 90's, all adenosine receptor subtypes were individually cloned and identified for a number of species. The adenosine A_1 receptor has been cloned from tissues from rat[16], cow[17], rabbit[18], mouse[19], guinea pig[20] and human[21,22]. Interestingly, a slight (5%) difference in sequence homology between bovine and human adenosine A_1 receptors accounts for considerable interspecies differences in ligand binding[23]. The adenosine A_{2A} receptor has been cloned from rat[24,25], mouse[19], guinea pig[26], and human tissues[27]. The rat adenosine A_{2B} receptor was cloned in 1992, and appeared to share only 50% sequence homology with the rat adenosine A_1 and A_{2A} receptor[28] which explained its different pharmacological profile like a low affinity for the reference A_{2A} receptor antagonist CGS21680. In addition, human[29], mouse[19] and chicken[30] adenosine A_{2B} receptors have been cloned as well. Whereas the existence of adenosine A_1, A_{2A} and A_{2B} receptors was based on pharmacological experiments and later confirmed by cloning of the respective genes, the opposite was true for the adenosine A_3 receptor. After cloning of this receptor from rat testis[14], its pharmacology was determined[15]. Subsequently, the A_3 receptor was also cloned from sheep[31], rabbit[32] and human[33]. The sequence homology among adenosine A_3 receptors from different species is rather low e.g. the rat adenosine A_3 receptor shows only 74% sequence homology with the human receptor, which is also reflected in its pharmacology. For instance xanthine-based antagonists have a high affinity for human and sheep A_3 receptors but a much lower affinity for rat A_3 receptors[34].

Occurrence of adenosine receptors

The different adenosine receptors are distributed widely in varying levels throughout the body, thus explaining the plethora of effects of adenosine [35,36]. The adenosine A_1 receptor is highly expressed in the central nervous system (CNS) and adipocytes. The adenosine A_{2A} receptor is found mostly in the striatum, spleen, thymus, leukocytes and blood platelets. The adenosine A_{2B} receptor is expressed in low levels in the brain, but can be found in high levels in the colon and bladder. The adenosine A_3 receptor is hardly expressed in the CNS, however, and in the periphery the highest levels have been found in the lung and liver.

Receptor Structure

The cloning of all four adenosine receptor subtypes and alignment of the amino acid sequences, revealed that the adenosine receptors indeed belong to the GPCR superfamily, sharing the characteristic 7 transmembrane (TM) domain structure[37,38]. The human adenosine A_1, A_{2A}, A_{2B}, and A_3 receptors consist of 326, 412, 332 and 318 amino acids, respectively[21,27,31,32,39]. The TM domains consist of α helices containing 21 to 28 amino acids and are connected by three extracellular and three intracellular hydrophobic loops. The A_{2A} receptor is the only subtype with an extraordinary long C-terminus of 122 amino acids, explaining its larger size compared to the other adenosine receptor subtypes[40]. The N-terminus of each protein is on the extracellular side, and the C-terminus is located on the cytoplasmic side of the membrane. The α helices are arranged in such a way that they form a core for ligand binding. Certain conserved amino acids contribute to ligand specificity within the binding pocket, e.g. Glu16 in TM1, Asp55 in TM2[41] and Thr277[42] and His278[17] in TM7 (numbering according to the human adenosine A_1 receptor).

Fusion proteins

Activation of G protein-coupled receptors results in an interaction of these receptors with their respective G proteins. Since it is known that upon activation the G_α subunit also interacts with intracellular domains including the C-terminus of receptors, constructs in which Gα subunits are physically linked to the C-termini of receptors have been prepared. One of the first fusion proteins, the β_2-adrenergic receptor fused to its $G_s\alpha$-subunit, was prepared by Bertin et al. in 1994. This β_2- $G_s\alpha$ fusion protein was shown to be functional in both ligand binding and in cAMP experiments[43]. Since then, many fusion proteins have been engineered between various receptors and their G proteins[44], e.g. the α_{2A}-adrenergic with $G_{i1}\alpha$ or $G_0\alpha$ subunits[45-47], the serotonin 5-HT_{1A} receptor with $G_{i1}\alpha$ or $G_0\alpha$ subunits[48-50], the δ opioid receptor with a $G_{i1}\alpha$ subunit[51] and the adenosine A_1 receptor with $G_{i1}\alpha$ or $G_0\alpha$ [52-54].

Milligan and coworkers constructed nine different fusion proteins between the human adenosine A_1 receptor and different ^{351}Cys-mutated G_{i1} α-subunits. They used these fusion proteins to investigate the ternary complex formation between agonist, receptor and G protein, and also demonstrated that there is no selectivity for any particular adenosine A_1 receptor – $G_{i1}\alpha/G_{0}\alpha$ combination, using different agonists[52,53].
Next to these G protein-receptor fusion proteins, fusion proteins between different receptors and fluorescent tags have been prepared to visualize the receptor. In 1997, Barak et al. were among the first to equip a GPCR with a fluorescent probe. They tagged the β_2-adrenergic receptor with a green fluorescent protein (β_2R-GFP), providing a tool to study intracellular trafficking and surface mobility of a functionally intact β_2-adrenergic receptor[55].
Concerning adenosine receptors, the human adenosine A_{2A} receptor was recently C-terminally tagged with the green fluorescent protein (GFP) by Niebauer et al. This provided an easy and useful tool to investigate the expression levels over time of the adenosine A_{2A}R expressed in yeast[56].

Desensitization and Internalization

Desensitization is generally defined as the phenomenon in which previous or continued exposure of receptor to agonist results in a diminished functional response of the receptor upon prolonged agonist treatment. Signal duration, intensity or quality is attenuated upon receptor desensitization. This does not necessarily mean that the receptor will disappear from the plasma membrane, it can remain their in the inactive state. Desensitization of a signal can occur at different levels of the signal transduction cascade, thereby terminating cellular responses. The process of desensitization follows a series of sequential steps. For most receptors, desensitization is initiated by the phosphorylation of serine and threonine residues in the third intracellular loop and C-terminus of the receptor. Subsequently, the phosphorylated receptors recruit the protein β-arrestin, which sterically inhibits G protein coupling and is also able to target the receptor to clathrin coated pits for internalization. During internalization, the receptor moves away from the plasma membrane and is targeted to endosomes or lysosomes, depending on which β-arrestin is attracted. Upon internalization, receptors can either be rapidly recycled to the plasma membrane, targeted to larger endosomes and slowly recycled, or degraded in lysosomes[57,58].
The adenosine receptor subtypes display different rates of desensitization. For example, adenosine A_1 receptors are not (readily) phosphorylated and internalize slowly which takes several hours. At the other extreme, adenosine A_3 receptors which are also G_i-coupled, require only a few minutes to internalize. The A_{2A} and A_{2B} receptors, which are both G_s-coupled, show a fast downregulation with kinetics

usually less than 1h for short-term desensitization. Apparently, receptor trafficking is regulated via different pathways, which are specific for receptor type, cell type, metabolic state of the cell, cell-specific factors etc[59].

Agonists for the adenosine A_1 receptor: traditional and non-ribose agonists.

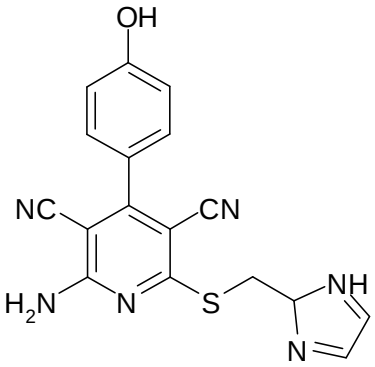

Figure 1.3. Structure of LUF5834, one of the non-adenosine compounds

As mentioned before, adenosine is the endogenous ligand for all adenosine receptor subtypes. Adenosine is composed of a purine group linked to a ribose moiety, see Figure 1.2. Examples of potent and selective adenosine A_1 agonists are: N^6-cyclopentyladenosine (CPA), 2-chloro-N^6-cyclopentyladenosine (CCPA), N^6-cyclohexyladenosine (CHA), (*R*)-N^6-(2-Phenylisopropyl)adenosine (*R*-PIA) and N^6-cyclopentyl-2-(3-phenylaminocarbonyltriazene-1-yl)adenosine (TCPA). All these prototypic A_1 receptor agonists are based on the endogenous ligand adenosine, and the ribose group of adenosine has long been considered essential for agonistic activity on adenosine receptors. However, Rosentreter *et al.,* (2003, 2004) recently described a new class of adenosine receptor ligands, the 2-amino-4-(3,4 substituted phenyl)-6-(2-hydroxyethylsulfanyl)-pyridin-3,5-dicarbonitriles, displaying significant affinity and efficacy towards different adenosine receptor subtypes[60-62]. One of those new, non-adenosine compounds, LUF5834 (Figure 1.3), was characterized as a full agonist for the human adenosine A_1 receptor with a very high affinity comparable to the $K_{i,\ high}$-value of CPA (2.2 nM), and as a partial agonist for the A_{2B} receptor with high affinity[63]. Another non-adenosine compound, LUF5831, appeared to be a partial agonist with an affinity of 18 $\pm$ 1 nM for the adenosine A_1 receptor[64]. From this study it also appeared that allosteric modulators such as PD81,723 and GTP seem to have no or much less effect on the binding of LUF5831 than they have on the binding of the traditional adenosine analogue CPA[64]. We have further investigated the properties of promising non-ribose agonists which have revealed some remarkable novel features.

Allosteric Modulation

The ligand binding site of the endogenous receptor ligands is also referred to as the orthosteric binding site. Next to such an orthosteric modulation more recently allosteric modulation of receptors was discovered. The term ‘allosteric modulation’ with respect to receptor binding, was first mentioned in literature in the early eighties[65]. The word ‘allosteric’ is derived from Greek and composed of two words: ‘allo’ and ‘stere’ which literally mean ‘other’ and ‘site’ or ‘shape’. Allosteric modulation

is thus achieved by adding compounds which act on a site of the receptor distinct from the orthosteric binding site, thereby inducing a conformational change of the receptor (Figure 1.4). Allosteric modulators for the adenosine receptors have been described in literature. Amiloride analogues and sodium ions were demonstrated to be common allosteric modulators for at least three subtypes of the adenosine receptors, A_1, A_{2A}, and A_3[66]. Next to these non-selective allosteric modulators also subtype selective modulators have been identified. The most well-known selective allosteric modulator is probably PD81,723, a benzoylthiophene derivative. It was demonstrated that several benzoylthiophene derivatives were selective enhancers of agonist binding to the adenosine A_1 receptors, with little or no effect on other adenosine receptor subtypes. Allosteric enhancers at the adenosine A_1 receptor have received attention as anti-arrhythmic and anti-lipolytic agents. In addition, they may also have therapeutic potential as analgesics and neuroprotective agents[67]. Allosteric modulation of A_{2A}R has so far only been described for the already mentioned sodium ions and amiloride analogues. In 2004, Van den Nieuwendijk et al, described a class of small organic molecules, 2,3,5-substituted [1,2,4]thiadiazoles, that seemed to allosterically modulate the A_{2A}R[68]. However, investigation of the mechanism of action of these compounds revealed that they acted as sulfhydryl modifiers rather than as allosteric modulators of the receptor[69]. Recently, two classes of A_3 AR allosteric modulators were characterized: 3-(2-pyridinyl)isoquinolines (e.g. VUF5455) and 1H-imidazo-[4,5-c]quinolin-4-amines (e.g. DU124183), which selectively decrease the agonist dissociation rate at the human A_3AR but not at A_1- and A_{2A}ARs. Allosteric enhancers for the adenosine A_3 receptors may be useful against ischemic conditions[70]. At this moment, allosteric modulators for adenosine receptors receive increasing interest and as described above may have therapeutic advantages over orthosteric ligands[71].

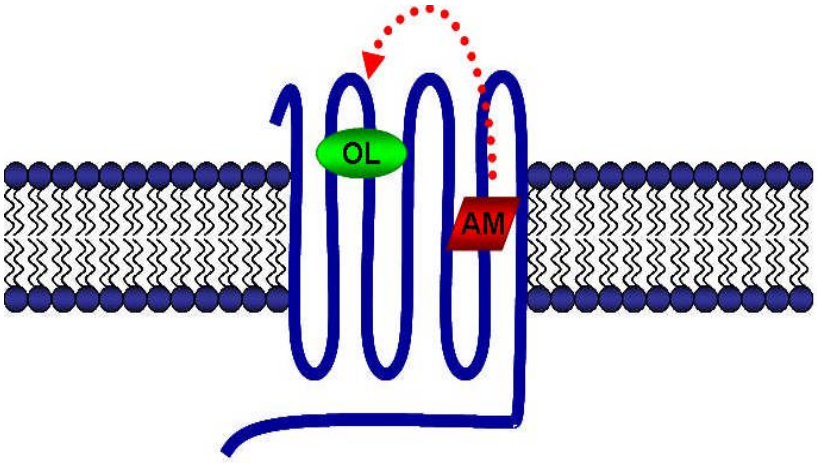

Figure 1.4. Schematic representation of the mechanism of allosteric modulation. OL = orthostheric ligand, AM = allosteric modulator.

Therapeutic potential of adenosine A_1 receptor regulation

As mentioned, the adenosine A_1 receptor is abundantly expressed in the brain with the highest levels in the hippocampus and the cerebral cortex. In addition, the

adenosine A_1 receptor is widely distributed and expressed at varying levels throughout the body, e.g. vas deferens, testis, white adipose tissue, stomach, spleen, adrenal gland, heart, aorta, liver, eye and bladder. Lower levels were found in the lung, kidney and small intestine[72-74]. In view of this broad distribution pattern, many therapeutic applications of adenosine A_1 receptor compounds have been proposed. For example, adenosine A_1 agonists or the high release of adenosine during cerebral ischaemia have been shown to reduce neuronal damage in cerebral ischaemia by inhibiting glutamate release[75]. These findings provide hope for the treatment of neurological disorders observed in Huntington's disease and Alzheimer[76]. Next to the neuroprotective effects of adenosine A_1 receptor activation in the central nervous system, also sedation, decreased locomotor activity and anticonvulsant effects have been observed. Compounds that are able to block the receptor from receiving its endogenous ligand in the CNS (antagonists) may be beneficial to counteract the sedation and negative locomotor effects of adenosine. Antagonists have also been found to enhance cognition, leading to an improvement in memory performance[77-79]. They may therefore be potentially useful in the treatment of neurological disorders such as Alzheimer's disease. The depth and levels of sleep are also dependent on the amount of adenosine present in the brain. Furthermore, adenosine plays an important role via adenosine A_1 receptors in analgesia in the periphery and at spinal sites. The expression of the adenosine A_1 receptor on atrial tissue suggests a role for this receptor in cardiovascular diseases. Activation of adenosine A_1 receptors was demonstrated to play a protective role upon myocardial ischaemia and subsequent reperfusion[80]. Patients with congestive heart failure often suffer from fluid retention caused by elevated adenosine levels in the kidneys. Administration of A_1 antagonists prevents renal failure and increases the urine flow in these patients[81].

The Scope and Content

In the previous paragraphs, an introduction into the history, occurrence, functioning, trafficking and therapeutic potential of adenosine receptors and in particular the adenosine A_1 receptors was given. In earlier work we have demonstrated our ability to design selective adenosine A_1 receptor ligands with high affinity. Moreover, we extended this ligand repertoire to include allosteric modulators and non-ribose agonistic ligands. In this thesis we have expanded our knowledge space by investigating the interaction of these compounds with the adenosine receptors in more detail. To study in addition the role of these compounds in novel concepts such as constitutive activity and trafficking we have applied recombinant DNA techniques, among others to construct fusion proteins that can serve as tracers. In chapter 2, the state of affairs is established with a review of the current literature concerning the desensitization and internalization of adenosine receptors. Results from *in vitro, ex*

vivo and *in vivo* studies are summarized, followed by the molecular mechanisms involved in adenosine receptor desensitization and internalization. The role of accessory proteins and the influence of receptor mutations are also taken into account.

In chapters 3 and 4, the use of fusion proteins between the human adenosine A_1 receptors and $G_{i\alpha}$-subunits or a yellow fluorescent protein as tools to investigate inverse agonism or receptor trafficking is described, respectively. Furthermore, we analysed if these constructs were still subject to allosteric modulation[54,82]. In Chapter 5, 6 and 7, the structure-activity relationships of differently substituted 2-amino-4-(substituted)phenyl-6-(substituted)sulfanyl-pyridine-3,5-dicarbonitriles are reported. These series of compounds display a wide variety of intrinsic efficacies, ranging from inverse agonists, to partial agonists and very potent full agonists. I present the first evidence that the properties of these non-adenosine agonists are very different from the traditional agonists for the adenosine A_1 receptor concerning allosteric modulation and internalization.

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CHAPTER 2

Desensitization and Internalization of Adenosine Receptors

Until now, more than 800 distinct GPCRs are identified in the human genome. The four subtypes of the adenosine receptor (A_1-, A_{2A}-, A_{2B}- and A_3 receptor) belong to this large family of GPCRs that represent the most widely targeted pharmacological protein class. Since adenosine receptors are widespread throughout the body and involved in a variety of physiological processes and diseases, there is great interest in understanding how the different subtypes are regulated, as a basis for designing therapeutic drugs that either avoid or make use of this regulation. The major GPCR regulatory pathway involves phosphorylation of activated receptors by G protein-coupled receptor kinases (GRKs), a process that is followed by binding of arrestin proteins. This prevents receptors from activating downstream heterotrimeric G protein pathways, but at the same time allows activation of arrestin-dependent signaling pathways.

Upon agonist treatment, adenosine receptor subtypes are differently regulated. For instance, the A_1Rs are not (readily) phosphorylated and internalize slowly, showing a typical half-life of several hours, whereas the A_{2A}R and A_{2B}R undergo much faster downregulation, usually shorter than 1 hour. The A_3R is subject to even faster downregulation, often a matter of minutes. The fast desensitization of the A_3R after agonist exposure may be therapeutically equivalent to antagonist occupancy of the receptor. This review describes the process of desensitization and internalization of the different adenosine subtypes in cell systems, tissues and *in vivo* studies. In addition, molecular mechanisms involved in adenosine receptor desensitization are discussed.

Based upon Klaasse EC, IJzerman AP, de Grip WJ, Beukers MW., Purinergic Signal., ***2008****, 4:21-37.*

Introduction

Adenosine is an important neuromodulator involved in a variety of brain activities and it also serves many different functions in the periphery. This nucleoside, when extracellular, exerts its action via specific G protein-coupled receptors (GPCRs) of the P1 class, divided in four subtypes: A_1R, $A_{2A}R$, $A_{2B}R$ and A_3R[1]. GPCRs consist of a single polypeptide, containing 7 α-helices which are oriented perpendicular to the membrane. The N-terminus is located at the extracellular side of the cell and often contains one or more glycosylation sites. The C-terminus is located intracellularly and contains phosphorylation and palmitoylation sites, which are involved in regulation of receptor desensitization and internalization[2]. All adenosine receptors, with the exception of the $A_{2A}R$, contain a palmitoylation site near the C-terminus. The $A_{2A}R$ is the only subtype with an extraordinary long C-terminus, 122 amino acids versus 36 amino acids in e.g. the A_1R[3]. All the adenosine receptors are glycosylated on the second extracellular loop, although glycosylation does not appear to influence ligand binding. The third intracellular loop and/or the C-terminus are involved in coupling the adenosine receptors to G-proteins. Phosphorylation of in particular intracellular loop 3 is involved in desensitization and internalization of adenosine receptors[4,5,6].

Adenosine and analogues

Adenosine, consisting of a purine ring connected to a ribose group, is the endogenous ligand for the adenosine receptors (Figure 2.1). Under normal conditions, adenosine is continuously formed extracellularly by dephosphorylation of ATP, ADP and/or AMP to adenosine by NTPDases (ecto-nucleoside triphosphate diphosphohydrolases). However, the A_3R can also be activated by inosine, a breakdown product from adenosine[5]. Most adenosine receptor agonists are analogues of adenosine, modified by N^6, C2 and C8 substitutions at the adenine base, and C5' modifications of the ribose moiety[5,6]. Antagonists lack the ribose group and usually possess a mono-, bi- or tricyclic core-structure, e.g. caffeïne, which contains a xanthine as basic structure (Figure 2.1). For extended reviews on high affinity agonists and antagonists for adenosine receptors and their structure-activity relationships, see Palmer and Stiles[1], Fredholm et al.[5], Jacobson and Gao[7], Beukers et al.[8], Müller[9] and Klotz[10].

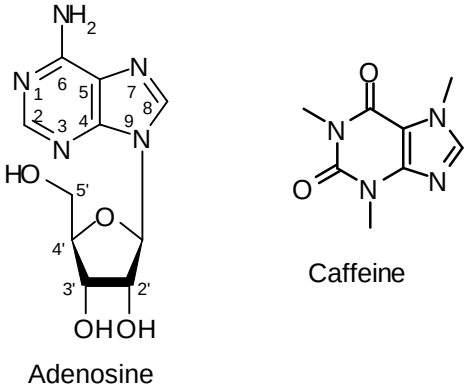

Figure 2.1. Chemical structures of the endogenous ligand adenosine and the antagonist caffeine.

Occurrence and Physiological functions of adenosine receptors

The adenosine receptors are widespread throughout the body, and exert many different functions both in the CNS and in the periphery.

The A_1R is particularly prevalent in the central nervous system, with high levels in the cerebral cortex, hippocampus, cerebellum, thalamus, brain stem and spinal cord. Numerous peripheral tissues also express the A_1R, including vas deferens, testis, white adipose tissue, stomach, spleen, pituitary, adrenal gland, heart, aorta, liver, eye and bladder. Low levels are found in the lung, kidney and small intestine[1,5,6]. The A_1R is involved in cardiovascular effects (e.g. reducing heart rate), inhibition of lipolysis and stimulation of glucose uptake in white adipocytes and the modulation of neurotransmitter release in the CNS[1]. The A_1R also plays a role in anxiety, hyperalgesia, bronchoconstriction and the glomerular filtration rate and renin release in the kidney[5,6,11].

In the CNS, the $A_{2A}R$ is highly expressed in the striatum and olfactory tubercle[1]. In the periphery, it is highly expressed in the spleen, thymus, leukocytes and blood platelets, and intermediate levels are found in the heart, lung and blood vessels[5,6]. The $A_{2A}R$ is involved in the onset of vasodilation, inhibition of platelet aggregation, exploratory activity, aggressiveness and hypoalgesia[1]. In addition, $A_{2A}R$ play a role in Parkinson's disease, Huntington's disease, Alzheimer's disease, ischemia, attenuation of inflammation, and neuroprotection, particularly in peripheral tissues[5,6]. A_{2A} receptor antagonists slow the neurodegeneration which occurs in Parkinson's- and Huntington's disease, and also prevent toxicity induced by beta-amyloid in the development of Alzheimer's disease[12-14].

The $A_{2B}R$ is widely expressed in the brain, but generally at very low levels. In the periphery, high levels of $A_{2B}R$ were detected in the cecum, large intestine and urinary bladder. Lower levels were observed in the lung, spinal cord, vas deferens, pituitary, adipose tissue, adrenal gland, kidney, liver and ovaries[5,6]. Since there is a lack of specific agonists for the $A_{2B}R$, little is known about the functional significance of this receptor. However, the $A_{2B}R$ plays a role in mediating vasodilation in a.o. the aorta, the renal artery and the coronary artery of different species. It is also involved in allergic and inflammatory disorders[5,6].

The A_3R is expressed in the CNS, but at relatively low levels, and only the hypothalamus and the thalamus have been reported to contain A_3R[15]. The highest levels of adenosine A_3R have been found in lung and liver, and somewhat lower levels were found in the aorta[1]. In addition, the A_3R was found in eosinophils, mast cells, testis, kidney, placenta, heart, spleen, uterus, bladder, jejunum, aorta, proximal colon and eye, although with pronounced differences in expression level between species[5,6,16]. The A_3R has been implicated in mediating allergic responses, airway inflammation and apoptotic events, however, the latter is dependent on the cell type

involved and/or the type of activation[5,16]. Furthermore, the A_3R is involved in the control of the cell cycle and inhibition of tumour growth both *in vitro* and *in vivo*[6]. In fact, adenosine A_3 receptors have been demonstrated to be more highly expressed in tumours than in healthy cells, suggesting a role for A_3R as a tumour marker[17].

Signal transduction of adenosine receptors

G protein-coupling and second messengers

Heterotrimeric G proteins are guanine-nucleotide regulatory protein complexes composed of α and $\beta\gamma$ subunits. They are responsible for transmitting signals from G protein-coupled receptors to effectors, e.g. adenylyl cyclase. Until now, 16 α, 5 β and 14 γ isoforms have been reported[18]. G proteins are divided in several subclasses with a specific activity profile: G_s proteins stimulate adenylyl cyclase, G_i proteins inhibit adenylyl cyclase and stimulate GIRK channels, G_0 proteins stimulate K^+ ion channels, $G_{q/11}$ proteins activate phospholipase C, G_{12} proteins activate Rho guanine-nucleotide exchange factors (GEFs) and the olfactory G protein, G_{olf}, stimulates adenylyl cyclase. Upon receptor activation, both the α subunit and the $\beta\gamma$ subunit can signal, but to different effectors[19-21]. For a recent review on G proteins, see Milligan and Kostenis[18].

The A_1R is usually coupled to a pertussis toxin-sensitive $G_{i\alpha}$ protein, which mediates inhibition of adenylyl cyclase and regulates calcium and potassium channels[1,3,5,6,11]. Both the third intracellular loop and the C-terminal tail of the A_1R are involved in $G_{i\alpha}$ coupling[5]. In addition, it has been reported that under certain conditions the A_1R couples to G_s to stimulate adenylyl cyclase, or to $G_{q/11}$ to stimulate inositol phosphate production. Apparently, the specific activity state of the receptor or the nature of the agonist determine which G protein class is activated by the A_1R[22,23]. The $A_{2A}R$ in the periphery is coupled to cholera toxin-sensitive $G_{s\alpha}$ proteins, which increase adenylyl cyclase activity upon receptor activation. The $A_{2A}R$ in the striatum is presumably coupled to G_{olf}[5,6]. The third intracellular loop, but not the C-terminus of the $A_{2A}R$, is involved in $G_{s\alpha}$ coupling[5]. The $A_{2B}R$ is coupled to $G_{s\alpha}$ proteins leading to stimulation of adenylyl cyclase upon receptor activation[6]. There is quite some evidence that $A_{2B}R$ can activate phospholipase C as well, via $G_{q/11}$ proteins[5]. The A_3R is coupled to pertussis toxin-sensitive $G_{i\alpha}$ proteins, which mediate inhibition of adenylyl cyclase. In addition, A_3R can stimulate phospholipase C via $G_{q/11}$ proteins[5,6]. For an extensive overview of adenosine receptor G protein-coupling, see Fredholm et al.[24].

Desensitization and internalization – general principles and players

Mechanisms to dampen GPCR signaling exist at every level in the cell. In this paragraph attention will be paid to the underlying principles of desensitization and internalization and the protein partners involved in these processes.

Receptor localisation

Depending on the localisation signal, GPCRs in the plasma membrane can be targeted to lipid rafts[1] or caveolae[2]. Different regions of GPCRs can influence not only the targeting to either lipid rafts or caveolae but may also enable an interaction of the receptor with constituents of these rafts and caveoli. For instance the extracellular part of the receptor might interact with GM1 gangliosides (glycosphingolipids) present in lipid rafts/caveoli. In addition, the C-terminal fatty acid acylation or palmitoylation may also affect targeting of GPCRs to either lipid rafts or caveoli. Finally, transmembrane regions may interact with cholesterol in the lipid rafts resulting in a change in conformation of the α-helices. Since the conformation of α-helices depends on the activation state of GPCRs, it may well be that agonist binding to the receptor may affect its localisation in lipid rafts by means of molecular transitions leading to receptor activation[25,26].

Desensitization

Desensitization reduces receptor activity and plays a role in signal duration, intensity and quality. Desensitization is initiated by phosphorylation of serine and/or threonine residues in the third intracellular loop and C-terminus of the receptor. Two types of desensitization occur, heterologous and homologous desensitization, and both are the result of receptor phosphorylation. Heterologous desensitization is induced by phosphorylation of the receptor by protein kinase A or C – sometimes even without agonist occupancy. On the other hand, homologous desensitization is specific for agonist-occupied receptors, and consists in most cases of two steps. Firstly, the receptor is phosphorylated by one of the G protein-coupled receptor kinases (GRKs 1-7); then it binds to β-arrestin, of which two subtypes exist, that exhibit high affinity for agonist-occupied, phosphorylated receptors. β-Arrestin serves to sterically inhibit

[1] **Lipid rafts** are planar domains of 25-100 nm in cell membranes enriched in specific lipids and proteins. They are in particular characterised by a high cholesterol and glycosphingolipid content in the outer leaflet of the lipid bilayer that gives them a gel-like liquid-ordered organisation in comparison with the surrounding phospholipid-rich disordered membrane[26].

[2] **Caveolae** are flask-shaped invaginations located at or near the plasma membrane with a 50-100 nm diameter. They are considered to be a non-planar subfamily of lipid rafts. The shape and structural organisation of caveolae are due to the presence of caveolin-1, -2 and -3, that self-assemble in high-mass oligomers to form a cytoplasmic coat on the membrane invaginations[25,26].

G protein coupling, thereby terminating the G protein activation, and may also target the receptor to clathrin-coated pits[3] for internalization (Figure 2.2)[19,25,27,28].

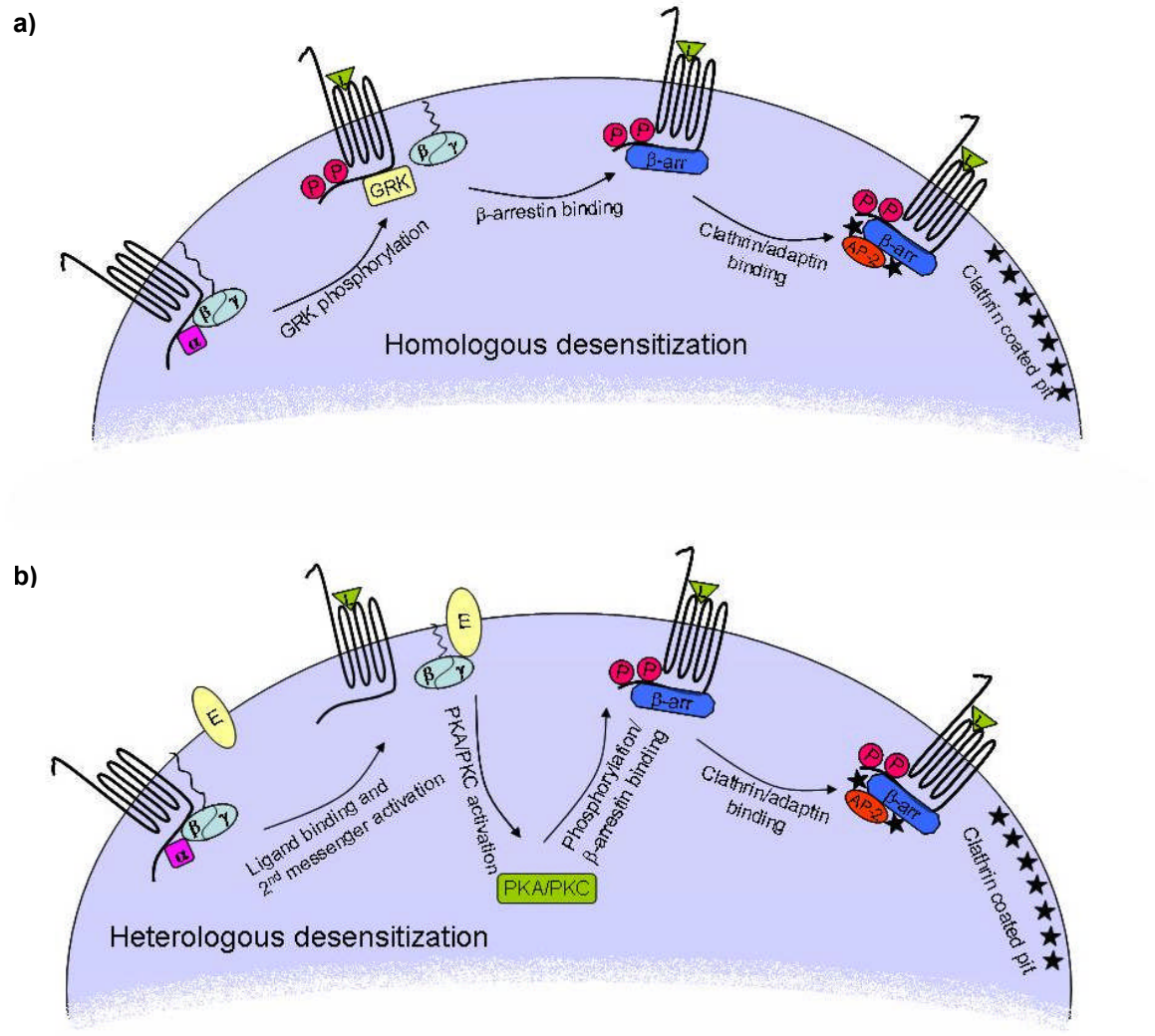

Figure 2.2. a) Homologous and b) heterologous desensitization and subsequent internalization of GPCRs. $\alpha = G_{i\alpha}$ subunit, $\beta\gamma = G_{i\beta\gamma}$ subunit, L = ligand, GRK = G protein-coupled receptor kinase, P = phosphorylated amino acids, β-arr = β-arrestin, AP-2 adaptin, E = effector, second messenger, PKA = protein kinase A, PKC = protein kinase C.

Internalization

Receptor desensitization, initiated by phosphorylation of the receptor by different protein kinases (A or C) or GRKs, can be subsequently followed by receptor internalization. Upon phosphorylation, β-arrestin 1 or 2 is attracted to the receptor[31]. β-Arrestins not only interact with the phosphorylated receptor, but also bind to the heavy chain of clathrin, to the β2-adaptin subunit of the clathrin adaptor protein AP-2,

[3] **Clathrin-coated pit** is a specialized region of the cell surface that mediates the internalization of extracellular macromolecules and GPCRs. Coated pits derive their name from the presence of a distinctive polygonal lattice that decorates the inner surface of the membrane. This lattice is composed of multiple triskelion-shaped subunits that contain three clathrin heavy chains (180 kD) and three clathrin light chains (30-40 kD) plus a family of associated poteins with molecular sizes of 50 kD and 110 kD [29,30].

and to phosphoinositides. These interactions direct the phosphorylated receptor to punctate clathrin-coated pits in the cell membrane, which are internalized by action of the GTPase dynamin. Upon internalization, receptors can either be rapidly recycled to the plasma membrane, targeted to larger endosomes and slowly recycled, or degraded in lysosomes. The final destination of the internalized receptors largely depends on the β-arrestin subtype (1 or 2) that is recruited by the receptor upon phosphorylation and the duration of β-arrestin binding. In this way, internalization may regulate receptor resensitization and contributes to a positive regulation of receptor signalling[19,25,31].

Internalization pathways

From internalization studies with several receptors, it appears that the internalization pathway is specific for receptor type, cell type, metabolic state of the cell, cell-specific factors etc. Receptor trafficking can be regulated in different ways (Figure 2.3): a) the receptor resides mainly in lipid rafts/caveolae and enters the cell via this pathway by default; b) the receptor is in lipid rafts, but leaves these upon agonist binding to be internalized via clathrin-coated pits; c) the receptor moves into lipid rafts upon agonist binding and is internalized via this pathway; d) the receptor moves into lipid rafts after agonist binding to activate certain signalling events, but is eventually moved out of the lipid rafts to be internalized via clathrin-coated pits. Internalization can even be achieved via uncoated vesicles or by a combination of two or more of the afore mentioned pathways. For example, β1-AR is internalized via both lipid rafts and clathrin-coated pits. PKA phosphorylation directs β1-AR to a clathrin-coated pit, whereas GRK phosphorylation directs the receptor to lipid raft-mediated internalization[19,25,26].

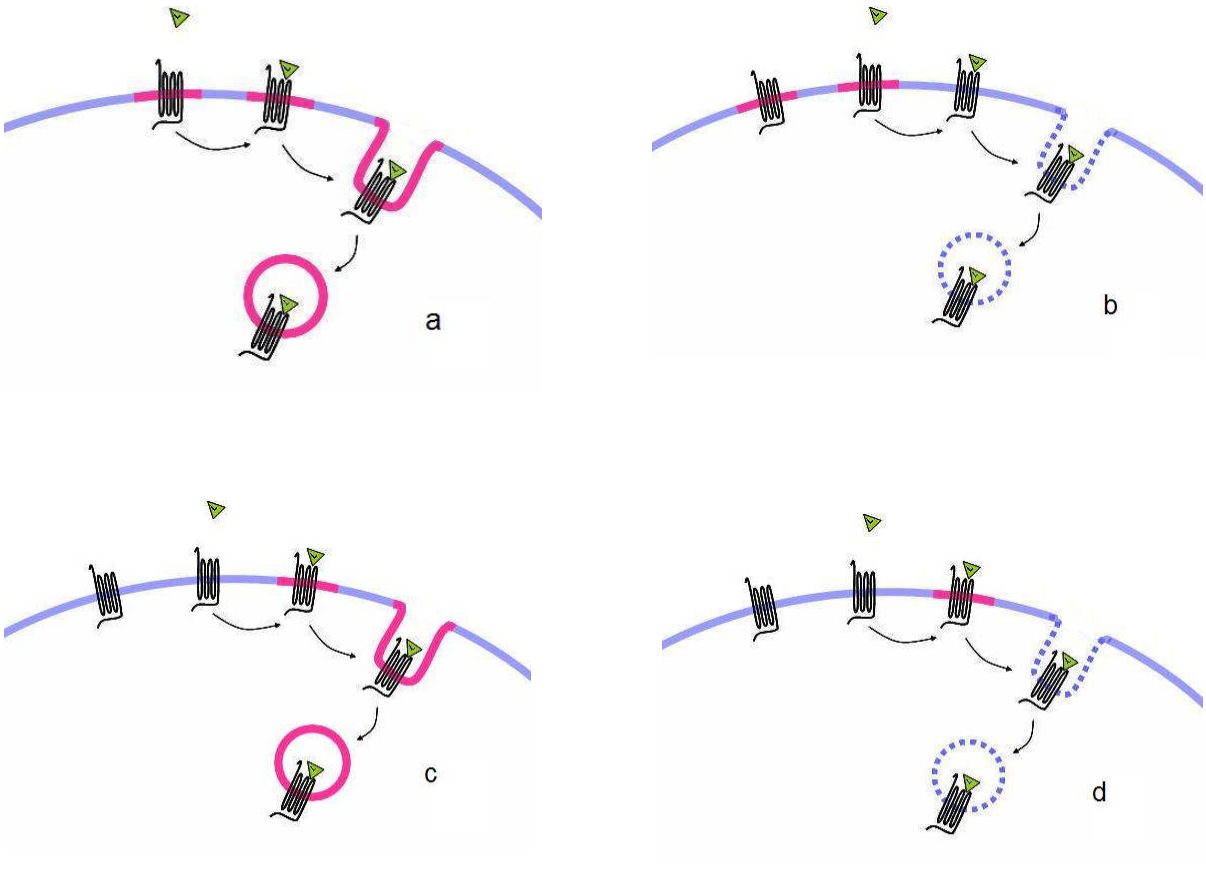

Figure 2.3. Different internalization pathways, adapted from Chini and Parenti, 2004[25]. Internalization via a) lipid rafts/caveolae b) upon agonist binding, the receptor moves to clathrin-coated pits to be internalized c) the receptor moves into lipid rafts upon agonist binding and is internalized d) the receptor moves into lipid rafts upon agonist binding to activate certain signalling events, but is eventually moved out to be internalized via clathrin-coated pits. Ligand (L, green triangle), clathrin-coated pits (dotted blue lines) and lipid rafts (solid pink lines) are indicated.

Function of lipid rafts/caveolae

The existence of lipid rafts/caveolae serves different functions. First of all, lipid rafts act as 'stations' in which GPCRs accomplish specific signalling tasks by meeting a selected set of signalling molecules, e.g. G proteins and adenylyl cyclases. Another possible function of lipid rafts is the protection of receptors from rapid constitutive or agonist-induced internalization, thus allowing their coupling to specific signalling pathways. In addition, caveolin may regulate the constitutive activity of receptors[32]. Finally, the endocytic pathways that GPCRs choose may depend on cell-specific factors. Switching the internalization pathway from lipid rafts/caveolae to clathrin-coated pits may alter the final receptor destination[19,25,26]. Research on adenosine receptors probably provided the first account of receptor internalization via caveolae and lipid rafts, as alternative to the well described β-arrestin pathway[33].

G protein-coupled receptor kinases (GRKs)

Upon agonist binding to GPCRs, not only G proteins are recruited to the activated receptor conformation, but also two other protein families, namely the G protein-coupled receptor kinases (GRKs) and the β-arrestins[31]. The GRK family consists of seven different genes. The GRKs have been divided into three protein subfamilies, based on sequence similarity. GRK1 and -7 belong together. The second subfamily consists of GRK2 and -3 since their membrane recruitment depends on interaction with Gβγ subunits and phosphatidylinositol 4,5-bisphosphate. GRK2 and -3 are primarily responsible for agonist-dependent receptor phosphorylation, β-arrestin recruitment and functional uncoupling of the receptor. GRK4, -5 and -6 form the third subfamily and are constitutively associated with the membrane. GRK1 and -7 are expressed mainly in the retinal rods and cones, whereas GRK4 expression is limited to the cerebellum, testis and kidney. In contrast, GRK2, -3, -5 and -6 are widely expressed in mammalian tissues[31].

β-arrestins

β-arrestins belong to the arrestin family of which four members have been identified. Arrestin-1 and arrestin-4 are expressed in the visual system in retinal photoreceptor cells; arrestin-2 (also called β-arrestin1) and arrestin-3 (also called β-arrestin2) are more widely expressed and are involved in the regulation of nonvisual GPCRs. The β-arrestins were originally discovered as molecules that bind to and desensitize the activated and phosphorylated form of G protein-coupled receptors as described before[19,25,27,28,31]. The β-arrestin protein consists of an N and a C domain, which are almost entirely composed of antiparallel β sheets, connected by a linker of 12 amino acids. A ‘polar core’ is embedded between those two domains, and its disruption by the phosphorylated C-terminus of the activated receptor leads to conformational changes in the β-arrestin. The C tail of the β-arrestin is then released, exposing both the clathrin and the AP2-binding domains. Recently, it appeared that β-arrestins not only are involved in the desensitization of G protein-coupled receptors, but also act as signal transducer on their own. As a consequence, they emerge as multifunctional adaptor/scaffold proteins which mediate cellular processes such as chemotaxis, apoptosis and metastasis besides receptor signalling and trafficking. For an extensive and recent review on the interactions of β-arrestins with other cellular proteins, see Lefkowitz et al.[34].

GRKs and β-arrestins orchestrate GPCR activities at three different levels: 1) silencing: the functional uncoupling of the receptor from its G protein by a mechanism known as ‘homologous desensitization’, 2) trafficking, which involves receptor internalization, resensitization and/or degradation, 3) cross-signalling: activation or

inhibition of intracellular signalling pathways, independent of heterotrimeric G proteins[31].

Dampening adenosine receptor signals

In this section we will discuss the evidence for and mechanisms of desensitization and internalization of the four adenosine receptor subtypes. For each receptor we will first summarize results from *in vitro*, *ex vivo* and *in vivo* studies. This will be followed by a more biochemical approach in which we will focus on the molecular mechanisms of adenosine receptor desensitization and internalization by paying attention to the role of accessory proteins, the influence of receptor mutations, etc. We will refer to earlier seminal publications, but mostly focus on more recent evidence for adenosine receptor desensitization and internalization.

A_1 receptor

Cellular and physiological studies

The early evidence for adenosine A_1 receptor desensitization was largely obtained from primary cells, cell lines, tissues or tissue slices, and intact animals that were exposed to varying concentrations of adenosine receptor agonists (either A_1-selective or not), often examined over several time periods.

Stiles and co-workers were among the first to study A_1 receptor desensitization in detail (1991). Ramkumar et al. pretreated DDT1 MF-2 cells, a smooth muscle cell line expressing both A_1 and A_{2A} receptors, with (*R*)-*N*^{6}-(2-Phenylisopropyl)adenosine (*R*-PIA) for up to 24 h, after which the adenylyl cyclase activity was reduced by approx. 50%. This was associated with a significant decrease in cell membrane-bound A_1 receptors, and a concomitant increase of receptor number in intracellular compartments. The authors also showed an increase in receptor phosphorylation, nicely paralleling the time course of adenylyl cyclase modulation[35]. In a later study, Nie et al. reported similar findings in the same cell line, although desensitization occurred at a somewhat faster pace (4 h)[36]. Interestingly, Palmer et al. were unable to demonstrate desensitization in CHO cells expressing the human A_1R[37]. Klaasse et al., however, were able to show internalization of the human A_1R tagged with a C-terminal yellow fluorescent protein (hA_1YFP-R), stably expressed in CHO cells. Exposure of these cells for 16 h to 400 nM or 4 μM CPA resulted in 25% and 40% receptor-internalization, respectively. Addition of 10 μM of the allosteric enhancer PD81,723 did not accelerate the internalization process, but lowered the threshold-

concentration at which internalization occurred. Under those conditions a small degree of internalization was observed already at a concentration of 40 nM CPA, and at 400 nM CPA, 59% of the receptors internalized[38].

Exposure of cerebellar granule cells, endogenously expressing A_1R, to 100 nM *R*-PIA for 2 to 48 h led to a blunting of the inhibition of adenylyl cyclase. Along with this observation, a decrease of [^{3}H]cyclohexyladenosine ([^{3}H]CHA) binding to intact cerebellar granule cells, and an increase of [^{3}H]CHA binding in microsomes was detected. Simultaneously, a decrease in the steady-state level of $G_{i\alpha}$ in plasma membrane and microsomes was observed. These findings point not only to homologous desensitization, but also to subsequent internalization of the A_1R in the microsomal fraction of cerebellar granule cells upon long-term agonist exposure. However, no change in mRNA level was observed, suggesting that post-transcriptional regulation underlies receptor desensitization[39].

In hippocampal neurons, A_1R are present on both presynaptic and postsynaptic terminals, as well as on the cell body and dendrites where they exert different actions. To address the question whether desensitization was influenced by subcellular localization, neurons were exposed to the agonist 2-chloroadenosine (CADO, 20 μM) from 2 up to 96 h. It was found that upon agonist exposure in cultured hippocampal neurons, the presynaptic A_1Rs desensitize less quickly (>12 h) than the postsynaptic A_1Rs (2 h). In accordance, the recovery of desensitized presynaptic A_1Rs also requires more time (48 h vs. 8 h for postsynaptic A_1R). Desensitization of the postsynaptic A_1Rs apparently occurs at the level of the receptor, because the other elements of the signal transduction machinery appeared to be fully functional upon receptor desensitization. All in all, these results suggest that the extent and the kinetics of agonist-induced desensitization of A_1Rs depend on the subcellular localization of the receptors[40].

Using a more intact preparation, Coelho et al. found that the density of A_1R in rat hippocampal slices was decreased with 30% upon 60 min of imposed hypoxia. This desensitization could be mimicked by adding the A_1R agonist CADO (10 μM) for 60 min, and was prevented by adding the A_1R antagonist DPCPX. These results suggest that hypoxia leads to an increase in extracellular adenosine levels, and a subsequent, quite rapid (<90 min) desensitization, possibly followed by subsequent internalization of the A_1R in nerve terminals[41].

Rat *in vivo* studies also demonstrated A_1R desensitization. In a study by Parsons and Stiles rats were chronically (6 d) infused with *R*-PIA. Examination of adipocyte membranes of both treated and control rats revealed a 40% lower inhibition of adenylyl cyclase in the treated animals, coinciding with a reduced number of A_1Rs (68% agonist-occupied receptors remaining) as determined by radioligand binding[42]. Upon further study it was noticed that the effects on adenylyl cyclase were not A_1R

specific but were controlled at the level of adipocyte G proteins. G_i levels appeared downregulated, whereas the amount of G_s in the preparation was increased, although not at the level of their mRNA, suggesting a heterologous form of desensitization[43].

Chronic exposure of rats to *R*-PIA (6 d) also led to A_1R desensitization in the brain, and subsequent reduced inhibition of adenylyl cyclase. This loss of response was accompanied by a significant decrease in both total numbers of A_1R and $G_{i\alpha}$ proteins in synaptic plasma membranes in the brain, paralleling the finding by Stiles and co-workers in adipocytes[44]. As a consequence, a significant increase of A_1R was observed in microsomes and coated vesicles, which suggested a role for coated vesicles in the internalization of A_1R. Similarly, chronic agonist exposure of rats to NECA (6 d) resulted in a significant decrease of A_1R in the high-affinity state in the rat brain, however without changes in adenylyl cyclase activity or inhibition of the $G_{i\alpha}$ proteins[45].

Molecular mechanisms

As discussed in the general introduction to this review, receptor posttranslational modifications, receptor phosphorylation, recruitment of arrestins, and the formation of clathrin-coated pits are elements of the molecular machinery of desensitization and internalization. In addition, other potential protein partners in the two processes have been studied for the various adenosine receptor subtypes, which will also be discussed.

Effect of receptor posttranslational modifications on desensitization and/or internalization

Gao et al. studied the effects of preventing palmitoylation of the A_1R. It appeared that the $Cys^{309}Ala$ mutation, thus removing the palmitoylation site of the human A_1R, had no effect on internalization[46]. These findings were later confirmed by Ferguson et al.[47].

GRKs and arrestins

A_1R phosphorylation by GRKs has been subject to debate. Palmer et al. and Ferguson et al. failed to demonstrate GRK-2 phosphorylation of A_1R; in the latter study it was found that nonphosphorylated A_1R redistributes arrestin-3 from the cytoplasm into punctate clusters at the plasma membrane[37,47]. Nie et al., however, reported that within 1 hr of exposure of DDT_1MF-2 cells to *R*-PIA, rapid translocation of GRKs was observed from the cytosol to the cytoplasm[36]. In a further biochemical approach with both purified receptor and GRK-2, a phosphorylated receptor was

obtained that showed enhanced affinity for arrestins over G proteins. The same receptor kinase, upon overexpression in FRTL-5 cells, influenced A_1R signaling, however, not via $G_{i\alpha}$-mediated adenylyl cyclase but through $G_{i\beta\gamma}$-mediated MAP kinase activation[48]. It may be concluded that under physiological conditions A_1R phosphorylation does not (readily) take place, which would be a rationale for the long time periods required for receptor internalization.

Other protein partners

The ecto-enzyme adenosine deaminase (ADA) regulates the extracellular adenosine concentration by converting excess adenosine into inosine. However, ADA also plays a role in the desensitization and internalization of A_1R in smooth muscle DDT_1MF-2 cells[49,50] and LLC-PK_1 epithelial cells[51], acting as a receptor activity modifying protein (RAMP). Upon agonist exposure (100 nM *R*-PIA, 2h), ADA and A_1R formed complexes on the cell surface, clustered and internalized together to intracellular compartments. Such clustering of adenosine A_1 receptors prior to internalization was also reported by Ciruela et al. and Saura et al.[49,52].The intracellular vesicles contained the lipid raft marker protein caveolin. Filipin, an agent that disrupts rafts or caveolae, inhibited A_1R internalization. In contrast, acidic treatment disrupting clathrin-coated vesicles, did not inhibit agonist-induced internalization of A_1R. These results indicate that ADA and A_1R form a stable complex in the cell membrane of LLC-PK_1 cells, internalizing upon agonist exposure via lipid rafts, in a clathrin-independent pathway. Furthermore, a direct interaction of the C-terminus of A_1R with caveolin was demonstrated[50]. These and other data suggest that the mode of receptor compartmentalisation in response to agonist stimulation may be governed by both receptor subtype and cell type[51].

Another accessory protein, the heat shock cognate protein 73 (hsc73), a member of the hsp70 family, was identified as a cytosolic component able to interact with the third intracellular loop of the A_1R. The interaction between hsc73 (30 nM) and A_1R led to a marked reduction in affinity of ligands for the A_1R and also prevented activation of the G proteins, even more so than the addition of GTP analogues or GTP itself (100 μM). These effects were completely prevented by the addition of 25 nM ADA. A high percentage of A_1R was coupled to hsc73 in cell lysate, according to immunoprecipitation experiments. A remarkable feature upon internalization of the receptor in DDT_1MF-2 cells was found; A_1Rs internalized via two different vesicle types, one in which A_1R and hsc73 are colocalized and another in which hsc73 was absent. These observations open the possibility that signalling via GPCRs is regulated at least to some extent by heat shock proteins[53].

Receptor-receptor interactions

Dunwiddie et al. found that activation of A_3R with a selective A_3R agonist resulted in subsequent heterologous desensitization of the A_1R, as observed in electrophysiological experiments in the CA1 region of rat hippocampus. Similar results were obtained after A_3R occupancy via a brief superfusion with a high concentration of adenosine[54].

Lopes et al. investigated how activated $A_{2A}R$ influenced A_1R function and whether this interaction was modified in aged rats. In hippocampal and cortical nerve terminals, the $A_{2A}R$ agonist CGS 21680 (30 nM) was able to lower the binding affinity of the A_1R selective agonist CPA, and this was taken as proof for A_1R desensitization. The effect was counteracted by the addition of the $A_{2A}R$ antagonist ZM 241385 (20 nM). This reduction in A_1R function could only be detected in young adult rats (6 weeks), but not in old rats (24 months). The addition of a PKC inhibitor, chelerythine (6 μM), also prevented the desensitization of A_1R[55]. Similarly, Ciruela et al. (2006) demonstrated that A_1R-$A_{2A}R$ heteromers were able to modulate the glutamatergic neurotransmission in the rat striatum. The main biochemical characteristic of this heteromer is the ability of the agonist-occupied $A_{2A}R$ to reduce the agonist affinity of the A_1R[56].

By binding to cannabinoid CB_1 receptors in Purkinje fibers in the cerebellum, Δ^9-tetrahydrocannabinol inhibits adenylyl cyclase and consequently motor coordination. Long-term Δ^9-tetrahydrocannabinol treatment resulted in CB_1R downregulation, desensitization of the $G_{i\alpha}$ protein and desensitization of adenylyl cyclase. G protein activation by A_1R, however, was unaffected. Surprisingly, heterologous attenuation of A_1R-mediated inhibition of adenylyl cyclase was observed. These results indicate that long-term Δ^9-tetrahydrocannabinol administration produces a disruption of inhibitory receptor control of cerebellar adenylyl cyclase and suggest a potential mechanism of cross-tolerance to the motor effects of cannabinoid and A_1 agonists[57].

D_1R and A_1R have been shown to form functionally interacting heteromeric complexes in engineered cell lines and in cortical neurons in culture. Pretreatment with an A_1R agonist caused complex formation of both receptors. Combined pretreatment with selective agonists for both receptors (but not one agonist alone) substantially reduced cAMP accumulation induced via the D_1R, indicative for this receptor's desensitization[58].

A_{2A} receptor

Cellular and physiological studies

DDT_1 MF-2 cells, expressing both A_1R and $A_{2A}R$, were exposed to an A_{2A}-selective agonist, which resulted in a rapid loss ($t_{1/2}$=45 min) of agonist-stimulated cAMP production in these cells. This receptor desensitization, however, did not involve a reduction in cell membrane receptor number or a change in ligand affinity[35].

Prolonged exposure of PC12 cells, expressing both $A_{2A}R$ and $A_{2B}R$, to AR agonists led to a fast (30 min) and significant inhibition of $A_{2A}R$ stimulation by the $A_{2A}R$-selective agonist CGS21680. This effect appeared to occur at the level of adenylyl cyclase, since no change was observed in receptor number or in CGS21680's affinity for the receptor. This conclusion was corroborated by the finding of reduced activation of adenylyl cyclase by forskolin. Longer agonist exposure (12-20h) led to a reduction of $G_{s\alpha}$ levels, whereas no changes occurred in the short-term protocol[59].

NG108-15 neuroblastoma x glioma hybrid cells express both $A_{2A}R$ and $A_{2B}R$[60,61]. Treatment of the cells with the non-selective agonist NECA followed by its washout, led to a rapid ($t_{1/2}$=20 min) and pronounced reduction in cAMP production by the $A_{2A}R$-selective agonist CGS21680 when compared to vehicle-treated cells.

Palmer et al. (1994) generated CHO cells solely expressing the recombinant (canine) $A_{2A}R$ and examined the desensitization process of this receptor. Cells exposed to NECA showed a rapid desensitization of $A_{2A}R$-stimulated adenylyl cyclase activity with no obvious difference between pretreatment of 30 min or 24 h. This was associated with a slightly reduced affinity of the receptor for the $A_{2A}R$-selective radiolabeled agonist [^{3}H]CGS21680. Cell surface receptor numbers only diminished significantly (up to 40%) upon longer-term pretreatment ($t_{1/2}$ = 8 h)[62].

Using a tissue preparation, Conti et al. investigated whether prolonged exposure of $A_{2A}R$ to the non-selective agonist NECA, or to the selective $A_{2A}R$ agonists CGS21680 and 2HE-NECA, influenced $A_{2A}R$ desensitization. The authors used the porcine coronary artery as a sensitive vascular model, expressing among others $A_{2A}Rs$. The arteries were first precontracted by adding 3 μM PGFα. NECA, CGS21680 and 2HE-NECA showed high affinities for the $A_{2A}R$ (EC_{50}'s of 72, 40 and 20 nM respectively) inducing vasorelaxation. Next, coronary arteries were pretreated with 10 μM NECA for 30 min or 2 h. After a two hour washout period, the functional response was assessed. It appeared that preincubation with NECA did not hamper the vasorelaxing effects of CGS21680 and 2HE-NECA. However, NECA response curves were shifted to the right after NECA pretreatment[63]. These results seem inconclusive, since NECA pretreatment might 'hit' other adenosine receptor subtypes.

A_{2A}Rs, next to A_1R, are present on presynaptic baroreceptor afferent terminals within the nucleus tractus solitarius (NTS) in the brain[64]. It was observed that these A_{2A}R modulate 5-HT release as a mechanism of baroreflex control mediated by the NTS. Low concentrations of the non-selective agonist NECA (0.3-3 nM), briefly exposed (5 min) to NTS brain slices, induced the release of 5-HT caused by the activation of the A_{2A}R present at presynaptic nerves. This effect could be blocked by the addition of the adenosine A_{2A}R antagonist 8-(3-chlorostyryl)caffeine (CSC; 100 nM). Longer exposure of NECA (20 min) to the NTS slices resulted in inhibition of the 5-HT release, probably caused by quick desensitization of the A_{2A}R (15 min) and subsequent involvement of the A_1R[65]. These findings were corroborated in a similar study with CGS21680, an A_{2A}R-selective agonist[66].

The effects on AR fate upon chronic agonist exposure were studied *in vivo* in rat brain[45]. After 6 days of NECA treatment, the adenylyl cyclase activity in synaptic plasma membranes was decreased, suggesting a desensitization of A_2Rs, although the authors did not specify or study which A_2R subtype was involved. G_s protein levels were decreased indicating G_s down-regulation as the mechanism of desensitization.

Interestingly, Rekik and Mustafa (2003) showed that chronic *antagonist* treatment (3 days) of porcine coronary arteries with ZM241,385 led to a decreased agonist responsiveness. Although A_{2A} receptor expression went up, it appeared that the levels of G_s had decreased, altogether leading to a functional desensitization of the relaxing response by e.g. CGS21680[67]. It should be kept in mind that ZM241,385 is also a potent antagonist for the adenosine A_{2B} receptor.

Molecular mechanisms

Effect of receptor C-terminus on desensitization and/or internalization

To investigate the importance of the (120 amino acid residues) long C-terminus of the A_{2A}R receptor in inducing desensitization and internalization, Palmer and Stiles introduced several mutations and deletions into the receptor tail. It appeared that deletion of the last 95 amino acids of the C-terminus, containing 10 possible phosphorylation sites, did not have any effect on radioligand binding, adenylyl cyclase activity or desensitization kinetics compared to the wild-type A_{2A}R. However, when two possible phosphorylation sites (Thr 298 and Ser 305) just upstream the 95 deleted amino acids were mutated to Ala, short term (30 min) agonist-induced desensitization was attenuated, while the long-term (24h) desensitization was not affected. Single mutations revealed that mutation of Thr 298 alone was sufficient to reduce receptor phosphorylation and agonist-induced short-term desensitization.

This study also shows that short-term and long-term desensitization have distinct structural requirements and do not occur via the same mechanism[4].

GRKs and arrestins

Which GRK isoforms are involved in the phosphorylation of the $A_{2A}R$ is not entirely clear yet. However, a putative role for GRK2 and/or GRK5 has been suggested[4]. The role of GRK2 in agonist-induced phosphorylation and subsequent desensitization of $A_{2A}R$ and $A_{2B}R$ was thoroughly investigated by Kelly and coworkers. Wild-type GRK2 was stably expressed in NG108-15 cells, which endogenously express $A_{2A}R$ and $A_{2B}R$. The acute stimulation of adenylyl cyclase by activation of $A_{2A}R$ was markedly reduced in NG108-15 cells overexpressing wild-type GRK2. This was probably caused by GRK2-dependent pre-desensitization of the $A_{2A}R$ by extracellular adenosine. This effect could be reversed by pretreating the cells 24h with 0.5 unit/mL ADA[68]. The same research group investigated the effect of a dominant-negative GRK2 mutant on the desensitization of the $A_{2A}R$[60]. Stable transfection of a GRK2Lys220Arg mutant in NG108-15 cells reduced desensitization of the $A_{2A}R$ by 50% following a 30 min treatment with the adenosine agonist NECA.

TNF-α treatment of human monocytoid THP-1 cells expressing the $A_{2A}R$ prevented desensitization of this receptor, occurring under control conditions upon pretreatment with CGS21680 or NECA[69]. It was discovered that the TNF-α treatment prevented GRK2 translocation to and decreased GRK2 association with the plasma membranes of these cells as a consequence of the activation of a sphingomyelinase-dependent pathway.

In another study, Mundell and Kelly investigated the effect of inhibitors of receptor internalization on desensitization and resensitization of ARs in NG108-15 cells[70]. Before agonist exposure, cells were pretreated with hypertonic sucrose or concanavalin A, both inhibitors of internalization. This pretreatment did not affect the agonist-induced desensitization of the $A_{2A}R$. However, the resensitization of the $A_{2A}R$ upon agonist removal was abolished in the presence of con A or sucrose.

Arrestins-2 and -3 have been implicated in the downstream desensitization process. CGS21680 stimulation of a tagged $A_{2A}R$ transiently transfected in HEK293 cells induced the translocation of GFP-tagged arrestins-2 and -3 towards the plasma membrane. A dominant-negative arrestin-2 mutant inhibited agonist-induced internalization[71].

Other protein partners

The long C terminus of the $A_{2A}R$ has been coined a ‘coincidence detector’ as it recognizes quite a number of other proteins such as α-actinin and ARNO, the Arf nucleotide site opener (for a review see Gsandtner and Freissmuth[72]). α-Actinin may

play a role in receptor internalization, not unlikely for a protein involved in cytoskeletal arrangements[71], however ARNO does not[73].

Receptor-receptor interactions

The $A_{2A}R$ colocalizes with D_2 dopamine receptors in the basal ganglia, and their interaction has been documented on several occasions[74]. For instance, the protein-protein interaction between $A_{2A}R$-D_2R was confirmed in HEK293T cells. The most likely mode of interaction is that helix 5 and/or helix 6 and the N-terminal portion of I3 in the D_2R approach helix 4 and the C-terminus of the $A_{2A}R$[75]. Within the scope of this review fits the observation that activation of the D_2 receptor actually sensitizes $A_{2A}R$-mediated increases in cAMP production, in CHO cells expressing both receptors[76] as well as in CAD and NS20Y neuroblastoma cells in which the D_2 receptor was expressed[77]. In SH-SY5Y neuroblastoma cells co-stimulation of $A_{2A}R$ and D_2R accelerated D_2R desensitization, probably by causing or enhancing D_2R internalization[80]. Recently it was found that in these SH-SY5Y neuroblastoma cells the $A_{2A}R$ also formed heteromers with the endogenously expressed CB_1R. The CB_1R is negatively coupled to adenylyl cyclase, and requires previous or simultaneous activation of $A_{2A}R$ to signal in these cells[79].

A_{2B} receptor

Cellular and physiological studies

The adenosine A_{2B} receptor ($A_{2B}R$) is endogenously expressed on most artificial cell lines, such as COS and HEK293 cells. The exception is formed by CHO cells that lack this adenosine receptor subtype.

The $A_{2B}R$, like the other three adenosine receptor subtypes, is subject to agonist-induced desensitization. This was measured on the level of cAMP production by Peters et al.[80]. Pretreatment of COS7 cells endogenously expressing A_{2B} receptors with the nonselective agonist NECA (1 μM) for 1 up to17 h, led to a significant reduction in cAMP production upon acute agonist challenge, already after 1 h of pretreatment. Maximal reduction of cAMP production was reached after 4 h of pretreatment. CHO cells stably expressing a 5' FLAG epitope-tagged A_{2B} receptor showed the same result, albeit that maximal reduction of cAMP response was already achieved after 1 h of pretreatment. Also mouse 3T3-L1 cells and human HEK293 cells, endogenously expressing the $A_{2B}R$, showed a decreased cAMP response after 2 h and 5.5 h of pretreatment with 1 μM NECA, respectively[80]. In NG108-15 cells, expressing both $A_{2A}R$ and $A_{2B}R$, the rate of desensitization for both receptor subtypes appeared similar, with a half-life of 15-20 min. Pretreatment with

NECA of these cells (0.1 mM, 30 min) reduced stimulation of adenylyl cyclase by $A_{2B}R$ by approx. 50%[60,61].

Rat pheochromocytoma PC12 cells also endogenously express both $A_{2A}R$ and $A_{2B}R$. Prolonged exposure (14 h) to 100 nM NECA or 1 μM CGS21680 significantly inhibited the cAMP response of the cells to subsequent stimulation with the A_{2A}-selective agonist CGS21680. Although a 100-fold higher NECA-concentration is needed to stimulate the $A_{2B}R$ compared to $A_{2A}R$, Chern et al. found that the A_{2B} receptor cAMP response was also desensitized upon desensitization of the A_{2A} receptor. This observation can be explained by the fact that the $G_{s\alpha}$ protein level and the adenylyl cyclase activity were diminished upon long-term desensitization of the $A_{2A}R$ by CGS21680[59].

The domain in which the receptor resides is also important for desensitization events, according to Sitaraman et al. NECA (10 μM) added to either the apical or basolateral side of T84 intestinal cells resulted in desensitization of the $A_{2B}R$ on the corresponding side within 2-3 h. However, whereas applying NECA to the apical side of the membrane had no effect on the basolateral $A_{2B}R$, basolateral NECA induced a complete desensitization of the apical receptor. Since receptor trafficking may play a role in this cross-desensitization process, this effect may contribute to the desensitization and subsequent downregulation of the $A_{2B}R$[81].

Trincavelli et al. observed a very rapid desensitization ($t_{1/2}$=5 min) of $A_{2B}R$ in a human astrocytoma cell line. Both G protein coupling efficiency and cAMP production were diminished after pretreatment of the cells with NECA in the presence of an $A_{2A}R$-selective antagonist[82].

Mundell et al. reported that acute exposure of human airway smooth muscle (ASM) cells to adenosine receptor agonists resulted in a rapid accumulation of cAMP, most probably via $A_{2B}R$. Treatment with adenosine deaminase (ADA) suggested that ASM cells produce adenosine which feeds back on the cells' $A_{2B}Rs$ thereby regulating basal cAMP levels and inducing a small degree of $A_{2B}R$ desensitization. Chronic treatment with adenosine agonists had a dual effect; both $A_{2B}R$ desensitization and adenylyl cyclase sensitization (an increased responsiveness of adenylyl cyclase upon stimulation) were observed[83].

Haynes et al. (1999) studied $A_{2B}R$ desensitization in both pulmonary artery smooth muscle cells and a more integrated preparation, the isolated perfused lung. Pretreatment of the smooth muscle cells with NECA for 45 min abrogated the increase in cAMP response otherwise observed for both NECA and isoproterenol, suggesting heterologous desensitization. Indeed, experiments with cholera toxin showed that the desensitization took place at the level of the G_s protein-adenylyl cyclase complex. In the lung preparation NECA, when dosed acutely, caused a rapid

vasodilation, which was not observed when the tissue was treated again with the same compound 45 min after the first dose[84].
Taken together, these results suggest that desensitization of the A_{2B} receptor is a robust phenomenon, rather independent from the cell type or tissue in which it is expressed.

Molecular mechanisms

Effects of receptor mutations on desensitization and/or internalization
Matharu et al. studied the regions of the $A_{2B}R$ which are responsible for the desensitization and internalization of the $A_{2B}R$[85]. For this purpose they introduced point mutations or deletions in the C-terminus of the $A_{2B}R$. Deleting the final two amino acids (Leu^{330}-stop) did not affect the internalization properties of the $A_{2B}R$. The Phe^{328}-stop and Gln^{325}-stop mutants however, were resistant to agonist-induced desensitization and internalization. GFP-tagged arrestin-2 did not translocate from the cytosol to the plasma membrane upon agonist stimulation of these two truncated receptors. From a single point mutation in the C-terminus, Ser^{329}Gly, it became clear that this serine residue is responsible for the rapid agonist-induced desensitization and internalization. Surprisingly, a further deletion mutant Ser^{326}-stop was able to undergo rapid agonist-induced desensitization and internalization, however, arrestin-2 was not attracted to the plasma membrane upon agonist stimulation. It appeared that internalization of this truncated receptor occurred via an arrestin- and clathrin-independent pathway, probably via caveolin-mediated internalization since the internalization of Ser^{326}-stop was dynamin-dependent. The destination of this truncated receptor was also different; a compartment close to the plasma membrane, instead of early endosomes. Whether the receptor will undergo rapid agonist-induced desensitization and internalization, or influence the rate, extent and mechanism of internalization depends on specific sites in the C-terminus of the $A_{2B}R$ and can be influenced by their mutation or deletion.

GRKs and arrestins
The $A_{2B}R$, endogenously expressed in NG108-15 cells, is desensitized through phosphorylation by GRK2. This GRK2 effect was selective for $A_{2A}R$ and $A_{2B}R$, since the other endogenously expressed receptors (secretin R and IP-prostanoid R) were not desensitized in this way[68]. In addition, stable transfection of a dominant negative GRK2(Lys220Arg) mutant in the NG108-15 cells reduced the NECA-induced (30 min, 100 μM) desensitization of the $A_{2B}R$ by 50%[60]. Peters et al. studied the role of phosphorylation by PKA and PKC rather than GRK2 in agonist-induced

desensitization in COS-7 cells transiently expressing the 5'-FLAG A_{2B}R. They concluded that these second messenger dependent kinases are not involved in A_{2B}R phosphorylation[80]. A similar conclusion for A_{2B}R phosphorylation in NG108-15 cells was reached by Mundell and Kelly[61]. Trincavelli et al. found that treatment of astrocytoma cells with TNF-α increased the A_{2B}R functional response, without changing receptor protein or mRNA levels. TNF-α treatment appeared to markedly reduce agonist-dependent receptor phosphorylation and also attenuated agonist-mediated A_{2B}R desensitization. These effects could be inhibited by the addition of the A_{2B}R antagonist MRS 1706[82]. These findings resemble data obtained with the A_{2A}R (*vide supra*).

Mundell et al. found that within the arrestin family, both arrestin-2 and arrestin-3 are involved in the internalization process of the A_{2B}-receptor. Within 1 minute of agonist exposure, both GFP-tagged arrestin isoforms were translocated to the plasma membrane. Longer agonist exposure (>10 min) however, revealed that only arrestin-2 colocalizes with the receptor in the early endosomes. One explanation for this may be a higher affinity of the A_{2B}R for arrestin-2 compared to arrestin-3. Interestingly, arrestins are not only involved in the internalization of the A_{2B}-receptor, but also in its recycling. It appeared that the expression of stable arrestin anti-sense constructs, reducing the levels of endogenous arrestins, not only resulted in less internalization of the receptors, but also impaired the recycling process significantly. In contrast to the internalization process, arrestin-3 induced a significantly faster rate of A_{2B}R recycling compared to arrestin-2[86].

Penn et al. also studied the behaviour of GFP-tagged arrestins, now in human airway smooth muscle cells. Arrestin-2 and arrestin-3 were able to reduce the cAMP production upon stimulation of endogenous A_{2B}R by NECA and stimulation of β_2-AR by isoproterenol. In addition, a punctate clustering was observed in the membrane upon exposure to either NECA (100 μM) or isoproterenol (1 μM), indicating that arrestins play a role in receptor trafficking. On the contrary, signaling and trafficking of the prostaglandin E_2-R, another endogenously expressed receptor, was not affected by arrestin-2 or arrestin-3[27].

A_3 receptor

Adenosine A_3 receptors (A_3R) evoke considerable interest as novel drug targets to address cerebral/cardiac ischemia, cancer, inflammation, asthma and chronic obstructive pulmonary disease. So far, potent and selective antagonists for the hA_3R have been identified; the disadvantage however is that those antagonists show extremely low binding affinity for the rodent A_3R, typically 1000 times lower than in

human. Since rodent models are essential for the pharmacological evaluation of new therapeutic agents, this forms a serious drawback.
In addition, the adenosine A_3 receptor (A_3R) sequence holds a nuclear localization signal in its C-terminal tail[87]. This typical stretch of 4 amino acids (KKFK) in helix 8 may direct the receptor to the cell nucleus, not necessarily as a consequence of desensitization or internalization. This should be kept in mind when appreciating the studies described below.

Cellular and physiological studies
Ramkumar et al. studied the characteristics of rat A_3R, endogenously expressed on RBL-2H3 mast cells. In one of their experiments the authors pretreated the cells with NECA, and found a partial desensitization of the initial Ca^{2+} response to NECA[88]. Trincavelli et al. studied the relationship between agonist-induced desensitization, internalization and resensitization of hA_3R in transfected CHO cells. Agonist-induced endocytosis of hA_3R was investigated by immunogold electron microscopy of plasma membranes and intracellular vesicles and shown to occur with a half-life of 17 min. Subsequent removal of the agonist led to recycling of 90% of the receptor population to the cell surface, with a half-life of 35 min. Short-term exposure to agonist caused rapid desensitization, as assessed in cAMP assays. Removal of the agonist led to resensitization of the cAMP signal to 90% of the original signal within 120 min. Internalization did not affect signal transduction, as was demonstrated after blockade of internalization and recycling. Internalization occurred via clathrin-coated pits. These results show that hA_3R undergoes agonist-induced endocytosis, which is not responsible for desensitization. Moreover, in the case of hA_3R, receptor sequestration rather than desensitization seems to be the first step in a cycle of internalization, dephosphorylation and recycling to the plasma membrane[89]. A similar cell line was used by Palmer et al. In stably transfected CHO cells prolonged treatment with NECA (10 μM, 20 h) induced uncoupling of recombinant hA_3R from G_i proteins and a functional desensitization. Upon this A_3R desensitization, an approximately 2-fold increase in adenylyl cyclase activity was found in the presence or absence of forskolin. This sensitization of adenylyl cyclase activity was not caused by an altered level of inhibitory or stimulatory G protein expression. The occurrence of the sensitization was also not due to new protein synthesis, but probably to an increased coupling efficiency between G_s and adenylyl cyclase. Compared to control cells, long-term exposure of stably transfected CHO cells to NECA caused an increase in phosphorylation of the cAMP-responsive element binding protein upon addition of suboptimal concentrations of forskolin. The sensitization of adenylyl cyclase activity upon long-term treatment might provide a molecular basis for the observation that for several adenosine receptor-mediated events, acute agonist

exposure produces opposite effects to those after chronic agonist treatment[90]. Ferguson et al. studied the fate of the rat A_3R expressed in CHO cells. The receptors internalized rapidly after treatment with NECA or *R*-PIA over a time frame ($t_{½}$=10 min) that followed receptor phosphorylation ($t_{½}$=1 min)[91].

The desensitization, internalization and down-regulation of hA_3R was also investigated in human astrocytoma cells, natively expressing hA_3R. Short-term (15 min) exposure of the cells to 100 nM Cl-IBMECA, an A_3R-selective agonist, caused rapid receptor desensitization, subsequently followed by receptor internalization within 30 min. With help of immunogold electron microscopy, the localization of the A_3R was revealed. After 10 min of exposure, the A_3R was found in smooth-surfaced pits and in uncoated vesicles in the cytoplasm. After 30 min of exposure, the A_3R was found in vesicular endosomes. Upon removal of the agonist, resensitization of the A_3R occurred within 120 min through receptor recycling to the cell surface. Long-term agonist exposure (1-24 h) resulted in a marked down-regulation of A_3R to 22 $\pm$ 3% of control after 24 h. In conclusion, multiple, temporally distinct and sequential processes are involved in the regulation of hA_3R upon short- and long-term exposure to agonists[92].

A_3Rs were found to be highly expressed on murine B16-F10 melanoma cells[93]. The authors examined the association between A_3R trafficking and receptor functionality and tumour growth inhibition upon activation with the A_3R-selective agonist IB-MECA. Exposure to 10 nM IB-MECA (5 min) led to rapid internalization of A_3R to the cytosol, upon which receptors were directed to the endosomes for recycling or to lysosomes for degradation. The addition of 100 nM MRS 1523 (5 min), an A_3R antagonist, was able to counteract the internalization process as well as the modulation of the Wnt pathway leading to proliferation, thereby emphasizing the involvement of A_3R in this process. When the melanoma cells were injected into nude mice, tumours rapidly developed. Tumour growth was significantly inhibited after administration of IB-MECA to the animals, paralleled by a decrease in A_3R expression in tumour lesions. In hypoxic human A172 and U87MG glioblastoma cell lines it was found that adenosine up-regulates the expression of hypoxia-inducible factor-1α (HIF-1α) and VEGF via the A_3 receptor. HIF-1α is a key regulator in the development of tumours. The addition of the A_3-antagonist MRE 3008F20 inhibited the adenosine-induced HIF-1α and VEGF accumulation in these hypoxic cells, thereby showing a putative role in inhibiting tumour growth [94].

To circumvent the species differences mentioned in the introduction to the A_3R paragraph, Yamano et al. generated A_3R humanized ($A_3AR^{h/h}$) mice in which the A_3R was replaced by its human counterpart. The expression level of hA_3R in the humanized ($A_3AR^{h/h}$) mice was equal to the expression levels of A_3R in WT mice. A_3R agonists were able to elevate the intracellular Ca^{2+} concentration in bone

marrow-derived mast cells from the humanized ($A_3AR^{h/h}$) mice. However, the rate of hA_3R internalization was markedly reduced compared with that of mA_3R in these mast cells[95].

Molecular Mechanisms

Receptor phosphorylation, GRKs and arrestins

Palmer and Stiles, and Ferguson et al. investigated which amino acid residues in the C-terminus are responsible and crucial for the rapid desensitization of the A_3R[96,91]. A triple mutant (Thr^{307}, Thr^{318} and Thr^{319} to Ala) exhibited dramatically reduced phosphorylation, desensitization and internalization of the rat A_3AR. Individual mutation of each Thr residue showed that Thr^{318} and Thr^{319} were the most important sites for phosphorylation. In addition, phosphorylation of Thr^{318} was necessary to observe phosphorylation of Thr^{319}, but not vice versa. Moreover, changing Thr^{318} for a negatively charged residue (Glu) was not sufficient to retain phosphorylation at Thr^{319}. Mutating two predicted palmitoylation-sites, $Cys^{302,305}$ to Ala resulted in agonist-independent basal phosphorylation of the rat A_3AR. Such findings strongly suggest that palmitoylation of these Cys residues is an important factor in controlling accessibility of the C-terminus of the A_3R in the process of recruiting GRKs. In fact, the palmitoylation sites are highly conserved between different species, e.g. rat, mouse, human, dog, sheep. Taking these results together, it appears that GRK-mediated phosphorylation of the A_3R C-terminus follows a sequential mechanism, with the receptor palmitoyl moieties in an important regulatory role, and phosphorylation of Thr^{318} being particularly crucial as an essential first step. In an earlier study Palmer et al. replaced the carboxyl terminus of the A_1R by that of the A_3R[37]. This chimaeric construct was able to undergo agonist-stimulated phosphorylation and functional desensitization, similar to A_3R. It was also found that purified GRK2, GRK3 and GRK5 were all able to enhance agonist-dependent phosphorylation of A_3R as well as the A_1-A_3 chimaera.

Trincavelli et al. studied the involvement of extracellularly regulated kinases (ERK1 and 2), members of the mitogen-activated protein kinase (MAPK) family, in A_3R phosphorylation. It was found that within 5 minutes of exposure to 10 μM NECA, ERK 1 and 2 were already phosphorylated in CHO cells stably expressing hA_3R. An inhibitor of MAPK activation (PD98059) also caused inhibition of A_3R phosphorylation, desensitization and internalization, probably by preventing the membrane translocation of GRK2. These results indicate that the MAPK cascade is involved in A_3R regulation by a feedback mechanism which controls GRK2 activity and probably involves direct receptor phosphorylation[97].

The rat basophilic leukaemia cell 2H3 cell line (RBL-2H3 cells) endogenously expresses equal levels of arrestin-2 and arrestin-3. Both arrestin isoforms also have a high and comparable affinity for clathrin, thereby promoting agonist-induced internalization. RBL-2H3 cells also endogenously express a high level of A_3R, which, however, did neither recruit arrestin-3 nor arrestin-2 upon stimulation with NECA. Also no changes in A_3R distribution were observed. One explanation is that A_3R follow an endocytic mechanism upon agonist stimulation that does not involve arrestin-mediated clathrin-coated pit internalization. Another possible explanation is that arrestin recruitment was below the limit of detection in the RBL-2H3 cells[98]. Ferguson et al. observed that upon phosphorylation of A_3R by GRK arrestin-3 is redistributed into punctate vesicles both at the plasma membrane and within the cytoplasm. Nevertheless, these authors also noticed that there was no colocalization between A_3R and arrestin-3[47].

Conclusions

The adenosine receptor subtypes are differentially regulated when exposed to agonist treatment. A_1Rs are not (readily) phosphorylated and slowly internalize with a typical half-life of several hours. This feature may cause less than average tachyphylaxis upon chronic agonist administration, for instance in type II diabetes. A_{2A}R and A_{2B}R show much faster downregulation with similar kinetics, usually < 1h for short-term desensitization. Agonists for the A_{2A} receptor, currently profiled in wound healing, may thus suffer from declining efficacy upon chronic administration, in contrast to those for the A_1 receptor. The A_3R undergoes even faster downregulation, often a matter of minutes. The latter receptor also holds a nuclear localization signal in its carboxy terminal tail, possibly obscuring true agonist-induced downregulation. The fast desensitization of the A_3R after agonist exposure may be therapeutically equivalent to antagonist occupancy of the receptor.

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CHAPTER 3

Allosteric modulation and constitutive activity of fusion proteins between the adenosine A_1 receptor and different ^{351}Cys-mutated $G_i\alpha$-subunits

We studied fusion proteins between the human adenosine A_1 receptor and different (^{351}Cys) mutated $G_{i1}\alpha$ subunits (A_1-$G_i\alpha$) with respect to two important concepts in receptor pharmacology, i.e. allosteric modulation and constitutive activity/inverse agonism. The aim of our study was twofold. We first analysed whether such fusion products are still subject to allosteric modulation, and, secondly, we investigated the potential utility of the fusion proteins to study constitutive receptor activity. We determined the pharmacological profile of 9 different A_1-$G_i\alpha$ fusion proteins in radioligand binding studies. In addition, we performed [^{35}S]GTPγS binding experiments to study receptor and G protein activation of selected A_1-$G_i\alpha$ fusion proteins. Compared to unfused adenosine A_1 receptors, the affinity of N^6-cyclopentyladenosine (CPA) at wild-type A_1-$G_i\alpha$ fusion proteins (^{351}Cys) increased more than eight-fold, while the affinity of 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) did not change significantly. Furthermore, we showed that the allosteric enhancer of agonist binding, PD81,723 (2-amino-4,5-dimethyl-3-thienyl-[3-(trifluoromethyl)-phenyl]methanone), 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, presumably because they exhibit their effect through uncoupling of the R-G complex. From [^{35}S]GTPγS binding experiments we learned 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. Moreover, the maximal CPA-induced stimulation of basal [^{35}S]GTPγS binding was increased for the five A_1-$G_i\alpha$ fusion proteins tested, whereas the inhibition induced by 8-cyclopentyltheophylline (CPT) was more pronounced at ^{351}Cys, ^{351}Ile, and ^{351}Val A_1-$G_i\alpha$ fusion proteins. Thus, the maximal receptor (de)activation depended on the amino acid at position 351 of the $G_i\alpha$-subunit. In conclusion, A_1-$G_i\alpha$ fusion proteins, especially with ^{351}Cys and ^{351}Ile, can be used as research tools to investigate inverse agonism, due to their increased readout window in [^{35}S]GTPγS binding experiments.

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Introduction

Adenosine A_1 receptors belong to the superfamily of G protein-coupled receptors (GPCRs). They preferentially interact with G_i and G_o proteins[1]. Various strategies have been employed to study the interaction between receptor and G protein. One method is the construction of so-called fusion proteins, in which both signalling partners, GPCR and G α-subunit, are physically linked to each other. In 1994, the first fusion protein (between β_2-adrenoceptor and G_s-subunit) was described and found to be functional in both ligand binding experiments and cAMP determinations[2].

The same strategy has been applied to adenosine A_1 receptors. Waldhoer et al. analysed the kinetic behaviour of the adenosine A_1 receptor linked to both G_i- and G_o-subunits[3]. The authors also introduced a fusion protein in which the G_i-subunit (of rat G_{i1}) was mutated at position ^{351}Cys. This cysteine residue is known to be ADP-ribosylated by pertussis toxin (PTX); changing it for another amino acid (glycine or isoleucine in this case) renders the fusion protein insensitive to the toxin[4]. Endogenous G proteins present in the cell which the fusion protein is expressed in, remain sensitive to PTX and can be 'knocked out'. Similar constructs were used in studies to analyse the action of both receptor ligands such as adenosine analogues and G protein inhibitors such as suramin and derivatives[5,6]. Curiously, Bevan et al. introduced a "trivalent" fusion protein between adenosine A_1 receptor, green fluorescent protein, and G_i-subunit, which behaved very much as the wild-type receptor in terms of ligand binding and G protein activation[7].

In the present study we performed a further analysis of such fusion proteins with two aims that have not yet been subject of investigation. Firstly, we wondered whether the constructs can still be influenced by allosteric modulators. In our laboratory, we have been investigating allosteric modulation of adenosine receptors by, e.g., sodium ions and PD81,723[8-10].

Secondly, we wanted to learn whether the fusion proteins might be useful tools in the analysis of constitutive activity and its inhibition by inverse agonists. This inhibition of constitutive activity is commonly observed at GPCRs (for review see de De Ligt et al.)[11]. Inverse agonism is not always easily detected, since basal receptor activity is, in general, not pronounced. We reasoned that fusion proteins, due to the proximity of both signalling partners, might have a higher spontaneous receptor activity and, consequently, might offer an enlarged "window" to study inverse agonism.

Materials and Methods

Chemicals

CPA, DPCPX, CPT, and PD81,723 were purchased from Research Biochemicals Inc. (Natick, USA). Adenosine deaminase (ADA), diethylaminoethyl (DEAE) dextran, chloroquine, and dithiothreitol were purchased from Sigma, while EDTA, $MgCl_2$, GDP,
3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate (CHAPS), and GTPγS were obtained from Boehringer (Mannheim, Germany). [^{3}H]DPCPX (120 Ci/mmol), [^{3}H]CCPA (2-chloro-*N*^{6}-cyclopentyladenosine, 55 Ci/mmol), and [^{3}H]cAMP (25 Ci/mmol) were purchased from NEN (DuPont Nemours, 's-Hertogenbosch, NL). [^{35}S]GTPγS (1250 Ci/mmol) was obtained from NEN (Cologne, Germany). BSA and BCA protein assay reagent were purchased from Pierce Chemical Company (Rockford, IL, U.S.A.). All other chemicals were obtained from standard sources. A fraction containing protein kinase A was isolated from bovine adrenal glands according to Leurs et al.[12]. Except for foetal calf serum (FCS, Greiner, Netherlands), all cell culture materials were taken from laboratory stocks. All other chemicals were obtained from standard sources, and were of the highest purity commercially available. The cDNAs encoding either the unfused adenosine A_1 receptor or a fusion protein between the human adenosine A_1 receptor and rat G_{i1} α-subunit were produced by one of us (G.M.). Full fusion constructs were excised from pCR-Script with *Eco*RI and *Xho*I and ligated into the mammalian expression vector pcDNA3 according to the instructions supplied by the vendor (Invitrogen, San Diego, USA).

Cell culture and transfection

African green monkey kidney (COS-7) cells were maintained at 37 °C in a humidified atmosphere with 7% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% FCS, penicillin (50 IU/ml), and streptomycin (50 μg/ml). COS-7 cells were transiently transfected with either the unfused adenosine A_1 receptor, or a fusion protein (A_1-$G_i\alpha$) using a slightly modified protocol of the 'in suspension' DEAE dextran method previously described by Brakenhoff et al.[13]. In short, COS-7 cells were subcultured one day prior to transfection. The next day cells were trypsinised, counted, and resuspended in RPMI 1640 amino acids solution supplemented with 2% FCS and 100 μM chloroquine (RSC), at a density of 2 x 10^6 cells/ml. DNA (4-10 μg/10^6 cells) and DEAE dextran (400 μg/ml) were mixed in a total volume of 4 ml RSC, and incubated at room temperature for two minutes. Then 0.5 ml (10^6 cells) of the cell suspension was added, and the total mixture was incubated for 60 min at 37 °C and 7% CO_2. Cells were subsequently spun down at

1000 x *g* for 5 min, resuspended in normal growth medium, and seeded in the appropriate plates.

Membrane preparation

After 48 hrs, transiently transfected COS-7 cells were harvested with a cell scraper, and recovered by a 5-min centrifugation at 1000 x *g*. Cells were then homogenised in ice-cold 50 mM Tris HCl buffer (pH 7.4) with a polytron homogeniser (5 sec, speed 8), and used for radioligand binding studies. To measure [^{35}S]GTPγS binding, the cell homogenates were purified with two additional centrifugation steps at 4 °C: a 10-min centrifugation at 1000 x *g* and the obtained supernatant for 30 min at 60,000 x *g* (Beckman L8-50 M/E Ultracentrifuge). The final pellet was resuspended in 3 ml ice-cold 50 mM Tris HCl buffer (pH 7.4), supplemented with ADA (2 U/ml). Protein concentrations were measured with the bicinchoninic acid method with BSA as a standard[14].

Radioligand binding studies

For displacement studies, membranes (10-30 μg) were incubated for 1 hour at 25 °C in 50 mM Tris HCl (pH 7.4) in the presence of ADA (1 U/ml), approximately 1.6 nM [^{3}H]DPCPX, and increasing concentrations of CPA, or DPCPX in a total volume of 400 μl. To study the modulatory effects of 1 M NaCl and 10 μM PD81,723, the indicated concentrations were added when appropriate. For displacement experiments in the presence of 1 M NaCl, approximately 0.5 nM [^{3}H]DPCPX was used.

Incubations were stopped by rapid dilution with 2 ml of ice-cold 50 mM Tris HCl buffer (pH 7.4) and bound radioactivity was subsequently recovered by filtration through Whatman GF/C filters using a Brandel harvester. Filters were then washed twice with 2 ml of the buffer described above. The retained radioactivity was measured by liquid scintillation counting (LKB Wallac, 1219 Rackbeta). Non-specific binding of [^{3}H]DPCPX was measured in the presence of 10 μM CPA.

Saturation experiments were carried out under similar conditions with increasing concentrations of [^{3}H]DPCPX (0-5 nM) or [^{3}H]CCPA (0-4 nM). Filters were washed five times with 2 ml of 50 mM Tris HCl buffer (pH 7.4) to remove all unbound radioligand. Non-specific binding of [^{3}H]CCPA was measured in the presence of 10 μM DPCPX.

[^{35}S]GTPγS binding

[^{35}S]GTPγS binding was measured in 100 μl containing 50 mM Tris HCl (pH 7.4), 1 mM EDTA, 5 mM $MgCl_2$, 10 μM GDP, 1 mM dithiothreitol, 100 mM NaCl, 5 U/ml ADA, 0.3 nM [^{35}S]GTPγS (~50,000 cpm), 0.004% CHAPS, and 0.5% BSA.

Incubations were started by addition of the membrane suspension (1-3 μg protein/tube) to the test tubes, and carried out in duplicate for 90 min at 25 °C. They were stopped by rapid filtration through Whatman GF/B filters, pre-soaked in 50 mM Tris HCl, 5 mM $MgCl_2$ (pH 7.4) containing 0.02% CHAPS. The filters were washed twice with 4 ml of the buffer mentioned before, and retained radioactivity was measured using liquid scintillation counting. Non-specific binding of [^{35}S]GTPγS was measured in the presence of 10 μM unlabelled GTPγS, and subtracted from total bound radioactivity.

Data and statistical analysis

All receptor binding data were analysed by using a non-linear regression computer program (Prism 3.0, GraphPad Software, Inc., San Diego, CA, USA). Statistical significance was evaluated with the Student's *t* test. Saturation experimental data (K_d and B_{max} values) were obtained by computer analysis of saturation curves. From displacement experiments IC_{50} values were calculated. All values obtained are means of at least three independent experiments performed in duplicate.

EC_{50}/IC_{50} values for stimulation or inhibition of [^{35}S]GTPγS binding were calculated from fitting experimental results to sigmoid dose-response curves with SigmaPlot (SPSS Science, Inc., Chicago, IL, USA), and are given as geometric means with 95% confidence limits from at least three experiments.

Results

First, we established B_{max} and K_d values in saturation experiments with [^{3}H]DPCPX on COS-7 membranes expressing either the adenosine A_1 receptor alone or fusion proteins (A_1-$G_i\alpha$) between the adenosine A_1 receptor and three (mutated) $G_i\alpha$ proteins (^{351}Cys (= wild-type), ^{351}Gly, and ^{351}Ile). Saturation experiments were performed both in the absence and presence of 1 M NaCl (Table 3.1). The affinity of [^{3}H]DPCPX for these A_1-$G_i\alpha$ fusion proteins was similar to its affinity for the unfused adenosine A_1 receptor. Moreover, the A_1-$G_i\alpha$ fusion proteins were expressed at similar receptor densities, which were lower than those for the adenosine A_1 receptor alone. The other A_1-$G_i\alpha$ fusion proteins (^{351}Phe, ^{351}His, ^{351}Pro, ^{351}Arg, ^{351}Ser, and ^{351}Val) were tested in a single saturation experiment with [^{3}H]DPCPX in the absence of sodium ions. They showed K_d values in a similar range as observed for the adenosine A_1 receptor alone (1.2 - 2.1 nM), while receptor expression varied to some extent (0.4 - 1.0 pmol/mg of protein).

Table 3.1. K_d and B_{max} values from saturation experiments with [^{3}H]DPCPX on COS-7 cell homogenates transfected with the human adenosine A_1 receptor or various (mutated) A_1-$G_i\alpha$ fusion proteins (^{351}Cys (= wild-type $G_i\alpha$), ^{351}Gly, and ^{351}Ile). The experiments were carried out in the presence or absence of 1 M NaCl. Data are expressed as means ± S.E.M. from three independent experiments performed in duplicate.

	control		**1 M NaCl**	
Construct	K_d (nM)	B_{max} (pmol/mg protein)	K_d (nM)	B_{max} (pmol/mg protein)
unfused A_1	1.8 ± 0.31	2.18 ± 0.13	0.83/0.83[a]	2.71/2.81[a]
A_1G_i ^{351}Cys	1.5 ± 0.04	1.29 ± 0.38	0.50 ± 0.06[b]	1.59 ± 0.41
A_1G_i ^{351}Gly	1.9 ± 0.38	1.22 ± 0.21	0.41 ± 0.05[c]	1.39 ± 0.27
A_1G_i ^{351}Ile	1.5 ± 0.03	1.53 ± 0.48	0.50 ± 0.02[b]	1.58 ± 0.38

[a] n=2; [b] p <0.005, [c] p <0.001, significant difference versus control

Next, we determined the IC_{50} values of CPA and DPCPX for the nine A_1-$G_i\alpha$ constructs and compared the values obtained with their IC_{50} for the unfused adenosine A_1 receptor. We also studied the modulatory effects of 1 M NaCl and 10 μM PD81,723 on ligand binding to these A_1-$G_i\alpha$ fusion proteins. We could not convert the IC_{50} values into K_i values, since we did not determine radioligand K_d values for all fusion products under all three conditions, i.e. without or in the presence of 1 M NaCl or PD81,723.

The results from radioligand binding experiments with the adenosine A_1 receptor agonist, CPA, are listed in Table 3.2. CPA had significantly higher affinities for five of the A_1-$G_i\alpha$ fusion proteins (^{351}Cys, ^{351}Gly, ^{351}Ile, ^{351}Ser, and ^{351}Val) than for the unfused adenosine A_1 receptor. In the presence of 1 M NaCl, CPA's affinity for the unfused adenosine A_1 receptor decreased three-fold. Contrarily, the A_1-$G_i\alpha$ fusion proteins did not seem to be affected very much by sodium ions, exhibiting no significant shifts in the presence of 1 M NaCl, except for ^{351}Arg and ^{351}Pro. In these two cases, the affinity of CPA decreased five- and eight-fold, respectively, and resembled the affinity of CPA for the unfused adenosine A_1 receptor in the presence of sodium ions (Table 3.2). Furthermore, in the presence of 10 μM PD81,723 all A_1-$G_i\alpha$ fusion proteins exhibited an increased affinity for CPA. This PD81,723-induced enhancement of CPA binding was also observed at unfused adenosine A_1 receptors, which had a four-fold higher affinity for CPA in the presence of 10 μM PD81,723. Yet,

under these conditions all nine A_1-$G_i\alpha$ fusion proteins had a higher affinity for CPA (13-76 nM) than the unfused adenosine A_1 receptor (111 nM, Table 3.2).

Table 3.2. IC_{50} values (nM) for CPA binding to COS-7 cell homogenates transfected with the human adenosine A_1 receptor or different ^{351}Cys-mutated A_1-$G_i\alpha$ fusion proteins. The experiments were carried out in the presence or absence of 1 M NaCl or 10 μM PD81,723. Shifts were calculated by dividing the IC_{50} (in the presence of modulator) by IC_{50} (control). Data are expressed as means ± S.E.M. from at least three independent experiments performed in duplicate (n = 3-5).

	control	**1 M NaCl**		**10 μM PD81,723**	
Construct	IC_{50} (nM)	IC_{50} (nM)	shift	IC_{50} (nM)	shift
unfused A_1	436 ± 118	1355 ± 173	3.11[a]	111 ± 18	0.25[b]
A_1-Gi ^{351}Cys	51 ± 9	78 ± 18	1.53	25 ± 9	0.49[b]
A_1-Gi ^{351}Gly	167 ± 17	171 ± 46	1.02	32 ± 8	0.19[c]
A_1-Gi ^{351}Ile	95 ± 17	65 ± 10	0.68	13 ± 2	0.14[a]
A_1-Gi ^{351}Phe	328 ± 74	601 ± 179	1.83	49 ± 13	0.15[a]
A_1-Gi ^{351}His	279 ± 72	456 ± 67	1.63	76 ± 15	0.27[b]
A_1-Gi ^{351}Pro	270 ± 59	2194 ± 609	8.13[b]	54 ± 7	0.20[b]
A_1-Gi ^{351}Arg	222 ± 17	1135 ± 281	5.11[b]	42 ± 7	0.19[c]
A_1-G_i ^{351}Ser	135 ± 23	121 ± 33	0.90	22 ± 7	0.19[a]
A_1-Gi ^{351}Val	87 ± 21	84 ± 5	0.97	16 ± 4	0.18[a]

Significant shift compared to unity, [a]P<0.005, [b]P<0.05, [c]P<0.001

The results from radioligand displacement studies with the adenosine A_1 receptor antagonist/inverse agonist DPCPX are presented in Table 3.3. The A_1-$G_i\alpha$ fusion proteins ^{351}Ile, ^{351}Ser, and ^{351}Val had slightly, but significantly lower affinities for DPCPX than the unfused adenosine A_1 receptor, while the other constructs showed statistically similar affinities for DPCPX. Moreover, sodium ions increased the affinity of DPCPX for the unfused adenosine A_1 receptor approximately two-fold. The binding of DPCPX to the A_1-$G_i\alpha$ fusion proteins ^{351}Phe, ^{351}Pro, ^{351}Arg, ^{351}Ser, and ^{351}Val also increased two- to threefold, while DPCPX binding to the other A_1-$G_i\alpha$ fusion proteins (^{351}Cys, ^{351}Gly, ^{351}Ile, and ^{351}His) was unaffected. In the presence of 10 μM PD81,723 the IC_{50} value of DPCPX at A_1-$G_i\alpha$ fusion protein ^{351}Arg increased almost two-fold ($p < 0.05$). However, the affinity of DPCPX for the other A_1-$G_i\alpha$ fusion

proteins, as well as for the unfused adenosine A_1 receptor, was not significantly changed in the presence of 10 μM PD81,723. Since we used 8-cyclopentyltheophylline (CPT) in our GTP□S binding studies (see below), we also determined its affinity for one of the fusion proteins. CPT displayed an IC_{50} value of 30 ± 12 nM on the A_1-G_i^{351}Ile product in the absence of any modulator, being sevenfold less active than DPCPX.

Table 3.3. IC_{50} values (nM) for DPCPX binding to COS-7 cell homogenates transfected with the human adenosine A_1 receptor or different ^{351}Cys-mutated A_1-G_iα fusion proteins. The experiments were carried out in the presence or absence of 1 M NaCl or 10 μM PD81,723. Shifts were calculated by dividing the IC_{50} (in the presence of modulator) by IC_{50} (control). Data are expressed as means ± S.E.M. from at least three independent experiments performed in duplicate (n = 3-5).

	control	**1 M NaCl**		**10 μM PD81,723**	
Construct	IC_{50} (nM)	IC_{50} (nM)	shift	IC_{50} (nM)	shift
unfused A_1	3.0 ± 0.34	1.6 ± 0.12	0.55[a]	4.2 ± 0.79	1.40
A_1-Gi ^{351}Cys	4.5 ± 0.92	5.1 ± 0.44	1.13	8.8 ± 2.40	1.96
A_1-Gi ^{351}Gly	4.6 ± 0.94	3.3 ± 0.37	0.72	5.4 ± 0.64	1.17
A_1-Gi ^{351}Ile	4.5 ± 0.66	4.6 ± 1.12	1.02	3.3 ± 1.10	0.73
A_1-Gi ^{351}Phe	3.7 ± 0.80	1.7 ± 0.34	0.46[a]	5.1 ± 0.53	1.38
A_1-Gi ^{351}His	2.3 ± 0.58	1.4 ± 0.14	0.61	4.1 ± 0.72	1.78
A_1-Gi ^{351}Pro	4.3 ± 0.95	1.4 ± 0.17	0.33[a]	4.4 ± 0.45	1.02
A_1-Gi ^{351}Arg	3.6 ± 0.31	1.3 ± 0.21	0.36[b]	6.1 ± 0.99	1.69[a]
A_1-G_i ^{351}Ser	6.4 ± 0.69	1.8 ± 0.07	0.28[b]	6.0 ± 0.29	0.94
A_1-Gi ^{351}Val	4.7 ± 0.74	1.5 ± 0.21	0.32[b]	7.9 ± 1.80	1.68

Significant shift compared to unity, [a]P<0.05, [b]P<0.005

To study the functionality of the A_1-$G_i\alpha$ fusion proteins, we determined [^{35}S]GTPγS binding on membranes prepared from transfected COS-7 cells, expressing the A_1-$G_i\alpha$ fusion proteins of interest. In these studies, CPT, a more water-soluble analogue of DPCPX, was used as reference adenosine A_1 receptor inverse agonist in [^{35}S]GTPγS binding experiments. Figure 3.1 shows the results of [^{35}S]GTPγS binding to COS-7 membranes, expressing the wild-type A_1-$G_i\alpha$ fusion protein (^{351}Cys). The agonist CPA increased basal [^{35}S]GTPγS binding more than seven-fold, while CPT decreased [^{35}S]GTPγS binding to 52% of the basal level. To obtain a reasonable window to observe inhibition of basal [^{35}S]GTPγS binding by the adenosine A_1 receptor inverse agonist CPT, we used more protein (3 μg) than in experiments with the agonist CPA (1 μg of membrane protein).

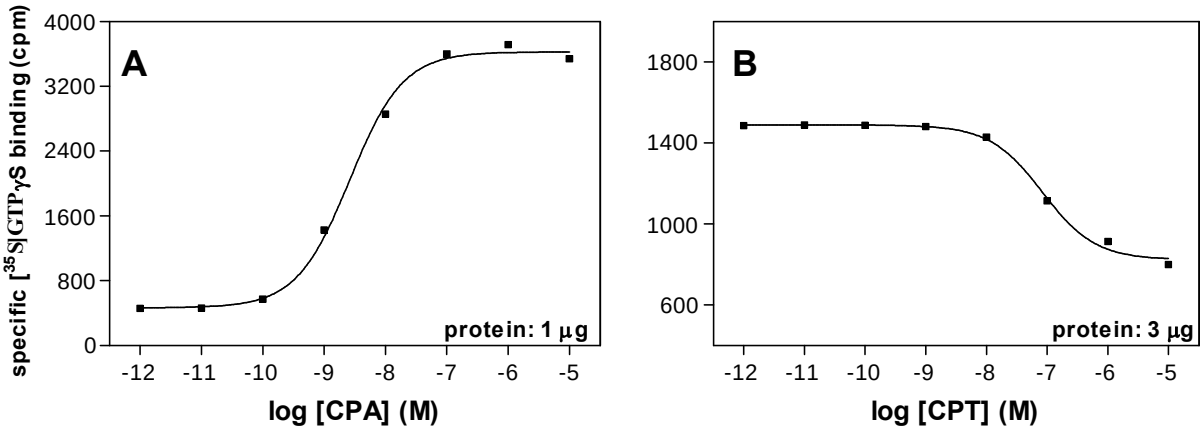

Figure 3.1. Stimulation by CPA (panel A) and inhibition by CPT (panel B) of basal [^{35}S]GTPγS binding in membranes prepared from COS-7 cells transfected with the A_1-$G_i\alpha$ fusion protein ^{351}Cys. Data are from a representative experiment performed in duplicate.

We performed similar experiments with COS-7 membranes expressing the unfused adenosine A_1 receptor (10 μg of protein) and the A_1-$G_i\alpha$ fusion proteins ^{351}Cys, ^{351}Gly, ^{351}Ile, ^{351}Pro and ^{351}Val (1-3 μg of protein). For the latter two A_1-$G_i\alpha$ fusion proteins, we tested only a single concentration of 1 μM CPT. It should be noted that less protein was needed in experiments with the A_1-$G_i\alpha$ fusion proteins to give similar basal [^{35}S]GTPγS binding. The results from these [^{35}S]GTPγS binding experiments are summarised in Table 3.4.

Table 3.4. Modulation by CPA or CPT of basal [^{35}S]GTPγS binding to the unfused adenosine A_1 receptor and various A_1-$G_i\alpha$ fusion proteins (^{351}Cys, ^{351}Gly, ^{351}Ile, ^{351}Pro, and ^{351}Val). EC_{50}/IC_{50} values are expressed with 95% confidence limits (n = 3). Effects of CPA and CPT are expressed as percentage of basal [^{35}S]GTPγS binding. Basal [^{35}S]GTPγS binding on membranes with the A_1-$G_i\alpha$ fusion proteins was ~600 cpm and ~1600 cpm in experiments with CPA and CPT, respectively. Basal [^{35}S]GTPγS binding on membranes expressing the unfused adenosine A_1 receptor was ~800 cpm for both CPA and CPT.

construct	CPA		CPT	
	EC_{50} (nM)	effect (% of basal)	IC_{50} (nM)	effect (% of basal)
unfused	0.25 (0.15-0.43)	165	14 (7-27)	75
A_1-G_i ^{351}Cys	3.74 (2.55-5.50)	744	91 (71-117)	52
A_1-G_i ^{351}Gly	49.2 (36.8-65.9)	580	148 (89-244)	84
A_1-G_i ^{351}Ile	5.94 (4.40-8.01)	1014	89 (41-192)	51
A_1-G_i ^{351}Ile+PTX	6.03 (5.71-6.36)	996	n.d.	49
A_1-G_i ^{351}Pro	1.70 (1.56-1.86)	437	n.d.	76
A_1-G_i ^{351}Val	9.03 (8.23-9.90)	967	n.d.	55

n.d. not determined

First, the extent of the CPA-induced stimulation of basal [^{35}S]GTPγS binding differed for the various membrane preparations. Membranes expressing the unfused adenosine A_1 receptor showed only a modest effect of CPA, i.e. 165% stimulation over basal [^{35}S]GTPγS binding, whereas the membrane preparations with the selected A_1-$G_i\alpha$ fusion proteins all had larger CPA-induced increases in basal [^{35}S]GTPγS binding (Table 3.4). Similar observations were made when the inverse agonist, CPT, was present. In all cases, CPT decreased basal [^{35}S]GTPγS binding, and to a different extent depending on the A_1-$G_i\alpha$ membrane preparation used. The CPT-induced decrease of basal [^{35}S]GTPγS binding was most prominent in experiments with COS-7 cell membranes expressing A_1-$G_i\alpha$ fusion protein ^{351}Cys or ^{351}Ile, i.e. a reduction to 52 and 51% (49% in the presence of PTX) of the basal levels, respectively. On membranes with the A_1-$G_i\alpha$ fusion protein ^{351}Val, CPT lowered basal [^{35}S]GTPγS binding to 55% of control values. Finally, the inhibitory effect of CPT on basal [^{35}S]GTPγS binding measured at the A_1-$G_i\alpha$ fusion proteins, ^{351}Gly and ^{351}Pro, or at unfused adenosine A_1 receptors, was less pronounced (Table 3.4).

Discussion

In 1994, the first fusion protein between the β_2-adrenoceptor and its cognate G_s α-subunit was described[2]. The authors showed that this fusion protein (β_2-$G_s\alpha$), when expressed in S49 cyc$^-$ cells, was functional in both ligand binding experiments and cAMP determinations. Since then, fusion proteins have been engineered between various receptors (e.g. α_{2A}-adrenoceptor, adenosine A_1, serotonin 5-HT_{1A} receptor) and G proteins (e.g. $G_{i1}\alpha$, $G_o\alpha$) (for review see Milligan)[15]. Because of the physical connection between receptor and G α-subunit, fusion proteins may resemble the precoupled receptor-G protein complex (RG and/or R*G) (Lefkowitz et al., and references therein)[16,17]. These R-G_α fusion proteins may also enable the examination of receptor interactions in a system with a fixed R:G (1:1) ratio.

As outlined in the introduction the adenosine A_1 receptor has also been linked to G protein α-subunits. Interestingly, this receptor subtype has been shown to be a target for allosteric modulation, a relatively new concept in receptor theory. Agonist binding can be enhanced (PD81,723) or inhibited (e.g., by sodium ions) in an allosteric manner (Van der Klein et al., Kourounakis et al. and references therein)[8,10]. Constitutive activity, another recent receptor concept, has also been demonstrated at adenosine A_1 receptors, but only at high levels of receptor expression and with a relatively poor “window”[18]. Therefore, we decided to use this new instrument of fusion proteins to study allosteric modulation and constitutive activity/inverse agonism further.

In the present and other studies, mutated G α-subunits were used. As demonstrated by Molinari et al. R-G_α fusion proteins might also couple to and activate endogenously expressed G proteins[19]. Treatment with pertussis toxin (PTX) assures that receptor activation arises solely through a mutated G α_i-subunit of the fused R-G construct, since the α-subunits of endogenously expressed G proteins are ADP-ribosylated by the toxin, and hence inactivated. Interestingly, we did not find significant differences in modulation of [^{35}S]GTPγS binding between PTX-treated and untreated membranes of COS-7 cells transfected with A_1-$G_i\alpha$ (^{351}Ile) fusion proteins (Table 3.4). In our hands, it thus seemed that the fusion proteins hardly activated endogenously expressed G α-subunits, if at all. Therefore, we did not use PTX-treated membranes in our further experiments.

Saturation experiments with the radiolabelled antagonist/inverse agonist [^{3}H]DPCPX taught us that its affinity was similar for the fusion proteins and the adenosine A_1 receptor itself (see Table 3.1 and text in Results section). Consequently, we found no significant differences in the IC_{50} values of DPCPX determined in radioligand binding displacement studies with COS-7 membranes expressing the various A_1-$G_i\alpha$ fusion proteins (control column in Table 3.3). On the other hand, the affinity of CPA was higher for most A_1-$G_i\alpha$ fusion proteins compared to the unfused adenosine A_1

receptor (436 nM, Table 3.2). These findings are in line with the idea that fusion proteins of this nature resemble a precoupled state of the natural complex between receptor and G protein, for which agonists have an increased affinity, whereas antagonists/inverse agonist binding is virtually undisturbed. The relatively low affinity of CPA for the unfused receptor may be due to the low abundance of endogenous G proteins in COS-7 cell membranes, such that a precoupled complex hardly occurs.

After this characterization of the fusion proteins under control conditions we next examined allosteric modulation of ligand binding to the mutated A_1-$G_i\alpha$ fusion proteins by sodium ions and PD81,723, as our first objective of this study.

In the presence of 1 M NaCl, CPA's affinity for the unfused adenosine A_1 receptor decreased three-fold (Table 3.2). Sodium ions act through an aspartate residue in transmembrane helix II, which is highly conserved in GPCRs[20]. Their interaction with this aspartate residue, Asp^{55} in the human adenosine A_1 receptor, presumably induces a change in receptor conformation towards the inactive R state. Moreover, mutation of this residue into an asparagine (Asp^{55}Asn) diminished the sodium-induced decrease in agonist affinity[21]. In the latter study it was also shown that concentrations of NaCl lower than 1 M also affect agonist binding, although less outspoken. Remarkably, the affinity of CPA for most of the A_1-$G_i\alpha$ fusion proteins was unaffected by sodium ions. Although the adenosine A_1 receptor was not mutated in the A_1-$G_i\alpha$ fusion proteins, sodium ions appeared unable to elicit their effect. 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[17]. The latter explanation may make more sense, since the same concentration of NaCl did affect DPCPX binding at the wild-type receptor and at many of the fusion proteins (Table 3.1 and Table 3.3). Apparently, the binding of the antagonist/inverse agonist DPCPX remains sensitive to sodium ions. Surprisingly, sodium ions were able to decrease CPA binding at ^{351}Pro and ^{351}Arg A_1-$G_i\alpha$ fusion proteins (Table 3.2). Although speculative, the distinct characteristics of proline (ring structure) and arginine (positive charge) residues may have more profound effects on the conformation of the $G_i\alpha$-subunit, and of the fusion protein as a whole.

The effects of 10 μM PD81,723 on ligand binding were rather similar for both the unfused adenosine A_1 receptor and the different A_1-$G_i\alpha$ fusion proteins. In the presence of PD81,723, CPA's affinity for the unfused receptor increased four-fold. The allosteric modulator increased the affinity of CPA for the various A_1-$G_i\alpha$ fusion proteins to a varying extent, namely two- to six-fold (Table 3.2). It should be noted here that in the presence of 10 μM PD81,723, the affinity of CPA for the unfused

receptor was lower compared to its affinity for the different A_1-$G_i\alpha$ fusion proteins under similar conditions. Thus, PD81,723 is still able to enhance CPA's binding to the adenosine A_1 receptor when the $G_i\alpha$-subunit is physically linked to it. The molecular mechanism of action for PD81,723 has not been resolved yet, but these findings suggest that PD81,723 has a direct effect on the receptor itself and, subsequently, shifts the receptor equilibrium towards a more activated form (R*) of the receptor[17]. The binding of DPCPX (Table 3.3) was not very much affected by PD81,723, suggesting that the antagonist/inverse agonist binding is relatively insensitive to the presence of an allosteric enhancer of agonist binding.

The second objective of this research was to investigate whether these A_1-$G_i\alpha$ fusion proteins may be used to detect inverse agonism at adenosine A_1 receptors more easily. To observe inverse agonistic activity of e.g., DPCPX, a sufficient basal receptor activity is required[11]. Possibilities to increase basal receptor activation include receptor overexpression[18], or the design of constitutively active mutant receptors[22]. Assuming that these A_1-$G_i\alpha$ fusion proteins resemble a 'precoupled' and, therefore, an active receptor conformation, we reasoned that basal receptor activity of A_1-$G_i\alpha$ fusion proteins should be higher than the basal activity of unfused adenosine receptors. We indeed found far higher basal [^{35}S]GTPγS binding for all A_1-$G_i\alpha$ fusion proteins tested in [^{35}S]GTPγS binding experiments. Despite lower receptor densities (see Table 3.1 and text in Results section), membranes expressing A_1-$G_i\alpha$ fusion proteins showed more than seven-fold higher basal [^{35}S]GTPγS binding than that found in membranes with the unfused receptor, approx. 600 and 80 cpm/μg of protein, respectively. Moreover, for all A_1-$G_i\alpha$ fusion proteins tested, the maximal stimulation of basal [^{35}S]GTPγS binding was significantly larger than the maximal CPA-induced effect with unfused adenosine A_1 receptors (Table 3.4). For instance CPA caused a 14-fold increase [(1014%-100%)/(165%-100%)] in stimulation on the ^{351}Ile fusion protein compared to the unfused receptor. The same was observed for inhibition of basal [^{35}S]GTPγS binding by the adenosine A_1 receptor inverse agonist CPT. This compound decreased basal [^{35}S]GTPγS binding to the ^{351}Ile and ^{351}Cys fusion proteins very effectively (-49 and -48% vs. –25% for the unfused receptor expressed in COS-7 cells). Differences in absolute numbers are also substantial. We used up to 10-fold less protein when studying the fusion constructs, implicitly showing the enormous increase in "window".

The maximal stimulation and inhibition of basal [^{35}S]GTPγS binding appeared to depend on the nature of the amino acid at position 351 of the $G_i\alpha$-subunit (Ile > Val > Cys > Gly > Pro > unfused receptor, for agonist activation). These observations are in line with the report by Bahia et al.[23]. They found a good correlation between the hydrophobicity of the amino acid at this position and the maximal activation of the porcine α_{2A} adrenoceptor. Note that the authors co-expressed the mutated $G_i\alpha$

proteins and the α_{2A} adrenoceptor, while we used A_1-$G_i\alpha$ fusion proteins. Here, we show that their conclusions regarding the correlation mentioned above were also applicable to our mutated A_1-$G_i\alpha$ fusion proteins.

Conclusions

In the present study we analysed fusion proteins between the adenosine A_1 receptor and (mutated) G protein α_i-subunits with respect to two novel concepts in receptor pharmacology, i.e. allosteric modulation and constitutive activity.

Allosteric modulation of ligand binding was not always similar for A_1-$G_i\alpha$ fusion proteins compared to the unfused adenosine A_1 receptor. In most cases, sodium ions were unable to decrease CPA binding to membranes expressing A_1-$G_i\alpha$ fusion proteins. Their lack of effect may result from a change in receptor conformation induced by the coupling of the $G_i\alpha$-subunit. In contrast, allosteric modulation by PD81,723 was comparable for the unfused adenosine A_1 receptor and the A_1-$G_i\alpha$ fusion proteins. Thus, PD81,723 is able to shift the 'fusion' receptor equilibrium further towards R*G.

We also demonstrated that fusion proteins between the human adenosine A_1 receptor and various ^{351}Cys-mutated rat $G_i\alpha$ subunits can be used as a research tool to investigate inverse agonism at adenosine A_1 receptors. Despite lower receptor densities, basal [^{35}S]GTPγS binding to COS-7 membranes expressing A_1-$G_i\alpha$ fusion proteins was higher than in membranes with the unfused adenosine A_1 receptor. In addition, proper substitution of ^{351}Cys, for instance into an isoleucine residue, increased the readout window of CPA-stimulated [^{35}S]GTPγS binding substantially. The same fusion protein (^{351}Ile A_1-$G_i\alpha$) also showed increased inhibition of basal [^{35}S]GTPγS binding induced by CPT.

In conclusion, fusion proteins between GPCRs and their G proteins may be subject to allosteric modulation. They emerge as very useful research tools in the study of constitutive activity and inverse agonism.

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CHAPTER 4

Allosteric modulators affect the internalization of human adenosine A_1 receptors

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To study the effect of allosteric modulators on the internalization of human adenosine A_1 receptors, the receptor was equipped with a C-terminal yellow fluorescent protein tag. The introduction of this tag did not affect the radioligand binding properties of the receptor. CHO cells stably expressing this receptor were subjected during 16 h to varying concentrations of the agonist *N*^{6}-cyclopentyladenosine (CPA) in the absence or presence of 10 µM of the allosteric enhancer PD81,723 ((2-amino-4,5-dimethyl-3-thienyl)-[3-(trifluoromethyl)phenyl]methanone) or SCH-202676 (*N*-(2,3-diphenyl-1,2,4-thiadiazol-5(2*H*)-ylidene)methanamine). CPA itself was able to internalize 25% and 40% of the receptors at a concentration of 400 nM or 4 µM, respectively. Addition of either PD81,723 or SCH-202676 alone had no effect on internalization. However, with PD81,723 a slight amount of internalization was obtained already at 40 nM of CPA, and at 400 nM CPA 59% of the receptors internalized. SCH-202676 on the other hand effectively prevented CPA-induced internalization of the receptor.

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Introduction

Adenosine receptors are members of the superfamily of G protein-coupled receptors (GPCRs) and are pharmacologically classified into four distinct types, namely the adenosine A_1, A_{2A}, A_{2B} and A_3 receptor. Adenosine A_1 and A_3 receptors are coupled to a G_i protein, thereby inhibiting the production of cAMP via adenylyl cyclase. In contrast, adenosine A_{2A} and A_{2B} receptors are coupled to a G_s protein, thereby stimulating the production of cAMP (for review on adenosine receptors, see Fredholm et al.)[1].

Like other members of the GPCR family, adenosine receptors are subject to allosteric modulation (for reviews on allosteric modulation, see Soudijn et al.; Christopoulos and Kenakin)[2,3]. PD81,723 ((2-amino-4,5-dimethyl-3-thienyl)-[3-(trifluoromethyl)phenyl]-methanone) is an allosteric enhancer selectively exerting its action on the adenosine A_1 receptor. It potentiates the agonist binding, thereby enhancing the functional effects of adenosine or its analogues[4]. It has also been reported that PD81,723 potentiates constitutive activity of the adenosine A_1 receptor[5]. SCH-202676 (*N*-(2,3-diphenyl-1,2,4-thiadiazol-5(2*H*)-ylidene)methanamine) on the other hand inhibits both agonist and antagonist binding to a number of GPCRs such as human μ-, δ-, and κ-opioid, α- and β-adrenergic, muscarinic M_1 and M_2, and dopaminergic D_1 and D_2 receptors[6], including adenosine receptors[7,8]. Recent studies revealed that SCH-202676 and analogues are not strict allosteric modulators as originally thought, but rather sulfhydryl modifying agents that reversibly modify cysteine residues in proteins[9,10].

Adenosine receptors, like other GPCRs, undergo internalization upon agonist stimulation. Internalization or sequestration is described as the loss of cell surface receptor number, determined by the combined effects of endocytosis and recycling[11]. In the present study, we focus on the internalization of the human adenosine A_1 receptor. The process of internalization has been shown to be initiated by the functional desensitization of the adenosine A_1 receptor. After a short period of agonist exposure (5-15 min), the adenosine A_1 receptors uncouple from the G_i proteins due to phosphorylation, catalyzed by specific receptor kinases[12-15]. Especially serine and threonine residues close to the C-terminus of the receptor are susceptible to phosphorylation[12,16]. Upon phosphorylation of the adenosine A_1 receptor, β-arrestins are attracted, which couple to the phosphorylated receptor. β-arrestins do not only desensitize the receptor, but also function as clathrin adaptors thereby inducing the process of sequestration and internalization. Although phosphorylation of the adenosine A_1 receptor returned to its basal state within minutes, the desensitization continued for hours. Unlike the uncoupling, $t_{1/2}$ for the internalization process of the adenosine A_1 receptor is quite slow, 10 ± 1 h[14]. In contrast, $t_{1/2}$ for internalization of the other G_i coupled adenosine receptor (A_3 receptor) is short, only 10 min[16].

In the present study, we have examined whether allosteric modulators are able to influence the internalization of the human adenosine A_1 receptor. To visualize this process, we engineered a yellow fluorescent protein (YFP) C-terminal of the human adenosine A_1 receptor. Our findings indicate that the presence of the allosteric enhancer PD81,723 lowers the threshold of agonist concentration at which receptor internalization occurs. In contrast, the presence of SCH-202676 almost completely prevented internalization of the human adenosine A_1 receptor.

Materials and methods

Materials

N^6-cyclopentyladenosine (CPA) was obtained from Research Biochemicals Inc. (Natick, MA, U.S.A.). 8-cyclopentyl-1,3-cylcopentyladenosine ([^{3}H]DPCPX -specific activity 124 Ci/mmol) was purchased from NEN (Du Pont Nemours, 's Hertogenbosch, The Netherlands). G418 (neomycin) was obtained from Stratagene (Cedar Creek, U.S.A.). (2-amino-4,5-dimethyl-3-thienyl)-[3-(trifluoromethyl)phenyl]methanone (PD81,723) and (*N*-(2,3-diphenyl-1,2,4-thiadiazol-5(2*H*)-ylidene)methanamine (SCH-202676) were synthesized in our own laboratory as described by Van der Klein et al.[18] and Van den Nieuwendijk et al.[7] respectively. All other chemicals were of analytical grade and obtained from standard commercial sources.

Construction of human adenosine A_1YFP receptor

The human adenosine A_1 receptor cDNA (1.3 kb) was isolated from pcDNA3 (Invitrogen BV, Breda, The Netherlands) and inserted into the pBluescript II SK (-) vector (Stratagene, Cedar Creek, USA) using *Hind*III/*Xba*I sites. A reverse primer was designed to mutate the stop codon, introduce an extra base and an *Apa*I site at the 3' end: 3' gggtcttctctccggactactgc<u>cccggg</u>cgc 5' (*Apa*I restriction site is underlined). The human adenosine A_1 receptor cDNA was amplified by a polymerase chain reaction (PCR) using a universal forward primer (un-4) for pBlueScript II SK(-) and the designed reverse primer. The PCR product was purified on gel and subcloned into the peYFP-N1 vector digested with *Hind*III/*Apa*I (kindly provided by C. Backendorf, Leiden University), resulting in a human adenosine A_1YFP receptor construct.

Cell culture

CHO cells were cultured in a humidified atmosphere at 37 °C and 5% CO_2 in a 1:1 mixture of Dulbecco's Modified Eagle Medium (DMEM) and Ham's F12 medium, containing 10% newborn calf serum, 50 IU/ml penicillin, 50 µg/ml streptomycin and

0.8 µg/ml G418 for selection. CHO cells were stably transfected with the human adenosine A_1YFP receptor construct, using 1mg/ml *N*-(2,3-dioleoyloxy-1-propyl)trimethylammonium methyl sulfate (DOTAP) as described by Beukers et al.[19]. In short, 5 x 10^4 cells per well (24 wells plate) were seeded the day before transfection. For each well, 2.3 µL DOTAP (1 mg/ml) plus 0.7 µg human adenosine A_1YFP receptor cDNA was added to 50 µL DMEM without serum. Liposomes were formed during 20 min at room temperature. Cells were washed twice with DMEM without serum, next 50 µL DMEM without serum was added followed by the transfection mix. Cells were left for 2 h at 37 °C, 5% CO_2. Subsequently, the cells were washed with phosphate buffered saline (PBS) and treated with 5% Dimethyl Sulfoxide (DMSO) in PBS for 3 min. The DMSO mixture was removed and 500 µL of a 1: 1 mixture of DMEM/F12 medium, containing 10% newborn calf serum, 2 mM glutamax, 50 IU/ml penicillin and 50 mg/ml streptomycin was added per well. G418 (0.8 mg/ml) was added to the medium to select for cells that had taken up the plasmid. The medium was replaced every other day. After 10 days, individual colonies were selected and transferred to separate wells in a 24 wells plate. Radioligand binding experiments were performed to select a clone with a sufficiently high expression level of the human adenosine A_1YFP receptor. This clone was used for internalization experiments. Cells were subcultured twice a week (1:40).

Preparation of cell membranes

CHO cells stably expressing the wt human adenosine A_1 receptor were obtained from A. Townsend-Nicholson[20]. Confluent CHO cells expressing either the wt human adenosine A_1 receptor or the human adenosine A_1YFP receptor were scraped in PBS and centrifuged for 10 min at 250 g. The cell pellets were resuspended in ice-cold Tris-HCl buffer, 50 mM, pH 7.4, and homogenized on ice for 5 s at position 8 using an Ystral homogenizer. The homogenates were centrifuged for 45 min at 17000 g at 4 °C. The resulting pellets were resuspended in Tris-HCl buffer, 50 mM, pH 7.4, and 2 IU/ml adenosine deaminase was added. Aliquots were stored at –80 °C. The protein concentration of the membranes was measured using the BCA method[21].

Receptor binding assays

Radioligand displacement experiments were performed with minor modifications as described previously for the wt human adenosine A_1 receptor in CHO cells[22]. In brief, membranes (10 µg protein) were incubated in 400 µL Tris-HCl buffer, 50 mM, pH 7.4, in the presence or absence of 10 µM PD81,723 or 10 µM SCH-202676 and in the presence of 1.6 nM [^{3}H]DPCPX and increasing concentrations of CPA. Non-specific binding was determined in the presence of 1×10^{-4} M CPA. For saturation experiments, concentrations of [^{3}H]DPCPX ranged from 0.1 to 10 nM. Samples were

incubated at 25 °C for 1 h in a shaking water bath and the incubation was terminated by adding 1 ml of ice-cold Tris-HCl, 50 mM, pH 7.4 followed by rapid filtration over Whatman GF/B glass-fiber filters. Filters were washed three times with 2 ml of ice cold Tris-HCl buffer and placed in scintillation vials. For saturation experiments, filters were washed six times with 2 ml of ice cold Tris-HCl buffer. Scintillation fluid (Emulsifier Safe, Packard BioScience), 3.5 ml, was added and after 2 h extraction, radioactivity was counted in an LKB Wallac 1219 rackbeta scintillation counter. Experiments were performed in triplicate.

Internalization experiments

CHO cells stably expressing the human adenosine A_1YFP receptor were plated on coverslips in 24 wells plates at a density of 3 x 10^4 cells/well in a volume of 0.5 ml. The cells were allowed to attach for 24 h, after which the medium was aspirated and the cells were exposed for 16 h to 0 nM, 40 nM, 400 nM, 4 µM CPA in the presence or absence of 10 µM PD81,723 or 10 µM SCH-202676. Cells were washed once with 0.5 ml PBS, and fixed with 0.5 ml 4.0% formaldehyde (pH 7.0-7.2) for 10 min at room temperature. Coverslips containing the cells were washed another 3 times with 0.5 ml PBS and subsequently mounted on object glasses using Aqua Polymount® (Polysciences). Coverslips were allowed to dry on air in darkness, and stored at 4 °C for short term storage or –20 °C for long term storage. Images of the incubated cells were obtained using confocal microscopy (Nikon Eclipse TE 2000-U), excitation at 520 nm, emission at 532 nm, 60x oil enlargement.

Quantification of internalization

The computer program Image Pro Plus (MediaCybernetics, Germany) was used to quantify the amount of fluorescence in representative transsections of cells. The amount of fluorescence in the cell membrane was determined and expressed as percentage of the total fluorescence and was corrected for the differences in surface area of the transsection. Transsections of at least three different cells were analyzed.

Quantification of internalization through radioligand binding

In addition, the amount of internalization was quantified with help of radioligand binding experiments. Therefore, confluent 10 cm plates of CHO cells stably expressing the human adenosine A_1YFP receptor were incubated with or without 4 µM CPA for 16 hrs. Both were washed once with 5 ml PBS and subsequently scraped in 5 ml PBS. The preparation of the membranes was performed as described in section 2.4. The only modification was that the homogenates were centrifuged for 2 x 20 min at 17000 g at 4 °C. Radioligand binding studies were

performed on membranes of the control cells as well as the exposed cells as described in section 2.5.

Data analysis

Data of radioligand binding- and saturation experiments were analysed using the non-linear regression curve fitting program Prism v. 3.0 (GraphPad, San Diego, CA, USA). Apparent inhibitory binding constants (K_i values) were derived from the IC_{50} values according to the Cheng and Prusoff equation $K_i = IC_{50}/(1 + [L^*]/K_D)$ where [L*] is the concentration of the radioligand and K_D its dissociation constant[23].

Results

Binding profile of wt human adenosine A_1 receptors and human adenosine A_1YFP receptors, stably expressed in CHO cells.

First, we assessed the affinity and binding capacity of the radioligand [^{3}H]DPCPX for the human adenosine A_1YFP receptor. In Table 4.1, the K_d and B_{max} values of [^{3}H]DPCPX for the human adenosine A_1YFP receptor in the presence or absence of PD81,723 and SCH-202676 are shown. The K_d value of [^{3}H]DPCPX for the human adenosine A_1YFP receptor was hardly affected by the addition of PD81,723 and SCH-202676. The B_{max} value was slightly but not significantly lowered in the presence of PD81,723. Subsequently, radioligand binding studies were performed on membranes of human adenosine A_1YFP-CHO cells to characterize the binding properties of the newly constructed human adenosine A_1YFP receptor. In Figure 4.1, displacement curves for the human adenosine A_1YFP receptor are shown. Displacement experiments were performed using the reference agonist CPA alone, and CPA in the presence of 10 µM PD81,723 or 10 µM SCH-202676.

Table 4.1. Saturation parameters of [^{3}H]DPCPX on CHO-hA_1YFP cell membranes

Compound	K_d (nM)	B_{max} (fmol/mg)
Control	1.45 ± 0.05	862 ± 50
+ PD81,723 (10 µM)	1.51 ± 0.15	669 ± 55
+ SCH-202676 (10 µM)	1.21 ± 0.09	805 ± 127

B_{max} and K_d-values of [^{3}H]DPCPX in the absence or presence of PD81,723 (10 µM) or SCH-202676 (10 µM) on CHO-hA_1YFP cell membranes were determined. Values are the means (± S.E.M.) of three independent experiments, performed in duplicate. None of the determined values is significantly different compared to the control values, $P > 0.05$.

[^{3}H]DPCPX was used as radioligand. The binding properties of the human adenosine A_1YFP receptors were compared with the binding profile of the wt human adenosine A_1 receptors. Like the wt human adenosine A_1 receptor, the human adenosine A_1YFP shows a biphasic behaviour towards CPA binding, and is also susceptible to allosteric modulation. PD81,723, the allosteric enhancer, shifts the CPA-curve to the left (gain of affinity) whereas in the presence of SCH-202676 the CPA-curve is shifted to the right (loss of affinity). In addition, the high affinity state of the human adenosine A_1YFP receptor was shifted to the low affinity state in the presence of SCH-202676. K_i-values of CPA for the human adenosine A_1YFP receptor in the presence or absence of PD81,723 and SCH-202676 are shown in Table 4.2. The shifts in the K_i-values of CPA were 0.24 and 4.0 for the human adenosine A_1YFP receptor in the presence of PD81,723 or SCH-202676, respectively.

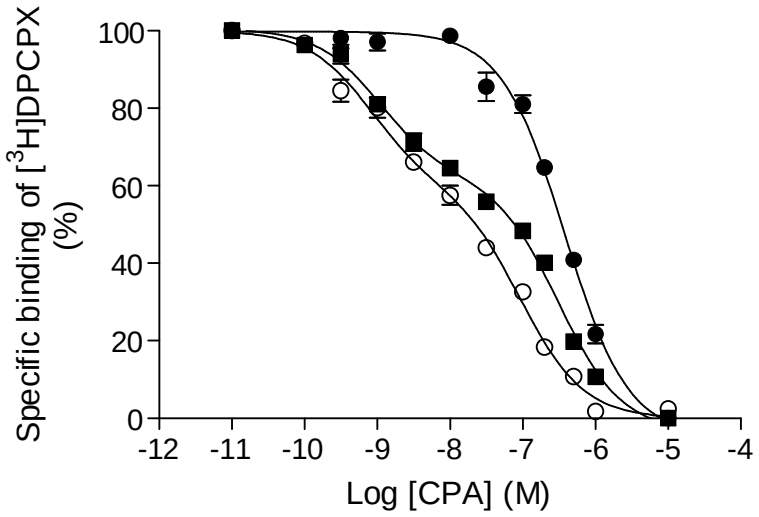

Figure 4.1. Displacement of [^{3}H]DPCPX with CPA (■) only, or in the presence of 10 µM PD81,723 (○) or 10 µM SCH-202676 (●). Membranes (10 µg) were incubated for 60 min at 25 °C as described in section 2.5. The graph is the mean of three displacement curves of the specific binding of [^{3}H]DPCPX to CHO-hA_1YFP membranes, each performed in duplicate.

Table 4.2. K_i values of CPA in the presence or absence of 10 µM PD81,723 or 10 µM SCH-202676 for hA_1YFP receptors expressed in CHO cells.

	CHO-A_1YFP				
K_i low (nM)	K_i low (nM)	K_i high (nM)	*Fraction high (%)*	K_i (nM)	*Shift*
CPA	154 ± 9.5	0.61 ± 0.20	*37 ± 2.0*	42.5 ± 7.2	
CPA + PD81,723	47 ± 7.8	0.40 ± 0.12	*40 ± 5.4*	10.4 ± 3.3	*0.24*
CPA + SCH-202676				172 ± 28	*4.0*

The affinity of CPA for the hA_1YFP-receptors in the presence or absence of different modulators was determined by its displacement of [^{3}H]DPCPX binding. Membranes (10 µg) were incubated for 60 min at 25 °C with CPA (in the presence or absence of PD81,723 and SCH-202676 as described in section 2.5. K_i values ± S.E.M. were calculated from three independent experiments. Shifts were calculated by dividing the K_i value in the presence of allosteric modulator by the K_i-value of CPA alone. To calculate the K_i values, the respective K_d values (Table 4.1) were used.

Internalization of the human adenosine A_1YFP receptors

The internalization of human adenosine A_1YFP receptors induced by CPA, and the influence of PD81,723 and SCH-202676 on this process, was investigated. First, we determined the time-course needed to observe proper internalization. We incubated CHO cells stably expressing the human adenosine A_1YFP receptor at increasing CPA concentrations for the following time points; 0, 2, 4, 6, 8, 16, 24 hours, and observed them with help of fluorescence microscopy. From these experiments it appeared that clear internalization was only observed after at least 16 hours of incubation (results not shown), which is in agreement with the fact that the $t_{1/2}$ for the internalization process of the adenosine A_1 receptor is 10 ± 1 h[14]. Additionally, we observed that the presence of the allosteric modulator PD81,723 (10 µM) did not accelerate the occurrence of internalization, but only increased the amount of internalized receptors (data not shown).

Subsequently, human adenosine A_1YFP-CHO cells were plated on coverslips and incubated for 16 hours with CPA in a concentration range of 0 nM, 40 nM, 400 nM and 4 µM, in the presence or absence of 10 µM PD81,723 or 10 µM SCH-202676. Several confocal images were made from each incubation, and representative transsections are shown in Figure 4.2. Control human adenosine A_1YFP-CHO cells show a clear, equally distributed membrane staining. The lowest CPA concentration (40 nM, approximate K_i-value) did not induce internalization of the human adenosine A_1YFP receptor. At 400 nM CPA (approx. 10 × K_i), internalization of the human adenosine A_1YFP receptor was apparent as green fluorescent dots in the cytoplasm. This effect was more distinct at the highest concentration CPA (4 µM, approx. 100 × K_i). In the presence of the allosteric enhancer PD81,723 (10 µM) at the lowest CPA concentration (40 nM), membrane staining was not as equally distributed as in the control cells. The internalization of the human adenosine A_1YFP receptor at CPA concentrations of 400 nM and 4 µM in the presence of PD81,723 was more substantial than internalization with CPA alone. Incubation of human adenosine A_1YFP-CHO cells in the presence of SCH-202676 (10 µM) almost completely prevented the internalization of the human adenosine A_1YFP receptors. No internalization was observed, except for the highest concentration of CPA (4 µM) where SCH-202676 was not able to completely prevent the internalization of the human adenosine A_1YFP receptors. PD81,723 or SCH-202676 alone did not induce internalization.

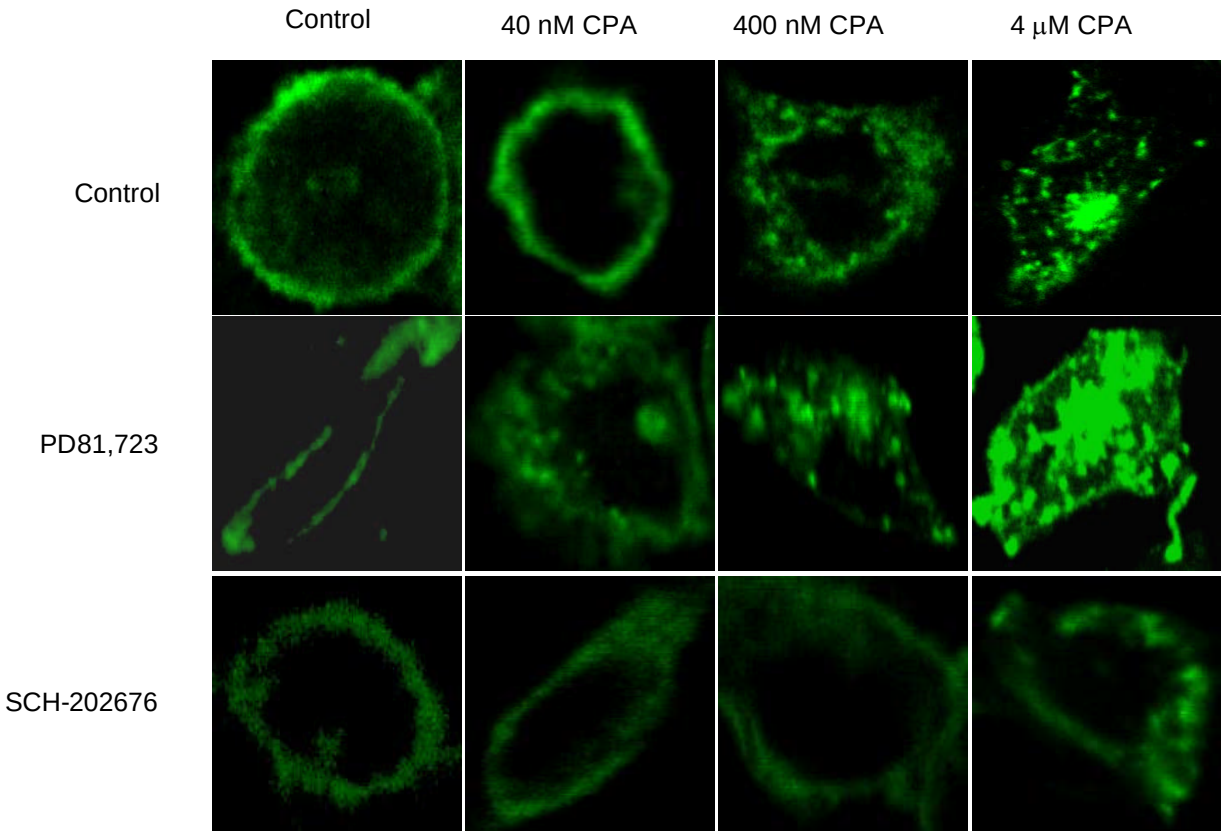

Figure 4.2. Confocal fluorescent pictures of internalized hA_1YFP receptors in CHO cells. hA_1YFP CHO cells were incubated for 16 hours with increasing concentrations of CPA in the presence or absence of PD81,723 (10 µM) and SCH-202676 (10 µM). Pictures were taken from a representative transsection of the cell.

Quantitative analysis of internalization

The amount of receptor internalization was quantified with help of the computer program Image Pro Plus. In Figure 4.3, the remaining fluorescence in the cell membrane is represented as a bar graph. At least three transsections of independent cells were quantified. Control human adenosine A_1YFP-CHO cells as well as cells exposed to either one of the compounds showed between 84% and 88% fluorescent membrane staining. The 40 nM CPA incubation showed only 11% internalization in the presence of 10 µM PD81,723, the membrane staining was reduced from 88% to 77%. However, this reduction was not significant. CPA alone (40 nM) and in the presence of SCH-202676 (10 µM) showed values comparable to control cells for membrane staining (resp. 87% and 84%). Exposure to 400 nM CPA reduced the membrane staining from 88% to 63% (25% internalization), whereas the addition of 10 µM PD81,723 reduced the membrane staining even further to 29% (59% receptor internalization). SCH-202676 counteracted the effect of 400 nM CPA, such that no internalization was observed (90% fluorescence in the membrane). Exposure to 4 µM CPA reduced membrane staining from 88% to 48%, whereas the addition of 10 µM PD81,723 reduced this percentage to 27% (61% internalized human adenosine A_1YFP receptors), yielding approximately the same amount of internalized receptors

as obtained after incubation with 400 nM CPA plus PD81,723. Incubation with 4 µM CPA in the presence of 10 µM SCH-202676 led to a marginal, non-significant, reduction of membrane staining from 84% to 74%.

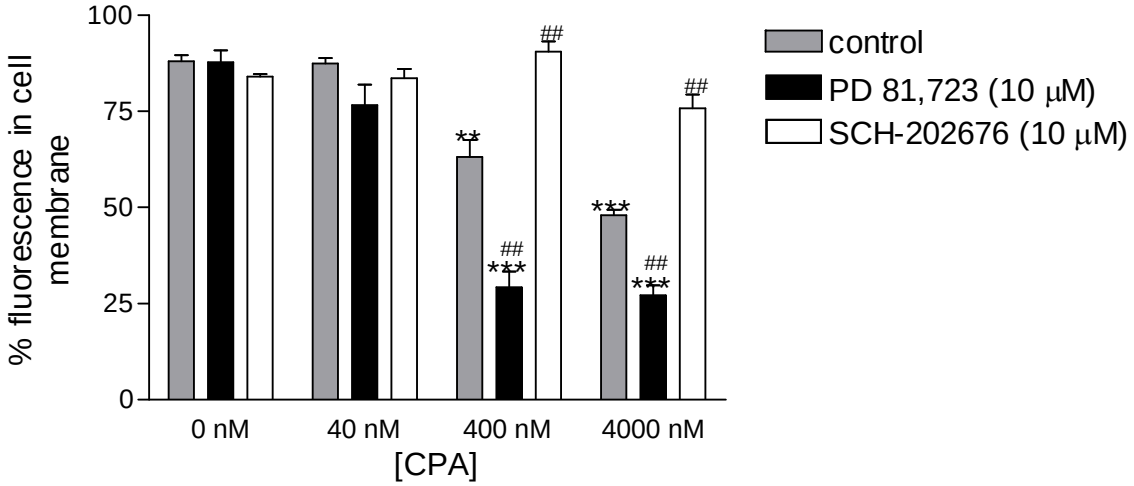

Figure 4.3. Quantitative analysis of the internalization of human adenosine A_1YFP-receptors in CHO cells. This bar graph corresponds with the confocal pictures shown in figure 2. The bars represent the percentage fluorescence in the cell membrane. Transsections of at least three different cells were analyzed. Values are means ± S.E.M. ** P ≤ 0.005, *** P ≤ 0.0005, percentage fluorescence compared to the percentages fluorescence of corresponding incubations in the absence of CPA. ## P ≤ 0.005 compared to the percentage fluorescence of the respective internal control (equal CPA concentration).

Confirmation of these results was obtained by performing radioligand binding studies on plasma membranes of human adenosine A_1YFP-CHO cells, exposed to 4 µM CPA for 16 hours. The percentage of internalization measured as decrease of radioligand binding compared to control (i.e. cells treated identically but without CPA present) was 32±1%, corresponding very well to the 40% internalization measured by confocal microscopy.

Discussion

The introduction of a yellow fluorescent protein at the C-terminus of the human adenosine A_1 receptor and stable transfection of this human adenosine A_1YFP receptor into CHO cells, provided us with a tool to study internalization of the human adenosine A_1 receptor. By making the human adenosine A_1 receptor visible, there is no need to add fluorescent antibodies or gold particles as has been described before to visualize the internalization of the adenosine A_1 receptors[12,14,24-26].

Saturation experiments revealed that the addition of YFP to the C-terminus of the adenosine A_1 receptor did not affect the K_d value of [^{3}H]DPCPX for the human

adenosine A_1YFP receptor (1.45 ± 0.05 vs 1.6 ± 0.1 nM for the wt human adenosine A_1 receptor[22]. The addition of the allosteric modulator PD81,723 or SCH-202676 also hardly affected the K_d-value (1.51 ± 0.15 and 1.21 ± 0.09 nM, respectively, see Table 4.1). However, in the presence of PD81,723, the B_{max} value was slightly lower, 669 ± 55 fmol/mg vs 862 ± 50 fmol/mg for control, although these values did not differ significantly. Similar effects of PD81,723 on B_{max} and K_d values for the wt human adenosine A_1 receptor had been found by Battacharya and Linden[27] and Kollias-Baker et al.[5].

As is shown in Figure 4.1 and Table 4.2, the addition of the C-terminal YFP did not markedly influence the binding properties of the adenosine A_1 receptor. The human adenosine A_1YFP receptor showed a two state binding curve, similar as described for the wild-type human adenosine A_1 receptor[28]. The fraction of high affinity receptors was 37– 40% for the human adenosine A_1YFP receptors, stably expressed in CHO cells. Slightly higher percentages of wild-type human adenosine A_1 receptors in the high affinity state were found by Musser et al.[4] and Dalpiaz et al.[28], 52% and 74% respectively. The addition of PD81,723 did not increase the number of human adenosine A_1YFP receptors receptors in the high affinity state, in accordance with experiments by Musser et al.[4].

The affinity of CPA for the human adenosine A_1YFP receptor is in the same order of magnitude as reported in the literature for the wt human adenosine A_1 receptor. The K_i value for the low affinity state was 154 ± 9.5 nM vs 76 ± 6 nM for the wt human adenosine A_1 receptor and the K_i value for the high affinity state was 0.61 ± 0.2 nM vs 2.8 ± 0.2 nM for the wt human adenosine A_1 receptor, respectively[28]. We observed a shift in affinity caused by the addition of PD81,723 of 0.30 for the low affinity state, and of 0.66 for the high affinity state, respectively. This corresponds well with the values found by Musser et al.[4], reporting shifts for the wt human adenosine A_1 receptor of 0.29 and 0.57, respectively. The addition of SCH-202676 caused a shift towards the low affinity state, resulting in a one-site binding curve with a K_i value of 172 ± 28 nM. This is in the same order of magnitude as the K_i value found by Heitman (personal communication) for binding of CPA in the presence of SCH-202676 to the wt human adenosine A_1 receptor, 225 ± 8 nM. SCH-202676 inhibited the binding of the agonist CPA to the human adenosine A_1YFP receptor with a shift of 4. This is in accordance with the results found by van den Nieuwendijk et al., who observed that the binding of the agonist radioligand [^{3}H] 2-Cl-*N*^{6}-cyclopentyladenosine ([^{3}H]CCPA) to human adenosine A_1 receptors was reduced to only 10% in the presence of 10 µM SCH-202676[7]. Gao et al. found that the dissociation of the antagonist [^{3}H]DPCPX was decreased almost 4 times by the presence of SCH-202676[8].

These binding experiments showed us that the human adenosine A_1YFP receptor retains its pharmacological profile and that the allosteric modulator PD81,723 and SCH-202676 have the same effect on the wt and the YFP-tagged adenosine A_1 receptor.

As reviewed in Chapter 2, desensitization and presumed internalization of adenosine A_1 receptors after long term exposure to (*R*)-*N*^{6}-(2-Phenylisopropyl)adenosine (*R*-PIA) was observed earlier in other tissues such as rat brain[13], DDT_1 MF-2 cells[12,15] and rat adipocytes[29]. Human adenosine A_1 receptors, stably expressed in HEK293 cells and pretreated with 10 µM CPA for 24 h showed a downregulation of 48%[16]. In these experiments, the desensitization was quantified using radioligand binding experiments on plasma membranes. Here, we describe the internalization of the human adenosine A_1YFP receptor in CHO cells induced by different concentrations of CPA and monitored by confocal microscopy, and confirm the results with radioligand binding experiments. After 16 hours of incubation with 400 nM CPA, which is approximately 10 $\times$ K_i of CPA, 25% of the receptors were internalized. It is striking that CPA is not able to induce receptor internalization at a concentration of 40 nM, close to CPA's K_i value (42.5 nM) at which half of the receptor population is assumed to be occupied. This is in contrast to DDT_1MF-2 cells exposed to 50 nM *R*-PIA for 12-24 h, which showed clear internalization[12]. The K_i value of *R*-PIA for the adenosine A_1 receptor in DDT_1MF-2 cells is 76 nM[15]. This difference may be explained to a different regulation of receptor internalization for each cell type adenosine A_1 receptors are expressed in. Apparently, over 50% of the human adenosine A_1YFP receptors have to be occupied by CPA before the receptors start to cluster, subsequently followed by internalization. Internalization became more apparent (40%) at a concentration of 4 µM CPA (approx. 100 $\times$ K_i). Radioligand binding experiments performed on membranes of this incubation confirmed this percentage (32 ± 1%). In the presence of the allosteric enhancer PD81,723 (10 µM), however, some internalization (11%) was already observed at a concentration of 40 nM CPA. The internalization at 400 nM and 4 µM became more distinct in the presence of 10 µM PD81,723 (59% and 61% respectively). Allosteric enhancers, such as PD81,723, have the property that they bind to a site distinct from the orthosteric ligand binding site. By doing so, they change the 3D-conformation of the receptor and facilitate ligand binding. In other words, they enhance the affinity of agonists for the receptor. Our data show that this change in receptor conformation, induced by PD81,723, also enhances clustering and internalization of de human adenosine A_1YFP receptor upon agonist binding, thereby increasing the amount of internalized receptors. This results in a higher percentage of internalized receptors in the presence of PD81,723 at the same CPA concentration. The action of PD81,723

is synergistic. PD81,723 (10 µM) alone has no effect on receptor distribution. This is in accordance with Bhattacharya and Linden[27] who did not observe a decrease in membrane binding sites after 24 h of pretreatment with 20 µM PD81,723. Pretreatment of CHO cells stably expressing recombinant human adenosine A_1 receptors with either 10 µM CPA or 10 µM CPA plus 20 µM PD81,723 resulted in a loss of membrane binding sites of over 40%.
Recently, it has been shown that the enzyme adenosine deaminase (ADA) acted as a receptor activity modifying protein (RAMP) on the adenosine A_1 receptor, and stimulated *R*-PIA induced A_1 receptor internalization[24]. Thus, ADA also appears to regulate A_1 receptor function allosterically. However, the role of ADA was different compared to that of PD81,723. ADA accelerated the internalization of adenosine A_1 receptors, $t_{1/2}$ decreased from 10 h to 2.9 h[14], whereas PD81,723 lowers the treshold of agonist concentration at which internalization occurs. ADA did not increase the number of receptors which are internalized[25], whereas PD81,723 increases the percentage of internalized receptors from 40% to 61% at the highest concentration of CPA tested (4 µM).

Next to the allosteric enhancer PD81,723 we also studied SCH-202676. In contrast to PD81,723, SCH-202676 (10 µM) completely prevented the internalization of the human adenosine A_1YFP receptors at all CPA concentrations, except for 4 µM CPA. At the highest CPA concentration, some clustering of human adenosine A_1YFP receptors was observed (10%). Even at a concentration of 4 µM CPA, the percentage of occupied receptors is not high enough to induce substantial internalization due to the presence of 10 µM SCH-202676. To the best of our knowledge the effects of this modulator on receptor processing have not been addressed before.

In conclusion, in this study we present the effect of the allosteric modulator PD81,723 and SCH-202676 on the internalization of the human adenosine A_1 receptor. PD81,723, an allosteric enhancer, is able to lower the threshold of agonist concentration for inducing internalization. Already at a concentration of 40 nM CPA, some internalization is observed. On the contrary, SCH-202676 is a strong inhibitor of agonist binding, thereby preventing internalization of the human adenosine A_1YFP receptors.

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CHAPTER 5

Structure-activity relationships of 2-amino-4-(substituted)phenyl-6-(substituted)sulfanyl-pyridine-3,5-dicarbonitriles reveal full agonists with picomolar affinity for the human adenosine A_1 receptor

Recently, we reported on selective agonists for human adenosine A_1 receptors with an unusual structure lacking the ribose moiety of traditional adenosine-like agonists. The synthesis, affinity and activity of twelve such non-ribose ligands, all 2-amino-4-(3 and/or 4-disubstituted phenyl)-6-(substituted)sulfanyl-pyridine-3,5-dicarbonitriles, are described in this study. The substitution pattern of the phenyl ring determined the affinity of the compounds for the adenosine A_1 receptor, whereas efficacy was determined by the substituents on both the phenyl ring and the sulfanyl chain.
The di-methoxyphenyl substituted compounds had very little affinity. The monosubstituted compounds recognized one binding state/site with higher nanomolar affinity. cAMP second-messenger 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 N^6-cyclopentyladenosine (CPA) in affinity and potency.

Based upon Klaasse EC, Roerink SF, van Veldhoven JPD, von Frijtag Drabbe Künzel JK, de Grip WJ, Brussee J, IJzerman AP, Beukers MW. Manuscript in preparation.

Introduction

Traditionally agonists for adenosine receptors are structurally related to the endogenous ligand adenosine, consisting of an adenine base coupled to a ribose moiety. In the search for selective, high affinity agonists for the human adenosine A_1 receptor, adenosine has been substituted at different positions. Especially the introduction of a hydrophobic substituent such as cyclopentyl at the N^6-position resulted in potent adenosine A_1 agonists[1].

We recently learned from patent literature about a new class of adenosine ligands, the 2-amino-4-(3,4-disubstituted-phenyl)-6-(2-hydroxyethylsulfanyl)-pyridine-3,5-dicarbonitriles, structurally unrelated to adenosine itself[2,3]. We synthesized a number of such compounds ourselves and observed that several compounds displayed a significant affinity and efficacy towards different adenosine receptor subtypes.[4,5]. In our hands it turned out that the substitution pattern at the phenyl ring R^1 greatly affected the efficacy of the compounds (for structures see Table 5.1). But what would happen to the affinity and efficacy of the compounds for the human adenosine A_1 receptor if the ethanol substituent R^2 would be modified? To address this issue, ethanol was replaced with methylethanol or ethylpropionate for four classes of phenyl-substituted compounds.

This synthetic program yielded a number of compounds with unprecedented high affinity and potency, some of which were significantly more potent (10 to 100-fold) than the decades-long reference agonist, N^6-cyclopentyladenosine (CPA).

Experimental section

Materials and Methods

N^6-cyclopentyladenosine (CPA) and N^6-cyclopentyl-9-methyladenine (N0840) were obtained from Research Biochemicals Inc. (Natick, MA, U.S.A.). Bovine serum albumin (BSA), 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), forskolin, DEAE dextran and chloroquine were from Sigma (St. Louis MO, U.S.A.). Adenosine deaminase (ADA) was purchased from Roche Biochemicals (Mannheim, Germany) and Bicinchonic acid (BCA) protein assay reagent was obtained from Pierce Chemical Company (Rockford, IL, U.S.A.). [^{3}H] 1,3-dipropyl-8-cyclopentylxanthine ([^{3}H]DPCPX with a specific activity of 124 Ci/mmol) was purchased from NEN (Du Pont Nemours, 's Hertogenbosch, The Netherlands). [^{3}H] cyclic adenosine monophosphate ([^{3}H] cAMP with a specific activity of 32.1 Ci/mmol) was obtained from Perkin Elmer Life Sciences Inc. (Boston, MA, U.S.A.). G418 (neomycin) was obtained from Stratagene (Cedar Creek, U.S.A.). Protein kinase A (PKA) was isolated from bovine adrenal glands according to Leurs et al.[6]. Chinese hamster ovary (CHO) cells stably expressing the human adenosine A_1 receptor were obtained from A. Townsend – Nicholson[7]. HEK293 cells expressing the human adenosine A_{2A} or the A_3 receptor

were kindly provided by Dr. J. Wang, Biogen, USA and Dr. K.-N. Klotz, University of Würzburg, Germany, respectively.

All other reagents were obtained from commercial sources and all solvents were of analytical grade. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC 200 (^{1}H NMR, 200 MHz; ^{13}C NMR, 50.29 MHz) spectrometer with tetramethylsilane as an internal standard. Chemical shifts are reported in δ(ppm) and the following abbreviations are used: s = singlet, d = doublet, t = triplet, m = multiplet, br = broad. Melting points were determined on a Büchi melting point apparatus and are uncorrected. Elemental analyses on all end products (**13-24**) were performed by the Leiden Institute of Chemistry and are within 0.4% of the theoretical values unless otherwise stated. Reactions were routinely monitored by TLC using Merck silica gel F_{254} plates.

Synthesis

General Procedure for functionalized malononitriles (1-4)

To malononitrile (1.32 mL, 20.8 mmol) dissolved in EtOH (14 mL) was added an aldehyde (20 mmol) followed by 3 drops of piperidine. This reaction mixture was then refluxed for 1 hour, and then allowed to cool to room temperature, upon which a precipitate was formed. The crude product was collected by filtration and used without any further purification.

2-(3,4-Dimethoxy-benzylidene)-malononitrile (1)

Off white solid, 53% yield. ^{1}H-NMR δ(DMSO): 8.39 (s, 1H, C*H*), 7.66-7.60 (m, 2H, phenyl-*H*), 7.27-7.22 (m, 1H, phenyl-*H*), 3.91 (s, 3H, CH_3), 3.83 (s, 3H, CH_3).

2-Benzo[1,3]dioxol-5-ylmethylene-malononitrile (2)

Off white solid, 46% yield. ^{1}H-NMR δ(DMSO): 8.39 (s, 1H, C*H*), 7.57-7.54 (m, 2H, phenyl-*H*), 7.24-7.19 (m, 1H, phenyl-*H*), 6.25 (s, 2H, -OCH_2O-).

2-(4-Dimethylamino-benzylidene)-malononitrile (3)

Off white solid, 45% yield. ^{1}H-NMR δ(DMSO): 8.08 (s, 1H, C*H*), 7.87 (d, 2H, J = 9.5 Hz phenyl-*H*), 6.89 (m, 2H, J = 9.5 Hz, phenyl-*H*), 3.13 (s, 6H, 2 × CH_3).

2-(3-Fluoro-benzylidene)-malononitrile (4)

Off white solid, 56% yield. ^{1}H NMR δ(DMSO): 8.59 (s, 1H, C*H*), 7.84-7.60 (m, 4H, phenyl-*H*).

General Procedure for 2-Amino-4-(substituted)-phenyl-6-phenylsulfanyl-pyridine-3,5-dicarbonitriles (5-8)

To a solution of the functionalized malononitrile (**1-4**) (10 mmol) in EtOH (10 mL), was added malononitrile (0.64 mL, 10 mmol), thiophenol (1.02 mL, 10 mmol) and triethylamine (50 µL), and the mixture heated at reflux for approximately 4 h. The reaction mixture was then allowed to cool to room temperature. The crude product precipitated upon cooling and was collected by filtration.

2-Amino-4-(3,4-dimethoxy-phenyl)-6-phenylsulfanyl-pyridine-3,5-dicarbonitrile (5)

Yellow solid, 28% yield. ^{1}H-NMR δ(DMSO): 7.77 (br s, 2H, NH_2), 7.65-7.50 (m, 7H, phenyl-*H*), 7.15 (d, 2H, J = 8.8 Hz, phenyl-*H*), 3.67 (s, 3H, CH_3), 3.63 (s, 3H, CH_3).

2-Amino-4-benzo[1,3]dioxol-5-yl-6-phenylsulfanyl-pyridine-3,5-dicarbonitrile (6)

Yellow solid, 34% yield. ^{1}H-NMR δ(DMSO): 7.80 (br s, 2H, NH_2), 7.64-7.60 (m, 2H, S-phenyl-*H*), 7.53-7.50 (m, 3H, S-phenyl-*H*), 7.20-7.04 (m, 3H, phenyl-*H*), 6.18 (s, 2H, -OCH_2O-).

2-Amino-4-(4-dimethylamino-phenyl)-6-phenylsulfanyl-pyridine-3,5-dicarbonitrile (7)

Yellow solid, 32% yield. ^{1}H-NMR δ(DMSO): 7.68 (br s, 2H, N*H*$_2$), 7.64-7.60 (m, 2H, phenyl-*H*), 7.54-7.48 (m, 2H, phenyl-*H*), 7.44 (d, 2H, J = 8.8 Hz, 4-NMe$_2$-phenyl-*H*), 6.86 (d, 2H, J = 8.8 Hz, 4-NMe$_2$-phenyl-*H*), 3.03 (s, 6H, 2 × C*H*$_3$).

2-Amino-4-(3-fluoro-phenyl)-6-phenylsulfanyl-pyridine-3,5-dicarbonitrile (8)

Yellow solid, 24% yield. ^{1}H-NMR δ(DMSO): 7.90 (br s, 2H, N*H*$_2$), 7.71-7.40 (m, 9H, phenyl-*H*).

General Procedure for 2-Amino-4-(substituted)-phenyl-6-mercapto-pyridine-3,5-dicarbonitriles (9-12)

The pyridine (**5-8**) (3 mmol) was dissolved in DMF (10 mL) and to this was added sodium sulfide (0.78 g, 10 mmol) and the mixture stirred at 80 °C for 2 h. Upon cooling to room temperature, 1 M HCl (20 mL) was added, resulting in the formation of a yellow precipitate. The crude product was collected by filtration.

2-Amino-4-(3,4-dimethoxy-phenyl)-6-mercapto-pyridine-3,5-dicarbonitrile (9)

Yellow solid, quantitative yield. ^{1}H-NMR δ(DMSO): 7.17-7.13 (m, 3H, phenyl-*H*), 3.85 (s, 3H, OC*H*$_3$), 3.81 (s, 3H, OC*H*$_3$).

2-Amino-4-benzo[1,3]dioxol-5-yl-6-mercapto-pyridine-3,5-dicarbonitrile (10)

Yellow solid, quantitative yield. ^{1}H-NMR δ(DMSO): 7.13-7.00 (m, 3H, phenyl-*H*), 6.17 (s, 2H, -OC*H*$_2$O-).

2-Amino-4-(4-dimethylamino-phenyl)-6-mercapto-pyridine-3,5-dicarbonitrile (11)

Yellow solid, quantitative yield. ^{1}H-NMR δ(DMSO): 7.41 (d, 2H, J = 8.8 Hz, phenyl-*H*), 6.83 (d, 2H, J = 8.8 Hz, phenyl-*H*), 3.03 (s, 6H, 2 × C*H*$_3$).

2-Amino-4-(3-fluoro-phenyl)-6-mercapto-pyridine-3,5-dicarbonitrile (12)

Yellow solid, quantitative yield. ^{1}H-NMR δ(DMSO): 7.65-7.59 (m, 1H, phenyl-*H*), 7.48-7.35 (m, 3H, phenyl-*H*).

General Procedure for 2-Amino-4-[(substituted)phenyl]-6-[(substituted)sulfanyl)]-pyridine-3,5-dicarbonitriles (13-24)

The free thiol (**9-12**) (1 mmol) was stirred with the bromo compounds (1 mmol), $NaHCO_3$ (0.34 g, 1 mmol) in DMF (2 mL) at room temperature for 2 h. Water (10 mL) was added to precipitate the crude product, which was collected by filtration. Purification by column chromatography on SiO_2, eluting with dichloromethane-methanol mixtures or ethylacetate-petroleum ether mixtures and/or ethylacetate, followed by recrystallisation gave the desired products (**13-24**) in approximately 40% yield.

2-Amino-4-(3,4-dimethoxy-phenyl)-6-(2-hydroxy-ethylsulfanyl)-pyridine-3,5-dicarbonitrile (13)

White Solid. mp: 209 ºC. ^{1}H-NMR δ(DMSO): 7.96 (br s, 2H, N*H*$_2$) 7.19-7.13 (m, 3H, phenyl-*H*), 5.02 (m, 1H, O*H*), 3.86 (s, 3H, OC*H*$_3$), 3.81 (s, 3H, OC*H*$_3$), 3.69-3.66 (m, 2H, OC*H*$_2$), 3.32-3.19 (m, 2H, C*H*$_2$S). ^{13}C-NMR δ(DMSO): 167.0, 159.7, 158.0, 150.4, 148.3, 126.0, 121.5, 115.7, 115.6, 112.3, 111.5, 92.7, 85.9, 59.5, 55.7, 55.6, 32.8. MS (ESI): 356.9. Anal. ($C_{17}H_{16}N_4O_3S$) C, H, N, S.

2-Amino-4-(3,4-dimethoxy-phenyl)-6-(2-hydroxy-propylsulfanyl)-pyridine-3,5-dicarbonitrile (14)

White Solid. mp: 198 ºC. ^{1}H-NMR δ(DMSO): 7.97 (br s, 2H, N*H*$_2$) 7.19-7.13 (m, 3H, phenyl-*H*), 4.99 (d, 1H, J = 5.1 Hz, O*H*), 3.86 (s, 3H, OC*H*$_3$), 3.81 (s, 3H, OC*H*$_3$), 3.88-3.80 (m, 1H, C*H*), 3.42-3.17 (m, 2H, C*H*$_2$), 1.20 (d, 3H, J = 6.6 Hz, C*H*$_3$). ^{13}C-NMR δ(DMSO): 167.3, 159.7, 158.0, 150.4, 148.3, 125.9, 121.6, 118.6, 112.3, 111.5, 93.6, 88.6, 68.1, 55.7, 55.6, 22.6. MS (ESI): 370.8. Anal. ($C_{18}H_{18}N_4O_3S$) C, H, N, S.

3-[2-amino-3,5-dicyano-4-(3,4-dimethoxy-phenyl)-pyridin-6-ylsulfanyl]-propionic acid ethyl ester (15)

White Solid. mp: 172 ºC. ^{1}H-NMR δ(DMSO): 8.04 (br s, 2H, N*H*$_2$), 7.19-7.13 (m, 3H, phenyl-*H*), 4.12 (q, 2H, J = 7.3 Hz, OC*H*$_2$), 3.86 (s, 3H, OC*H*$_2$), 3.81 (s, 3H, OC*H*$_3$), 3.46-3.39 (m, 2H, C*H*$_2$CO), 2.82 (t, 2H, J = 6.9 Hz, C*H*$_2$S), 1.22 (t, 3H, J = 6.9 Hz, C*H*$_3$). ^{13}C-NMR δ(DMSO): 171.4, 166.6, 159.8, 158.1, 150.4, 148.3, 125.8, 121.5, 115.6, 112.3, 111.5, 91.9, 88.8, 60.2, 55.7, 55.6, 33.4, 24.9, 14.1. MS (ESI): 412.7. Anal. ($C_{20}H_{20}N_4O_4S$) C, H, N, S.

2-Amino-4-benzo[1,3]dioxol-5-yl-6-(2-hydroxy-ethylsulfanyl)-pyridine-3,5-dicarbonitrile (16)

White Solid. mp: 192 ºC. ^{1}H-NMR δ(DMSO): 7.99 (br s, 2H, N*H*$_2$), 7.16-7.01 (m, 3H, phenyl-*H*), 6.17 (s, 2H, -OC*H*$_2$O-), 5.02 (t, 1H, J = 5.5 Hz, O*H*), 3.67 (m, 2H, OC*H*$_2$), 3.38-3.32 (m, 2H, C*H*$_2$S). ^{13}C-NMR δ(DMSO): 197.7, 167.0, 159.7, 157.9, 148.9, 147.4, 127.4, 123.0, 115.6, 115.4, 108.9, 108.5, 101.8, 93.8, 59.5, 32.6. MS (ESI): 341.1. Anal. ($C_{16}H_{12}N_4O_3S$) C, H, N, S.

2-Amino-4-benzo[1,3]dioxol-5-yl-6-(2-hydroxy-propylsulfanyl)-pyridine-3,5-dicarbonitrile (17)

White Solid. ^{1}H-NMR δ(DMSO): 7.98 (br s, 2H, N*H*$_2$), 7.12-7.00 (m, 3H, phenyl-*H*), 6.17 (s, 2H, -OC*H*$_2$O-), 4.99 (d, 1H, J = 4.7 Hz, O*H*), 3.94-3.83 (m, 1H, C*H*), 3.41-3.16 (m, 2H, C*H*$_2$), 1.20 (d, 3H, J = 6.2 Hz, C*H*$_3$). ^{13}C-NMR δ(DMSO): 167.3, 159.6, 157.9, 148.9, 147.4, 127.5, 123.0, 115.6, 115.4, 108.9, 108.6, 101.9, 93.8, 85.8, 65.1, 38.3, 22.7. Anal. ($C_{17}H_{14}N_4O_3S$) C, H, N.

3-(2-Amino-4-benzo[1,3]dioxol-5-yl-3,5-dicyano-pyridin-6-ylsulfanyl)-propionic acid ethyl ester (18)

White Solid. mp: 162 ºC. ^{1}H-NMR δ(DMSO): 8.05 (br s, 2H, N*H*$_2$), 7.16-7.01 (m, 3H, phenyl-*H*), 6.17 (s, 2H, -OC*H*$_2$O-), 4.12 (q, 2H, J = 6.6 Hz, OC*H*$_2$), 3.44-3.35 (m, 2H, C*H*$_2$CO), 2.85-2.78 (m, 2H, C*H*$_2$S), 1.22 (t, 3H, J = 7.3 Hz, C*H*$_3$). ^{13}C-NMR δ(DMSO): 171.4, 166.5, 159.8, 157.9, 148.9, 147.4, 127.4, 123.0, 115.3, 108.9, 108.6, 101.8, 93.7, 86.0, 60.2, 33.3, 24.9, 14.1. MS (ESI): 396.7. Anal. ($C_{19}H_{16}N_4O_4S$) C, H, N, S.

2-Amino-4-(4-dimethylamino-phenyl)-6-(2-hydroxy-ethylsulfanyl)-pyridine-3,5-dicarbonitrile (19)

White Solid. mp: 268 ºC dec. ^{1}H-NMR δ(DMSO): 7.87 (br s, 2H, N*H*$_2$) 7.39 (d, 2H, J = 8.8 Hz, phenyl-*H*), 6.82 (d, 2H, J = 8.8 Hz, phenyl-*H*), 4.99 (t, 1H, J = 5.5 Hz, O*H*), 3.69-3.60 (m, 2H, OC*H*$_2$), 3.38-3.32 (m, 2H, C*H*$_2$S), 3.00 (s, 6H, 2 × C*H*$_3$). ^{13}C-NMR δ(DMSO): 167.1, 160.0, 158.3, 151.5, 129.9, 120.0, 116.1, 116.0, 111.3, 93.2, 85.0, 59.6, 39.5, 32.6. MS (ESI): 339.8. Anal. ($C_{17}H_{17}N_5OS \cdot 0.2CH_2Cl_2$) C, H, N, S.

2-Amino-4-(4-dimethylamino-phenyl)-6-(2-hydroxy-propylsulfanyl)-pyridine-3,5-dicarbonitrile (20)

White Solid. mp: 245 ºC. ^{1}H-NMR δ(DMSO): 7.86 (br s, 2H, N*H*$_2$) 7.39 (d, 2H, J = 8.8 Hz, phenyl-*H*), 6.83 (d, 2H, J = 8.8 Hz, phenyl-*H*), 4.98 (d, 1H, J = 5.1 Hz, O*H*), 3.94-3.83 (m, 1H, C*H*), 3.40-3.10 (m, 2H, C*H*$_2$S), 3.02 (s, 6H, 2 × C*H*$_3$), 1.20 (d, 3H, J = 5.8 Hz, C*H*$_3$). ^{13}C-NMR δ(DMSO): 167.4, 160.0, 158.3, 151.6, 129.9, 120.1, 116.1, 111.4, 93.2, 84.9, 65.2, 39.6, 38.3, 22.7. MS (ESI): 353.9. Anal. ($C_{18}H_{19}N_5OS \cdot 0.2$ MeOH) C, H, N, S.

3-[2-Amino-3,5-dicyano-4-(4-dimethylamino-phenyl)-pyridin-6-ylsulfanyl]-propionic acid ethyl ester (21)

White Solid. mp: 186 ºC. ^{1}H-NMR δ($CDCl_3$): 7.47 (d, 2H, J = 8.8 Hz, phenyl-*H*), 6.76 (d, 2H, J = 8.8 Hz, phenyl-*H*), 5.71 (br s, 2H, N*H*$_2$), 4.18 (q, 2H, J = 7.3 Hz, OC*H*$_2$), 3.43 (t, 2H, J = 6.6 Hz, C*H*$_2$CO), 3.03 (s, 6H, 2 × C*H*$_3$), 2.76 (t, 2H, J = 6.6 Hz, C*H*$_2$S), 1.27 (t, 3H, J = 7.3 Hz, C*H*$_3$). ^{13}C-NMR δ($CDCl_3$): 171.6, 168.2, 159.6, 158.2, 151.9, 130.0, 119.5, 116.3, 115.7, 111.4, 85.8, 60.9, 39.9, 34.0, 25.4, 14.1. MS (ESI): 396.0. Anal. ($C_{20}H_{21}N_5O_2S$) C, H, N, S.

2-Amino-4-(3-fluoro-phenyl)-6-(2-hydroxy-ethylsulfanyl)-pyridine-3,5-dicarbonitrile (22)

White Solid. mp: 189 ºC. ^{1}H-NMR δ(DMSO): 8.09 (br s, 2H, N*H*$_2$), 7.70-7.59 (m, 1H, phenyl-*H*), 7.52-7.37 (m, 3H, phenyl-*H*), 5.05 (t, 1H, J= 5.5 Hz, O*H*), 3.72-3.64 (m, 2H, OC*H*$_2$), 3.40-3.33 (m, 2H, C*H*$_2$S). ^{13}C-NMR δ(DMSO): 167.8, 159.5, 130.3, 130.2, 123.7, 117.1, 116.7, 115.2, 114.8, 114.3, 105.6, 83.3, 59.8, 31.5. MS (ESI): 314.8 Anal. ($C_{15}H_{11}FN_4OS \cdot 0.3$MeOH) C, H, N, S.

2-Amino-4-(3-fluoro-phenyl)-6-(2-hydroxy-propylsulfanyl)-pyridine-3,5-dicarbonitrile (23)

White Solid. mp: 185 ºC. ^{1}H-NMR δ(DMSO): 8.09 (br s, 2H, N*H*$_2$), 7.61-7.57 (m, 1H, phenyl-*H*), 7.52-7.36 (m, 3H, phenyl-*H*), 5.00 (d, 1H, J= 5.1 Hz, O*H*), 3.91-3.82 (m, 1H, C*H*), 3.21 (d, 2H, J = 6.6 Hz, C*H*$_2$S), 1.18 (d, 3H, J = 5.8 Hz, C*H*$_3$). ^{13}C-NMR δ(DMSO): 167.3, 164.2, 159.5, 156.9, 136.3, 136.1,

131.2, 131.0, 124.8, 117.5, 117.1, 115.9, 115.5, 115.3, 115.1, 93.6, 85.7, 65.1, 22.7. MS (ESI): 328.7 Anal. ($C_{16}H_{13}FN_4OS \cdot 0.1CH_2Cl_2$) C, H, N, S.

3-[2-Amino-3,5-dicyano-4-(3-fluoro-phenyl)-pyridin-6-ylsulfanyl]-propionic acid ethyl ester (24)

White Solid. mp: 131 ºC. ^{1}H-NMR δ(MeOD): 6.86-6.75 (m, 1H, phenyl-*H*), 6.57-6.50 (m, 3H, phenyl-*H*), 3.39 (q, 2H, J = 7.3 Hz, OC*H*$_2$), 2.71 (t, 2H, J = 6.6 Hz, C*H*$_2$CO), 2.03 (t, 2H, J = 6.6 Hz, C*H*$_2$S), 0.49 (t, 3H, J = 7.3 Hz, C*H*$_3$). ^{13}C-NMR δ(MeOD): 173.3, 168.9, 166.4, 161.3, 158.5, 137.6, 132.1, 131.9, 125.7, 118.5, 118.1, 117.0, 116.5, 115.9, 95.5, 89.8, 61.9, 35.0, 26.4, 14.5. MS (ESI): 370.9. Anal. ($C_{18}H_{15}FN_4O_2S$) C, H, N, S.

Cell culture

CHO cells stably expressing the human adenosine A_1 receptor were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium containing 10% newborn calf serum, streptomycin (50 µg/mL), penicillin (50 IU/mL) and 0.2 or 0.8 mg/mL neomycin (G418), respectively. The cells were maintained in a humidified atmosphere at 37 ºC and 5% CO_2, and subcultured twice weekly.

Preparation of cell membranes

Membranes of CHO cells stably expressing the human adenosine A_1 receptor and of HEK293 cells stably expressing the human adenosine A_{2A} receptor or human adenosine A_3 receptor were prepared as previously described[8,9]. The protein concentration in the membrane preparation was determined using the BCA method with BSA as a standard[10].

Radioligand displacement assay

Radioligand binding studies were carried out as previously described[11]. In short, membrane aliquots containing 10 µg of protein were incubated in 400 µL of 50 mM Tris-HCl buffer, pH 7.4 at 25 ºC for 60 min in the presence of ADA (1 U/mL) and approximately 1.6 nM [^{3}H]DPCPX. Increasing concentrations of cold ligand were added to determine the affinity of the ligands. Non-specific binding was measured in the presence of 10 µM CPA. Incubations were stopped by rapid dilution with 1 mL ice-cold 50 mM Tris-HCl, pH 7.4 and bound radioligand was separated from free radioligand by rapid filtration through Whatman GF/B filters using a Brandel harvester. Filters were subsequently washed three times with 2 mL ice-cold buffer. Filter-bound radioactivity was measured by liquid scintillation counting (Tri-Carb 2900TR, Perkin Elmer) after the addition of 3.5 mL of Packard Emulsifier Safe. Experiments were performed in triplicate. Radioligand binding to membranes of HEK293 cells expressing the adenosine A_{2A} and A_3 receptors was performed in duplicate with [^{3}H]ZM241385 (K_D = 1.0 nM) and [^{125}I]I-ABMECA (K_D = 5.0 nM) as radioligands, respectively. Details of these experiments have been previously described[8].

cAMP assay

cAMP assays were performed according to Kourounakis et al. (2001)[12]. In short, cells stably expressing the human adenosine A_1 receptor were harvested using a trypsin solution (0.25% in PBS/EDTA), resuspended in medium and plated in 24-well plates at 500 µl or 2×10^5 cells/well. After 24 hr, the cells were washed two times with 500 µl DMEM containing 50 mM HEPES, pH 7.4. Subsequently, cells were incubated at 37 ºC in 250 µL DMEM + HEPES supplemented with ADA (2 IU/mL), rolipram (50 µM) and cilostamide (50 µM). After 30 min of preincubation, ligands (50 µL) were added, giving a final concentration between 1 to 20 µM based on the K_i value of the ligand. For the same reason, the reference compounds CPA, DPCPX and N0840 were tested at 1, 1 and 100 µM, respectively. The concentration was at least 100 × K_i, to achieve maximal receptor activation as reported e.g. by Beukers et al. (2004)[4]. In addition, concentration-effect curves were generated for CPA, LUF5853 and LUF6037 with concentrations ranging from 1 pM to 1 µM. After 10 min of incubation (37 ºC), 100 µL forskolin was added (final concentration 10 µM) and cells were incubated for an additional 15 min at 37 ºC. The assay was terminated by quick aspiration of the medium and cells were lysed by adding 200 µL of 0.1 M ice-cold HCl. Plates were stored at -20 ºC until further use. To determine the amount of cAMP produced, 100 µL PKA in buffer (150 mM K_2HPO_4; 10 mM EDTA; 0.2% BSA, pH 7.5), 50 µL [^{3}H]cAMP (20,000 dpm) in K_2HPO_4/EDTA buffer (150 mM K_2HPO_4; 10 mM EDTA, pH 7.7) and 50 µL of the cell lysate or cAMP solution for generation of a standard curve (0-16 pmol/200 µL 0.1 M HCl) were incubated on ice for 2.5 hr. Separation of bound and free radioligand was performed by rapid filtration through Whatman GF/C filters that were subsequently washed once with ice-cold buffer. Scintillation fluid (Emulsifier Safe, Packard Bioscience), 3.5 mL, was added and after 2 hr extraction, radioactivity was counted using a Tri-Carb 2900TR, Perkin Elmer scintillation counter.

Data analysis

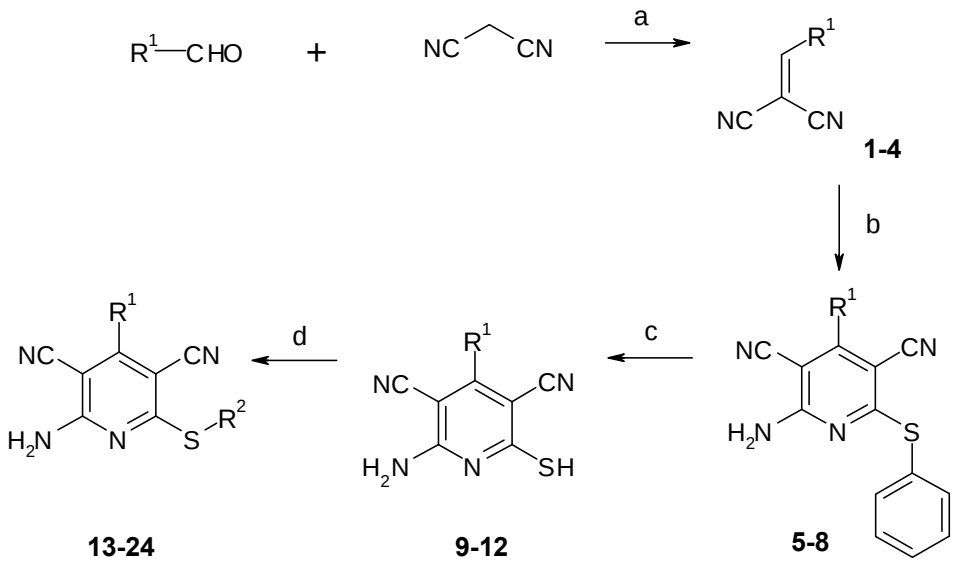
Data of radioligand binding and cAMP experiments were analyzed using the non-linear regression curve fitting program Prism v. 4.0.2 (GraphPad, San Diego, CA, USA). Radioligand displacement curves were fitted to one and two site binding curves. For LUF5853 (**16**), LUF6037 (**17**), LUF5854 (**18**) and CPA the data were best fitted to a two site binding model. K_i values were derived from the IC_{50} values according to the Cheng & Prusoff equation $K_i = IC_{50}/(1+[L^*]/K_d)$, where [L*] is the radioligand concentration, and K_d its dissociation constant[13].

Results

Chemical synthesis

Compounds **13-24** were synthesized according to Scheme 5.1 essentially as previously described[2,3,5]. Intermediates (**1-4**) were obtained in moderate to good yields (46-56%) via a straightforward Knoevenagel condensation of the respective aldehyde with malononitrile in the presence of a few drops of piperidine. The formation of the pyridine occurred according to the preparation previously described by Kambe et al. (1981) resulting in the phenyl protected sulfide at the 6-position of the ring[14]. The functionalized malononitrile was refluxed with another equivalent of malononitrile and an equivalent of thiophenol in ethanol and triethylamine, resulting in compounds **5-8** (24-34% yields). The free thiol in the 6-position of the pyridine ring was obtained by adding 3.3 equivalents of sodium sulfide in DMF at 80 °C for 2-3 hours and resulted in quantitative yields of compounds **9-12**. The yields stated correspond to the crude material, and it was this crude substance that was used in subsequent reactions. Purification of the crude product was not performed throughout the synthesis, nor were the reactions optimized. The final step was the reaction of the free thiol with the bromo compounds in the presence of $NaHCO_3$ in DMF at room temperature. The final compounds **13-24** were obtained in 35-43% yields. They were purified by chromatography and subsequent recrystallisation gave clean, fully characterized compounds.

Scheme 5.1. Synthetic route to 2-Amino-4-(substituted)phenyl-6-(substituted)sulfanyl-pyridine-3,5-dicarbonitriles **13-24**.[a]



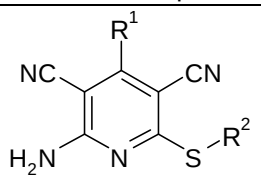
(a) piperidine, EtOH, 1 h reflux; (b) malononitrile, thiophenol, triethylamine, EtOH, 4 h reflux; (c) (i) Na_2S, DMF, (ii) 1M HCl; (d) bromo compound, $NaHCO_3$, DMF.

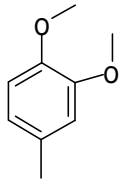
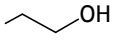
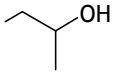
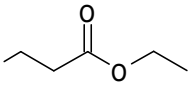
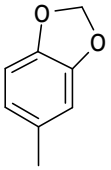
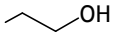
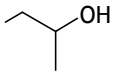
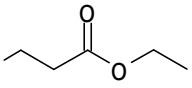
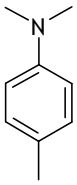
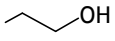
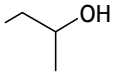
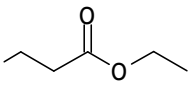
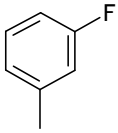
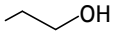
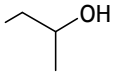
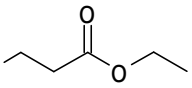
Affinities of the novel, non-ribose ligands for the human adenosine A_1 receptor

The affinity of the newly synthesized non-ribose ligands was assessed with help of radioligand binding studies on membranes of CHO cells stably expressing the human adenosine A_1 receptor. First of all, single point measurements at a concentration of 1 µM were performed for each ligand to determine the percentage inhibition of radioligand binding. For those compounds that gave more than 50% displacement at a concentration of 1 µM, whole displacement curves were generated, to determine the K_i value of the ligand. In Table 5.1, the percentages displacement at 1 µM or the K_i values in nM for each non-ribose ligand are presented. From this table it appears that within the different subsets – based on the R^1 group - a change in R^2 group from ethanol, to methylethanol or ethylpropionate did not affect the affinity of the ligand for the human adenosine A_1 receptor to a great extent.

On the other hand, modification of the R^1 substituent had striking effects on the affinity. LUF5850 – 5852 (**13-15**), containing a 3,4-dimethoxyphenyl-group as R^1 group, showed a displacement between 22% and 37% at a concentration of 1 µM, whereas introduction of a (4)-*N,N*-dimethylphenyl group as in LUF5874 (**19**), LUF5875 (**20**) and LUF5876 (**21**) resulted in affinities of 245, 340 and 231 nM, respectively. A 3-fluoro-phenyl group at the R^1 position resulted in ligands (**22-24**) with a rather high affinity for the human adenosine A_1 receptor (K_i of 40 – 69 nM).

Table 5.1. Reported K_i values or % displacement of compounds 13-24 for human adenosine A_1 receptors. The double figures for compounds 16-18 represent the $K_{i, high}$ and $K_{i, low}$ values.



R^1	R^2	Compound	K_i [nM][b] or Displacement at 1 μM [%]	
		LUF5850 (**13**)[a]	25 ± 1%	
		LUF5851 (**14**)	22 ± 6%	
		LUF5852 (**15**)	37 ± 9%	
		LUF5853 (**16**)	0.026 ± 0.020	12 ± 3.9
		LUF6037 (**17**)	0.021 ± 0.008	7.9 ± 2.8
		LUF5854 (**18**)	0.28 ± 0.18	45 ± 6.6
		LUF5874 **(19)**[a]	245 ± 103	
		LUF5875 (**20**)	340 ± 111	
		LUF5876 (**21**)	231 ± 93	
		LUF5877 (**22**)[a]	40 ± 10	
		LUF5878 (**23**)	43 ± 24	
		LUF5879 (**24**)	69 ± 17	

[a] Data from Chang et al. (2005) *J Med Chem* **48**:2045-2053[5].
[b] Mean of three experiments ± SEM

Much higher affinities were obtained with LUF5853 (**16**), LUF6037 (**17**) and LUF5854 (**18**), containing a 3,4-methylenedioxyphenyl as R^1 group. Interestingly, the three compounds recognized two binding states/sites on the receptor, whereas all other non-ribose compounds recognized just one binding site (see Figure 5.1). Moreover, the affinity of LUF5854 (**18**) for the high affinity site was 10 fold higher than CPA, 0.28 ± 0.18 nM and 2.2 ± 0.9 nM, respectively[15]. The affinities of LUF5853 (**16**) and LUF6037 (**17**) for the high affinity site were even higher, 100-fold, compared with CPA, 26 pM and 21 pM, respectively.

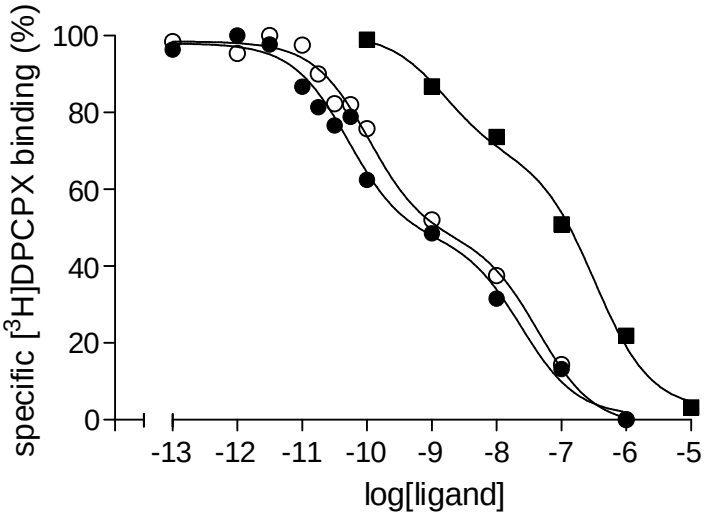


Figure 5.1. Displacement of ^{3}H]DPCPX from the human adenosine A_1 receptor expressed on CHO membranes by LUF5853 (**16**) (○), LUF6037 (**17**) (●) or CPA (■). A representative curve from one experiment performed in duplicate is shown.

Selectivity of the novel, non-ribose ligands for the human adenosine A_1 receptor

To determine the selectivity of the non-ribose ligands for the adenosine A_1 receptor the interaction of the compounds with the adenosine A_{2A} and adenosine A_3 receptors expressed on HEK293 cells was investigated. None of the compounds (**13-24**) was able to displace to a considerable extent either [^{3}H]ZM241385 or [^{125}I]I-ABMECA from the adenosine A_{2A} or adenosine A_3 receptors, respectively (Table 5.2).

Efficacies of the novel, non-ribose ligands on the human adenosine A_1 receptor

Subsequently, the functional activity of the novel, non-ribose ligands was tested in a cAMP assay. The final concentration varied between 1-20 µM, to obtain a concentration of at least 100 × K_i to ensure full receptor occupancy. LUF5850 – LUF5852 (**13-15**) were not considered in the activity assay, because of their low affinity. The basal cAMP concentration in the CHO cells was set at 0%, and the cAMP concentration in forskolin-stimulated cells was set at 100%. For comparison, three reference ligands, CPA (1 µM), DPCPX (1,3-dipropyl-8-cyclopentylxanthine, 1 µM) and N0840 (N^6-cyclopentyl-9-methyladenine, 100 µM) were also included. The agonist CPA inhibited the forskolin-stimulated cAMP production from 100% to 41 ± 9%. DPCPX, an inverse agonist, increased the cAMP production from 100% to 297 ± 37%. A somewhat smaller increase was observed for N0840 (210 ± 41%). The set of non-ribose ligands represented the whole range of full agonists to inverse agonists (Figure 5.2). LUF6037 (**17**) (29 ± 7%), LUF5853 (**16**) (31 ± 20%), LUF5854 (**18**) (50 ± 14%), and LUF5877 (**22**) (40 ± 14%) performed better than or equally well as CPA (41 ± 9%) in inhibiting the cAMP production, and are thus considered full agonists. LUF5874 (**19**) (68 ± 19%) and LUF5878 (**23**) (56 ± 13%) rather behaved as partial agonists in this test system. LUF5875 (**20**) and LUF5879 (**24**) can be regarded as neutral antagonists, given their slight influence on forskolin-stimulated cAMP

production, 107 ± 19% and 118 ± 9%, respectively. Finally, LUF5876 (**21**) appeared to be a (partial) inverse agonist, given its increase in cAMP production to 187 ± 31%.

Table 5.2. Reported% displacement of compounds **13-24** for human adenosine A_{2A} and A_3 receptors.

Compound	hA_{2A} Displacement at 1 μM	hA_3 Displacement at 1 μM
LUF5850 (**13**)[a]	25%	0%
LUF5851 (**14**)	19%	1%
LUF5852 (**15**)	26%	4%
LUF5853 (**16**)[a]	33%	13%
LUF6037 (**17**)	22%	1%
LUF5854 (**18**)	28%	2%
LUF5874 (19)[a]	0%	7%
LUF5875 (**20**)	3%	9%
LUF5876 (**21**)	5%	11%
LUF5877 (**22**)[a]	19%	8%
LUF5878 (**23**)	19%	1%
LUF5879 (**24**)	24%	16%

[a] Data from Chang et al. (2005) *J Med Chem* **48**:2045-2053[5].

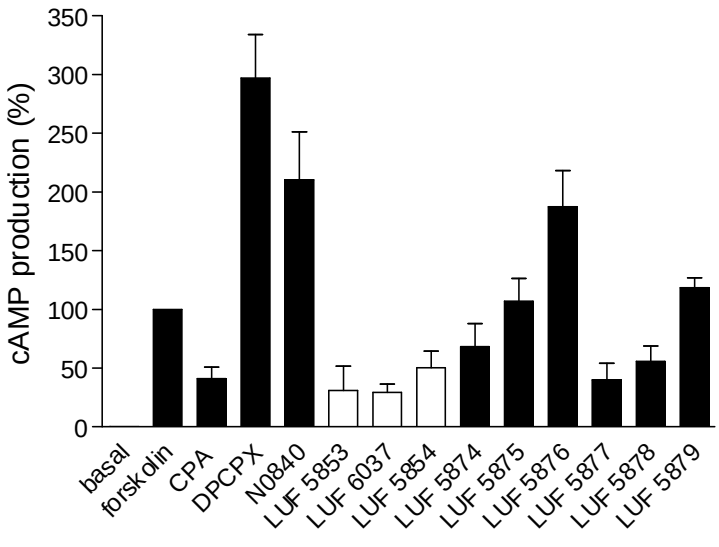


Figure 5.2. Modulation of forskolin-induced cAMP production in CHO cells stably expressing the human adenosine A_1 receptor, after exposure to reference ligands (CPA, DPCPX, N0840) and non-ribose ligands (LUF5853-5879, **16-24**) at a concentration between 1 to 100 μM to obtain full receptor occupancy (n = 3). Each subset of non-ribose ligands with identical R^1 group but different R^2 groups is labeled with the same bar pattern.

Full cAMP concentration-effect curves were generated for the high, picomolar, affinity non-ribose agonists LUF6037 (**17**) and LUF5853 (**16**). Representative curves for CPA, LUF6037 (**17**) and LUF5853 (**16**) are shown in Figure 5.3. The EC_{50} values were 2.1 ± 0.7 nM, 0.06 ± 0.05 nM and 0.1 ± 0.03 nM, respectively.

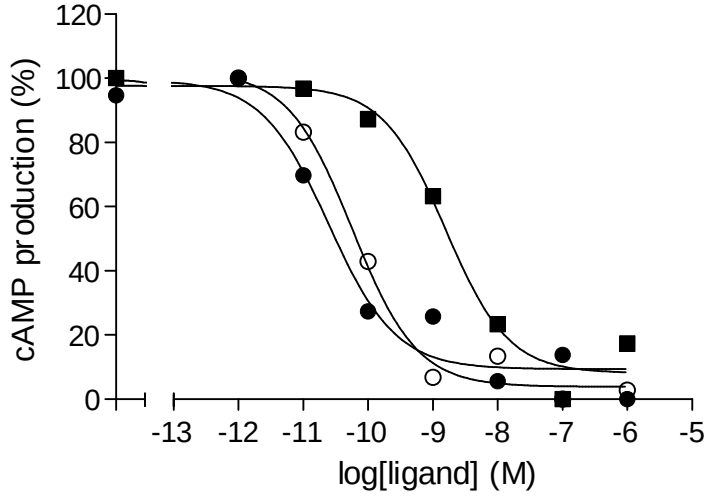


Figure 5.3. Representative curves of the inhibition of forskolin-stimulated cAMP production by CPA (■), LUF5853 (**16**) (○) and LUF6037 (**17**) (●) in CHO cells stably expressing the human adenosine A_1 receptor (n = 5, except for LUF5853 (**16**), n = 4).

Discussion

Structural analogues of adenosine, especially when substituted at the N^6 position are known to, often selectively, activate the human adenosine A_1 receptor[1]. Moreover the presence of a ribose moiety has always been considered essential for receptor activation[16,17]. The publication of several patents[2,3] describing a series of compounds as agonists for the adenosine receptors lacking a ribose moiety firmly challenged that hypothesis. These findings prompted us to synthesize a number of such ligands, all substituted 3,5-dicyanopyridine derivatives, that indeed showed a high affinity, activity and selectivity for the human adenosine A_1 receptor[5]. In the present study, we extended our research program by synthesizing and evaluating compounds **13-24**.

Affinity and selectivity of the novel, non-ribose ligands for the human adenosine A_1 receptor

The compounds with an ethanol substituent at the R^2 position were part of a larger series of non-ribose ligands, with more variation in the 3,4-substituents at the phenyl ring at the R^1 position[5]. It was found that increasing the electronegativity by introducing e.g. a fluorine atom or a hydroxyl group at especially the 3-position is important to increase the affinity of the ligands[5]. These findings were confirmed and extended in our current experiments, demonstrating that the R^2 group itself is important for affinity, but does not affect the rank order of potency that is dictated by the R^1 group. Moreover, three highly potent ligands, LUF5853 (**16**) and LUF6037 (**17**) and LUF5854 (**18**), were identified, all with unprecedented picomolar affinity. In fact

we had missed the very high affinity of LUF5853 (**16**) in our previous publication[5]. but due to our experimental observations with **17** and **18** we were prompted to reexamine these earlier findings. These compounds share a 3,4-methylenedioxy group at R^1 with an alcohol or ester function at R^2. Whereas these agonists recognized two binding states of the receptor, all other non-ribose compounds recognized just one binding state/site. This is in line with previous data on a similar compound, LUF5831, a partial adenosine A_1 receptor agonist that recognized a single binding state/site only[15]. Strikingly, the rigidity and somewhat smaller size provided by the closed ring structure of these three compounds is strongly preferred over the low affinity compounds **13-15**. The latter have a minor change in substitution pattern, namely two adjacent methoxy groups, which may cause too much steric hindrance in the receptor binding site.

To determine the selectivity of these compounds for the adenosine A_1 receptor the interaction with the adenosine A_{2A} and A_3 receptors was determined. In agreement with the previously reported data for a series of related analogues, none of the compounds (**13-24**) was able to interact with these receptors to a considerable extent[5].

Efficacy of the novel, non-ribose ligands for the human adenosine A_1 receptor

Whereas the affinity was mainly determined by the substitution pattern on the phenyl ring, different results were obtained with respect to efficacy. In this series of compounds (**13-24**) the efficacy was affected both by the substitution pattern on the phenyl ring, and by the substitution pattern on the sulfanyl chain. The effects of the substitution pattern on the phenyl ring were in line with previously reported data[5]. Interestingly, however, the substitution pattern on the sulfanyl side-chain was also important. The efficacy to activate the receptor decreased with increasing chain length. Thus a change from ethanol to methylethanol and ethylpropionate resulted in a decrease in efficacy from an agonist such as LUF5874 (**19**), a neutral antagonist such as LUF5875 (**20**), to an inverse agonist such as LUF5876 (**21**). In our hands and in this cAMP assay N0840, which is generally claimed to be a neutral antagonist and behaved as such in [^{35}S]GTPγS binding studies[18,19], was an inverse agonist (210 ± 41%), quite similar to LUF5876 (**21**).

In conclusion, structure-activity relationship studies documented very potent full agonists for the adenosine A_1 receptor in a series of 3,5-dicyanopyridines substituted at both the phenyl and sulfanyl position. These compounds lack any obvious similarity with the traditional ribose, adenosine-like compounds. Substitution at the phenyl ring mostly dictated affinity, whereas the substitution pattern at the sulfanyl position appeared to rather govern intrinsic activity. Compounds such as LUF5853 (**16**) and LUF6037 (**17**) largely superseded CPA in its affinity and potency.

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CHAPTER 6

LUF6037, a non-adenosine agonist with picomolar potency for the adenosine A_1 receptor is unable to internalize the receptor

We and others have recently shown that a novel class of non-adenosine compounds interacts with adenosine A_1 receptors. Here we report on a new derivative, LUF6037, a very potent non-adenosine A_1 receptor agonist. The striking structural differences between traditional adenosine-like agonists and LUF6037 prompted us to investigate its interaction with the receptor and its ability to induce internalization. Radioligand binding and cAMP studies were carried out with CHO cells expressing the human adenosine A_1 receptor. The ability of LUF6037 to internalize the receptor was determined with an adenosine A_1 receptor equipped with a yellow fluorescent protein (YFP) tag. LUF6037 recognized two binding states/sites on the human adenosine A_1 receptor with affinities of 44 ± 30 pM and 8.7 ± 3.7 nM, both much higher than the reference agonist cyclopentyladenosine (CPA) with affinities of 2.2 ± 0.9 nM and 338 ± 24 nM. The interaction of LUF6037 with the receptor did not change in the presence of the allosteric enhancer PD81,723, whereas PD81,723 increased CPA's affinity. LUF6037 behaved as a full agonist in thermodynamic radioligand binding experiments as well as in a second messenger assay, where it completely inhibited forskolin-stimulated cAMP production with an EC_{50} value of 60 ± 5 pM. Finally, whereas CPA dose-dependently induced receptor internalization, LUF6037 had no effect. LUF6037 fully activates the human adenosine A_1 receptor just like traditional agonists such as CPA. However, unlike CPA the activation by LUF6037 is insensitive to the allosteric modulator PD81,723. Moreover, LUF6037 does not induce receptor internalization. Apparently full agonism does not necessarily lead to receptor internalization. This behaviour may be therapeutically advantageous as drug resistance, as a result of receptor internalization, may less readily occur.

Based upon Klaasse EC, Roerink SF, van Veldhoven JPD, von Frijtag Drabbe Künzel JK, de Grip WJ, IJzerman AP, Beukers MW. Manuscript in preparation.

Introduction

During the past decades, several agonists with high affinity for the human adenosine A_1 receptor have been developed. These ligands are structurally related to the endogenous agonist adenosine, consisting of the adenine base coupled to a ribose moiety. To increase the affinity and the selectivity of the agonists for the human adenosine A_1 receptor, adenosine has been substituted at different positions. Especially substitutions at the N^6-position often lead to potent and selective adenosine A_1 receptor agonists[1].

Recently, a new class of adenosine ligands has been described in the patent literature, the 2-amino-4-(3,4 substituted phenyl)-6-(2-hydroxyethylsulfanyl)-pyridin-3,5-dicarbonitriles[2,3]. Although this class of ligands shows very little structural similarity to adenosine, it was observed that several compounds displayed a significant affinity and efficacy towards different adenosine receptor subtypes[2,3,4,5]. A more detailed pharmacological characterization of one of these compounds, LUF5831, revealed that this compound was a partial agonist with an affinity of 18 nM for the adenosine A_1 receptor[6].

In this report we characterized a new non-adenosine agonist, LUF6037 (Figure 6.1), and demonstrated it is a full agonist with very high picomolar affinity, largely surpassing the potency of the reference agonist, *N*^{6}-cyclopentyladenosine (CPA). However, unlike CPA, we found that this full agonist is insensitive to the allosteric enhancer PD81,723. LUF6037 was also tested for its ability to induce internalization of the human adenosine A_1 receptor. Again unlike CPA, LUF6037 did not cause any translocation of a YFP-tagged A_1 receptor from the cell membrane to the cytoplasm. This observation is first-time evidence that full activation of this receptor does not necessarily lead to internalization.

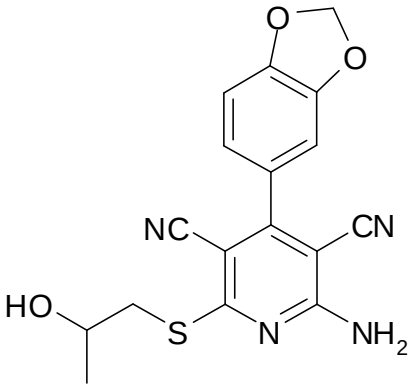

Figure 6.1. Structure of LUF6037

Methods

Materials

N^6-cyclopentyladenosine (CPA) was obtained from Research Biochemicals Inc. (Natick, MA, U.S.A.). 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), bovine serum albumin (BSA), forskolin, DEAE dextran and chloroquine were from Sigma (St. Louis MO, U.S.A.). Adenosine deaminase (ADA) was purchased from Roche Biochemicals (Mannheim, Germany) and Bicinchonic acid (BCA) protein assay reagent was obtained from Pierce Chemical Company (Rockford, IL, U.S.A.). [^{3}H] 1,3-dipropyl-8-cyclopentylxanthine ([^{3}H]DPCPX -specific activity 124 Ci/mmol) was purchased from

NEN (Du Pont Nemours, 's Hertogenbosch, The Netherlands). [^{3}H] cyclic adenosine monophosphate ([^{3}H] cAMP – specific activity 32.1 Ci/mmol) was obtained from Perkin Elmer Life Sciences Inc. (Boston, MA, U.S.A.). G418 (neomycin) was obtained from Stratagene (Cedar Creek, U.S.A.). (2-Amino-4,5-dimethyl-3-thienyl)-[3-(trifluoromethyl)phenyl]-methanone (PD81,723) was synthesized in our laboratory as described by Van der Klein et al.[7]. Protein kinase A (PKA) was isolated from bovine adrenal glands according to Leurs et al.[8]. LUF6037 was synthesized in our laboratory, as described for the 2-amino-4-(3,4 substituted phenyl)-6-(2-hydroxyethylsulfanyl)-pyridin-3,5-dicarbonitrile compounds[5]. Chinese hamster ovary (CHO) cells stably expressing the human adenosine A_1 receptor were obtained from A. Townsend – Nicholson[9]. CHO cells stably expressing the human adenosine A_1YFP receptor were made in our laboratory[10]. All other chemicals were of analytical grade and obtained from standard commercial sources.

Cell culture

CHO cells stably expressing the human adenosine A_1 receptor or the human adenosine A_1YFP receptor were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium containing 10% newborn calf serum, streptomycin (50 µg/mL), penicillin (50 IU/mL) and 0.2 or 0.8 mg/mL neomycin (G418), respectively. The cells were maintained in a humidified atmosphere at 37 ºC and 5% CO_2, and subcultured twice weekly.

Preparation of cell membranes

Membranes of CHO cells stably expressing the human adenosine A_1 receptor were prepared as previously described[10]. The protein concentration in the membrane preparation was determined using the BCA method[11] with BSA as a standard.

Radioligand displacement assay

Radioligand binding studies were carried out as previously described[12]. In short, membrane aliquots containing 10 µg of protein were incubated in 400 µL of 50 mM Tris-HCl buffer, pH 7.4 at 25 ºC for 60 min or at 0 ºC for 120 min in the presence of ADA (1 U/mL) and approximately 1.6 nM [^{3}H]DPCPX. Increasing concentrations of cold ligand were added in the presence or absence of 10 µM PD81,723. This concentration of PD81,723 was chosen based on its EC_{50} value of 9.8 µM[13]. Non-specific binding was measured in the presence of 10 µM CPA. Incubations were stopped by rapid dilution with 1 mL ice-cold 50 mM Tris-HCl, pH 7.4 and bound radioligand was separated from free radioligand by rapid filtration through Whatman GF/B filters using a Brandel harvester. Filters were subsequently washed three times with 2 mL ice-cold buffer. Filter-bound radioactivity was measured by liquid

scintillation counting (Tri-Carb 2900TR, Perkin Elmer) after the addition of 3.5 mL of Packard Emulsifier Safe. Experiments were performed at least in triplicate.

Thermodynamic data determination

The values of the thermodynamic parameters were obtained by measuring K_i values at 0 ºC and at 25 ºC, followed by van 't Hoff analysis[14]. The standard free energy, ΔG^0, was calculated according to $\Delta G^0 = -RT \ln K_A$ with $K_A = 1/K_i$. The van 't Hoff equation yields a graph of ln K_A versus $1/T$. The standard enthalpy, ΔH^0, can be calculated from the slope ($\Delta H^0/R$), whereas the standard entropy, ΔS^0, can be calculated either from the intercept ($\Delta S^0/R$) or from $\Delta S^0 = (\Delta H^0 - \Delta G^0)/T$, with R = 8.314 $JK^{-1}mol^{-1}$ and T = 298.15 K

cAMP assay

cAMP assays were performed according to Kourounakis et al.[15]. In short, cells stably expressing the human adenosine A_1 receptor were harvested using a trypsin solution (0.25% in PBS/EDTA), resuspended in medium and plated in 24-well plates at 500 µl or 2×10^5 cells/well. After 24 hr, the cells were washed two times with 500 µl DMEM containing 50 mM HEPES, pH 7.4. Subsequently, cells were incubated at 37 ºC in 250 µL DMEM + HEPES supplemented with ADA (2 IU/mL), rolipram (50 µM) and cilostamide (50 µM). After 30 min of preincubation, ligands were added, to obtain the concentrations as indicated. After 10 min of incubation (37 ºC), 100 µL forskolin was added (final concentration 10 µM) and cells were incubated for an additional 15 min at 37 ºC. The assay was terminated by quick aspiration of the medium and cells were lysed by adding 200 µL of 0.1 M ice-cold HCl. Plates were stored at -20 ºC until further use. To determine the amount of cAMP produced, 100 µL PKA in buffer (150 mM K_2HPO_4; 10 mM EDTA; 0.2% BSA, pH 7.5), 50 µL [^{3}H]cAMP (20,000 dpm) in K_2HPO_4/EDTA buffer (150 mM K_2HPO_4; 10 mM EDTA, pH 7.7) and 50 µL of the cell lysate or cAMP solution for generation of a standard curve (0-16 pmol/200 µL 0.1 M HCl) were incubated on ice for 2.5 hr. Separation of bound and free radioligand was performed by rapid filtration through Whatman GF/C filters, that were subsequently washed once with ice-cold buffer. Scintillation fluid (Emulsifier Safe, Packard Bioscience), 3.5 mL, was added and after 2 hr extraction, radioactivity was counted using a Tri-Carb 2900TR, Perkin Elmer scintillation counter. Experiments were performed at least in triplicate.

Internalization experiments and quantification

CHO cells stably expressing the human adenosine A_1YFP receptor were exposed to the reference agonist CPA, to LUF6037 and to the inverse agonist DPCPX at a concentration of 4 µM to observe if internalization occurred. Internalization experiments were performed as described before[10]. Briefly, 3×10^4 cells were plated

on coverslips in a 24 wells plate (500 µL), and allowed to attach for 24 hr. Subsequently, cells were exposed for 16 hr to the ligands, washed, fixed with 4.0% formaldehyde (pH 7.0 - 7.2) and mounted on object glasses using Aqua Polymount® (Polysciences). Images of representative transsections of the cells were obtained using confocal microscopy (Nikon Eclipse TE 2000-U) with excitation at 520 nm and emission at 532 nm under 60 × oil enlargement. In representative transsections of cells, total cell fluorescence and fluorescence in the cytosol were measured using the computer program Image Pro Plus (MediaCybernetics, Germany). Transsections of at least three different cells were analyzed. The percentage plasma membrane staining relative to total was subsequently calculated according to the method described in Klaasse et al.[10].

Data analysis

Data of radioligand binding and cAMP experiments were analyzed using the non-linear regression curve fitting program Prism v. 4.0.2 (GraphPad, San Diego, CA, USA). Radioligand displacement curves were fitted to one and two state/site binding curves. For LUF6037 and CPA the data were best fitted to a two state/site binding model. K_i values were derived from the IC_{50} values according to the Cheng & Prusoff equation $K_i = IC_{50}/(1+[L^*]/K_d)$, where $[L^*]$ is the radioligand concentration, and K_d its dissociation constant[16]. The K_d values of [^{3}H]DPCPX were 0.69 nM at 0 ºC, and 1.6 nM and 8.8 nM at 25 ºC in the absence and presence of PD81,723, respectively[6].

Results

Affinity of LUF6037 for the human adenosine A_1 receptor

To determine the affinity of LUF6037, radioligand binding studies were performed on membranes of CHO cells stably expressing the human adenosine A_1 receptor with [^{3}H]DPCPX as the radioligand (see Figure 6.2). LUF6037 recognized two binding states/sites with affinities of 44 ± 30 pM and 8.7 ± 3.7 nM for the high and low affinity sites, respectively (see Table 6.1). Both affinities are substantially higher than the previously determined affinities of the reference agonist CPA, which were 2.2 ± 0.9 nM and 338 ± 24 nM, for the high and low affinity sites, respectively[6].

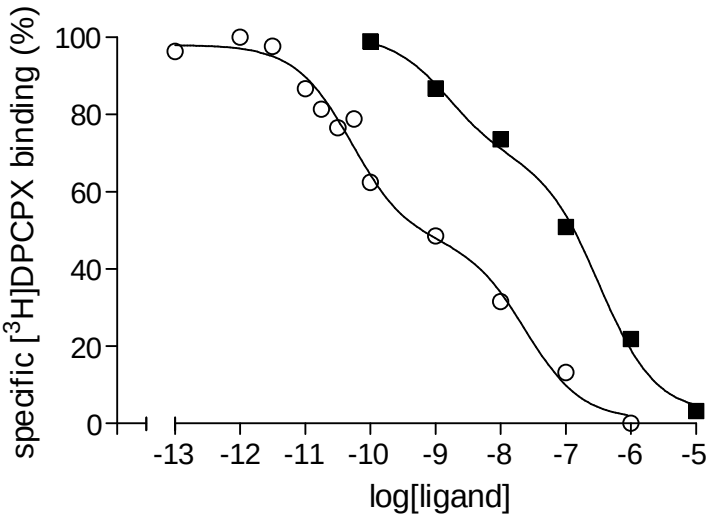

Figure 6.2. Displacement of [^{3}H]DPCPX from the human adenosine A_1 receptor expressed on CHO membranes by LUF6037 (○) or CPA (■). A representative curve from one experiment performed in duplicate is shown.

Effect of the allosteric modulator PD81,723 on the affinity of LUF6037

Radioligand binding experiments were performed in the presence and absence of 10 μM of the allosteric modulator PD81,723 (see Figure 6.3 and Table 6.1). In contrast to CPA (Heitman et al.[6], Table 6.1), LUF6037 was virtually insensitive to this compound. The $K_{i,H}$ and $K_{i,L}$ values were 73 ± 32 pM and 9.9 ± 4.3 nM, respectively, almost identical to the values reported in the absence of PD81,723 (Table 6.1).

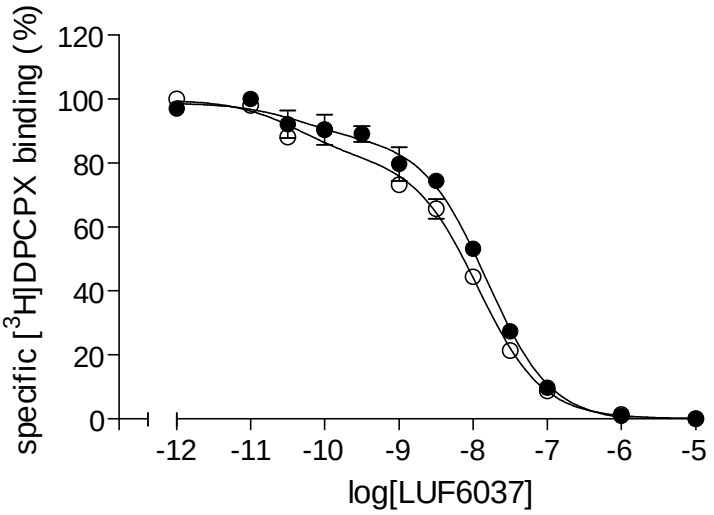

Figure 6.3. Displacement of [^{3}H]DPCPX from the human adenosine A_1 receptor expressed on CHO membranes by LUF6037 in the presence (closed symbols) or absence (open symbols) of 10 μM PD81,723.

Table 6.1. Effect of PD81,723 (10 μM) and temperature (25 ºC versus 0 ºC) on the high ($K_{i,H}$) and low ($K_{i,L}$) affinity binding of LUF6037 to the human adenosine A_1 receptor. Data for CPA are shown for comparison.

Condition	LUF6037		CPA[a]	
	$K_{i,H}$ in pM	$K_{i,L}$ in nM	$K_{i,H}$ in nM	$K_{i,L}$ in nM
25 ºC	44 ± 30	8.7 ± 3.7	2.2 ± 0.9	338 ± 24
25 ºC + PD81,723	73 ± 32	9.9 ± 4.3	2.3 ± 0.4	93 ± 22
0 ºC	55 ± 30	3.9 ± 0.3	7.0 ± 3.0	128 ± 64

[a]: data are from Heitman et al. 2006[6].

Effect of temperature on the affinity of LUF6037

To study the effect of temperature on the displacement of [^{3}H]DPCPX with LUF6037, radioligand binding studies were performed at 25 ºC and 0 ºC (see Table 6.1). Neither the affinity for the high affinity site nor the affinity for the low affinity site was strongly affected.

From these affinities the equilibrium binding association constants, K_A (=1/K_i), were calculated. The thermodynamic parameters ΔG^0 (Gibbs energy), ΔH^0 (standard enthalpy) and ΔS^0 (standard entropy) were determined with a van 't Hoff plot (Figure 6.4 and Table 6.2). The interaction of both CPA[6] and LUF6037 with the high affinity site is entropy driven and endothermic. The interaction with the low affinity state/site was also similar for the two compounds, being exothermic and partially entropy and partially enthalpy driven.

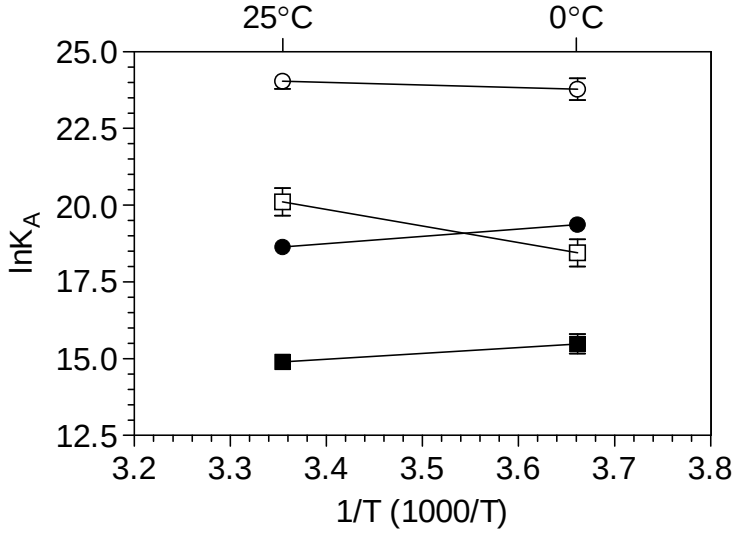

Figure 6.4. Van 't Hoff plots showing the effect of temperature on the equilibrium binding constants, K_A, for the high and low affinity binding sites of LUF6037 (open and closed circles, respectively) and CPA (open and closed squares, respectively). The thermodynamic parameters are presented in Table 6.2.

Table 6.2. Thermodynamic parameters, ΔG^0, ΔH^0 and ΔS^0, for the high and low affinity binding of LUF6037 to the human adenosine A_1 receptors. Data for CPA are shown for comparison.

Compound	ΔG^0 kJ mol^{-1}	ΔH^0 kJ mol^{-1}	ΔS^0 J mol^{-1} K^{-1}
LUF6037 (high)	-59 ± 2	7.0 ± 12	223 ± 40
LUF6037 (low)	-46 ± 9	-19.7 ± 5.7	89 ± 20
CPA (high) [a]	-50 ± 1	45 ± 17	318 ± 60
CPA (low) [a]	-37 ± 1	-16 ± 9	71 ± 31

[a]: data are from Heitman et al. 2006[6].

Efficacy of LUF6037

LUF6037 was further tested in cAMP experiments on CHO cells expressing the human adenosine A_1 receptor. For comparison the reference agonist CPA and the reference inverse agonist DPCPX were also tested. The compounds were tested at the concentrations indicated (see Figure 6.5). The basal cAMP production in the cells was set at 0%, whereas the amount of cAMP produced in the presence of 10 μM forskolin was set at 100%. The reference agonist CPA (30 nM) inhibited the cAMP production to 27 ± 7%, whereas the reference inverse agonist DPCPX (100 nM) increased the cAMP production to 143 ± 15%. LUF6037 (30 nM) was equipotent to CPA resulting in an inhibition of the cAMP production to 34 ± 5%. DPCPX at a concentration of 100 nM was able to antagonize the effect of both CPA and LUF6037 to a similar extent resulting in values of 102 ± 9% and 109 ± 9%, respectively.

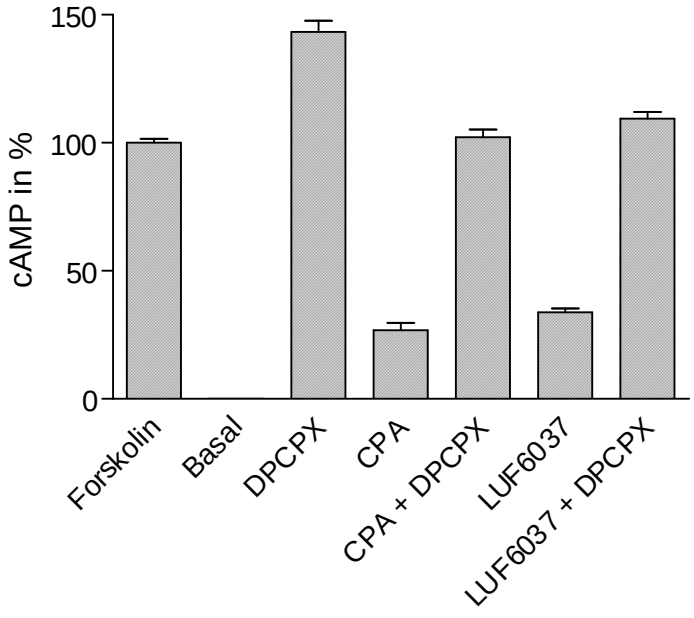

Figure 6.5. Modulation of forskolin-induced cAMP production in CHO cells stably expressing the human adenosine A_1 receptor, after exposure to reference ligands (CPA, DPCPX) or LUF6037. DPCPX was tested at a concentration of 100 nM, whereas CPA and LUF6037 were tested at a concentration of 30 nM.

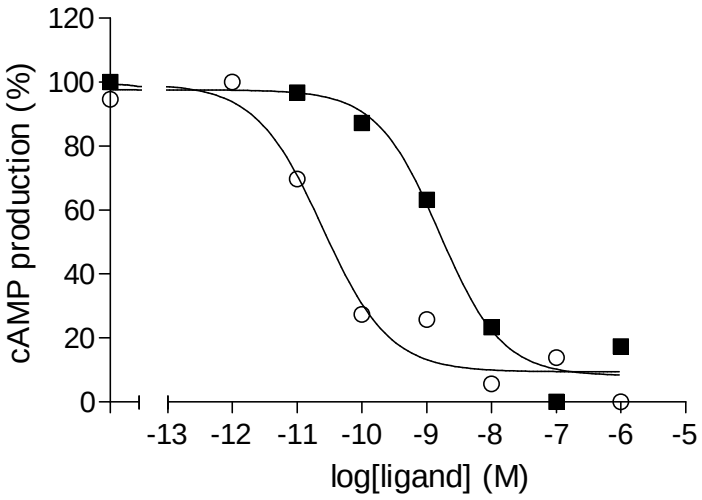

Figure 6.6. Representative curves of the inhibition of cAMP production by CPA (■) and LUF6037 (○) in CHO cells stably expressing the human adenosine A_1 receptor (n = 5).

In addition, full dose response curves for both CPA and LUF6037 were made (Figure 6.6). Analysis of these data revealed EC_{50} values for CPA and LUF6037 of 2.1 ± 0.7 nM and 60 ± 50 pM, respectively. These EC_{50} values are similar to the affinities of CPA and LUF6037 for the high affinity state/site of the receptor, as obtained in the radioligand binding studies.

Internalization of the human adenosine A_1 receptor by LUF6037

CHO cells stably expressing the human adenosine A_1YFP receptor were incubated for 16 h with CPA and LUF6037 at a concentration of 4 μM. Transsections of the cells were studied with confocal microscopy, and the amount of plasma membrane fluorescence was assessed. In Figure 6.7 the percentage fluorescence retained in the plasma membrane is shown for the ligand-free situation as well as upon incubation with either CPA or LUF6037. Whereas CPA significantly reduced the number of plasma membrane bound receptors, LUF6037 was without effect, very similar to a control experiment with the antagonist/inverse agonist DPCPX (4 μM). The respective percentages of fluorescence retained on the plasma membrane were 38 ± 5%, 91 ± 4%, 80 ± 3% and 85 ± 5% for CPA, DPCX, LUF6037 and the control situation, respectively. Representative transsections of confocal images are presented in Figure 6.8.

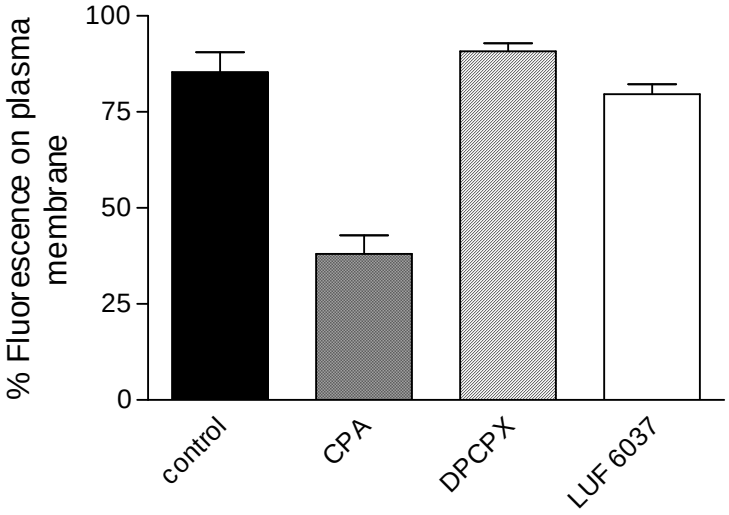

Figure 6.7. Percentage membrane fluorescence on plasma membranes of CHO cells stably expressing human adenosine A_1YFP receptors, after 16 h exposure to CPA, DPCPX or LUF6037 at a concentration of 4 μM, respectively.

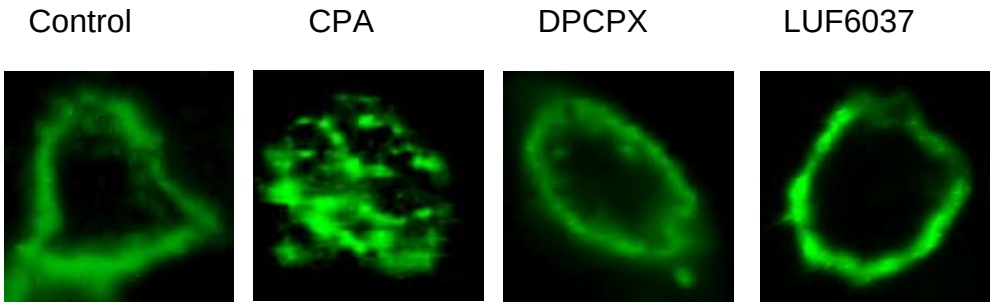

Figure 6.8. Confocal fluorescent pictures of internalized human adenosine A_1YFP receptors in CHO cells. The cells were incubated for 16 hours with CPA, DPCPX or LUF6037, each at a concentration of 4 μM. Pictures were taken from a representative transsection of the cell.

Apparently, LUF6037 is able to inhibit cAMP production as effectively as the full agonist CPA, but differs from CPA with respect to its sensitivity to PD81,723 and its ability to induce receptor internalization.

Discussion

Fuelled by a number of patent disclosures[2,3], we recently synthesized LUF6037. This compound is structurally unrelated to the endogenous ligand adenosine, the most striking feature being the absence of a ribose moiety, which had always been thought to be responsible for 'agonistic' behaviour and activation of the receptor[17,18]. In this study, LUF6037 was tested on its ability to bind, activate and induce internalization of the adenosine A_1 receptor.

Affinity of LUF6037 for the human adenosine A_1 receptor

Previously we reported on a structural analogue of LUF6037, LUF5831[6]. LUF5831 recognized a single state/site of the human adenosine A_1 receptor and was a partial

agonist. In line with the full agonism displayed by LUF6037 in the cAMP experiments (see below), LUF6037 recognized two states of the receptor, a high and a low affinity state just like the reference full agonist CPA. However, LUF6037 was almost 100-fold more potent than CPA, one of the typical high affinity reference agonists in this research field.

Effect of the allosteric modulator PD81,723 on the affinity of LUF6037

PD81,723 has been classified as an allosteric enhancer as it increases the affinity of CPA for the human adenosine A_1 receptor [10,13,15,19]. More precisely, addition of this allosteric enhancer at a concentration of 10 μM resulted in a leftward shift of the [^{3}H]DPCPX displacement curve affecting the low, but not the high affinity state/site of the receptor[6].

On the contrary, PD81,723 did not have an allosteric effect on LUF6037. The affinity of LUF6037 was not affected by the presence of PD81,723, indicating that the change in receptor conformation induced by PD81,723 is unable to enhance the binding of this non-adenosine compound to the human adenosine A_1 receptor. One explanation could be that the non-adenosine ligands bind to a different binding site, insensitive to PD81,723, since the binding of the partial agonist LUF5831 was not sensitive to PD81,723 either[6]. The ability of the non-adenosine ligands to displace [^{3}H]DPCPX binding, just like the adenosine derivatives, suggests however that these ligands share – at least partly – the same binding site. These findings also show the general importance of defining the object of allosteric enhancement. In this case, PD81,723 is an allosteric enhancer of CPA binding, not of LUF6037 binding, despite the agonistic nature of both CPA and LUF6037.

Effect of temperature on affinity of the affinity of LUF6037

Thermodynamic experiments can be used to characterize ligand binding at GPCRs[20]; for review see Borea et al.[21]. In these studies interaction of an agonist with the adenosine A_1 receptor appeared entropy driven whereas the binding of antagonist was essentially enthalpy driven. Partial agonists display intermediate behaviour both for adenosine derivates [14,21,22] and for the non-adenosine ligand LUF5831[6]. Based on these criteria LUF6037 should be classified as a full agonist as the thermodynamic experiments at 25 ºC and 0 ºC demonstrated that the interaction with the high affinity state is fully entropy driven. Indeed the interaction of LUF6037 largely mimics CPA with respect to interaction with both the low and high affinity sites, although the ΔH^o (enthalpy) values are quite different.

Efficacy of LUF6037

LUF6037 was tested on its ability to activate the human adenosine A_1 receptor. The second messenger cAMP was used as a read-out to determine the efficacy of LUF6037. In line with the displacement experiments and the thermodynamic analysis, LUF6037 was a very potent full agonist for the human adenosine A_1 receptor with a picomolar EC_{50} value, closely mimicking the value of $K_{i,H}$ obtained in the radioligand binding studies. Its effect at a single concentration of 30 nM could be reversed with the antagonist/inverse agonist DPCPX (100 nM), suggesting a competitive interaction between the two ligands.

Internalization of the human adenosine A_1 receptor by LUF6037

Stimulation of GPCRs with agonists often results in internalization of the receptor. In this process, the receptors are transported from the plasma membrane to endosomal compartments, thereby contributing to desensitization, a loss of functional response[23]. Several studies have confirmed that treatment with the agonist *R*-PIA, an adenosine derivative, induces internalization of the adenosine A_1 receptor [24,25,26]. Upon stimulation a decrease in number of membrane-bound receptors was found, but an increase of adenosine A_1 receptors in light vesicles, indicating that internalization was the underlying process of the desensitization of the receptor[24]. Recently, we engineered a yellow fluorescent protein (YFP) to the C-terminus of the human adenosine A_1 receptor. By making the receptor fluorescent we could easily detect and quantify internalization with confocal microscopy. In that study CPA dose-dependently promoted internalization of the human adenosine A_1 receptor upon incubation during at least 16 hrs. Moreover, addition of 10 μM PD81,723 did not accelerate the process of internalization, but rather lowered the minimal concentration of CPA needed to induce internalization[10].

Since LUF6037 behaved as a full agonist in the radioligand binding as well as the second messenger studies we also evaluated the ability of LUF6037 to induce internalization of the human adenosine A_1YFP receptor. In contrast to CPA, LUF6037 was not able to induce receptor internalization. These findings differ from more commonly observed results such as reported by Schlag et al.[27] in their research on the 5-HT_{2C} receptor, who observed internalization levels corresponding to agonist intrinsic activity.

Receptor activation versus internalization

Apparently the high affinity and efficacy displayed by LUF6037 do not necessarily lead to internalization of receptors after exposure to this ligand. Although LUF6037 acts as a full agonist, effectuating down-stream inhibition of adenylyl cyclase, thereby decreasing cAMP formation, β-arrestins necessary to induce internalization are most

probably not attracted by the ligand-receptor complex. In that case, the broad spectrum of signaling molecules recruited upon β-arrestin activation as recently reviewed by Lefkowitz and Shenoy[28] will also be by-passed.
Complex signaling behaviour, also coined “collateral efficacy”, in which receptor activation, phosphorylation and internalization are not always causally related, has been recently reviewed by Maudsley et al.[29] and Kenakin[30]. For instance agonists may effectuate receptor phosphorylation but are unable to either activate the second messenger system or to induce receptor internalization as observed with the angiotensin analogue [$Sar^{1,}$ Ile^{4}, Ile^{8}]AngII on the wild-type AT_{1A} receptor[31].
The opposite may also occur whereby the ligand mediates receptor internalization without activating the second messenger pathway. Such behaviour was reported for amino-truncated forms of parathyroid hormone[32] as well as for CCK analogues[33] that mediated internalization but not activation of their respective receptors. Keith et al.[34] demonstrated that both enkephalins and morphine activate δ- and μ-opioid receptors to inhibit adenylyl cyclase, but only enkephalins are able to induce rapid receptor internalization. Yu et al.[35] subsequently showed that a direct relationship existed between the efficacy of morphine and its analogues and their ability to induce phosphorylation and desensitization of the μ-opioid receptor. The $5\text{-}HT_{2C}$ receptor provides yet another example, in which the capacity of agonists to elicit desensitization was not related to their efficacy to activate signaling[36].
LUF6037 extends the list of examples of collateral efficacy in which a full agonist lacks the ability to induce receptor internalization.

In conclusion, a new agonist for the adenosine A_1 receptor with a structure completely different from the classic adenosine-like compounds was pharmacologically evaluated. This ligand, LUF6037, showed a very high affinity for the human adenosine A_1 receptor, with an affinity far extending beyond that of the reference full agonist CPA. 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, suggest that LUF6037 interacts with the receptor binding pocket in a partially different way from the known adenosine-like agonists. The data show the first proof of separation between activation and internalization of the adenosine A_1 receptor by a full agonist with 100-fold higher affinity and 35-fold greater potency with respect to the reference agonist CPA. The ability of LUF6037 to fully activate the receptor without inducing internalization is a novel example of collateral efficacy. This

behaviour may be therapeutically advantageous as drug resistance due to receptor internalization, as seen with traditional full agonists, might be avoided.

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CHAPTER 7

[^{3}H]LUF5834, a new non-adenosine radioligand for the adenosine A_1 receptor, revealing 3 binding sites for DPCPX on the A_1 receptor

Recently, non-adenosine ligands for the adenosine receptors were synthesized. One of these ligands, LUF5834, a high affinity full agonist for the A_1 receptor, was radioactively labeled. Here we report on the characterization of [^{3}H]LUF5834 as a new radioligand for the A_1 receptor. Radioligand binding studies were performed in the absence and presence of the allosteric enhancer PD81,723 and GTP on CHO cells stably expressing the A_1 receptor. [^{3}H]LUF5834 recognized two binding sites in kinetic and saturation studies. K_d-values of 0.16 ± 0.07 nM and 1.69 ± 0.05 nM were determined. This new radioligand [^{3}H]LUF5834 recognized the same number of receptors as [^{3}H]DPCPX. Surprisingly, unlabeled LUF5834 showed a one site displacement curve with an IC_{50}-value of 5.4 ± 1.3 nM. PD81,723 (10 μM) and GTP (1mM) were not able to shift its affinity. CPA yielded a two site displacement curve with IC_{50}-values of 4.8 ± 1.9 nM and 339 ± 150 nM, respectively. PD81,723 was able to increase only the $IC_{50,low}$-value of CPA. GTP did not affect the IC_{50}-values, but lowered the fraction of receptors in the high affinity state. DPCPX showed a three site displacement curve with IC_{50}-values of 1.2 ± 1.4 pM, 0.26 ± 0.24 nM and 127 ± 44 nM respectively. PD81,723 decreased all affinities, GTP on the other hand only shifted the percentages of receptors in the direction of the high affinity states. In conclusion, [^{3}H]LUF5834 recognizes two binding sites with very high affinity. Moreover, the displacement of [^{3}H]LUF5834 by DPCPX reveals not two, but three binding sites.

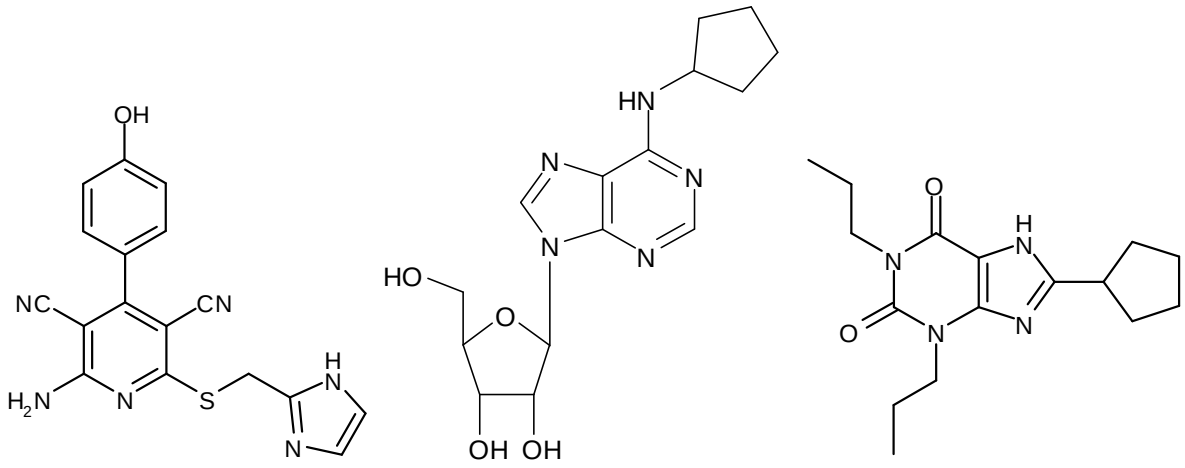
Based upon Klaasse EC, Chang LCW, de Vries H, de Grip WJ, IJzerman AP, Beukers MW. Manuscript in preparation.

Introduction

Recently, Rosentreter *et al.*[1,2] patented a new class of adenosine receptor ligands, the 2-amino-4-(3,4 substituted phenyl)-6-(2-hydroxyethylsulfanyl)-pyridin-3,5-dicarbonitriles. This class shows no structural similarity to adenosine, the endogenous ligand for the adenosine receptors. However, it was observed that several compounds of this class display a significant affinity and efficacy towards different adenosine receptor subtypes[1-4]. Until then, only modified analogues of adenosine substituted at different positions had been introduced as potent and selective ligands for the adenosine receptors. Especially substitutions at the N^6- and C2 positions of adenosine yielded potent adenosine A_1 agonists, e.g. N^6-cyclopentyladenosine (CPA), N^6-2-chloro-cyclopentyladenosine (CCPA) and N^6-cyclohexyladenosine (CHA)[5].

One of the new, non-adenosine compounds, LUF5831, appeared to be a partial agonist with an affinity of 18 $\pm$ 1 nM for the adenosine A_1 receptor[6]. From this study it also appeared that allosteric modulators such as PD81,723 and GTP seem to have no or much less effect on the binding of LUF5831 than they have on the binding of the traditional adenosine derivative CPA[6]. Another non-adenosine compound, LUF5834, was characterized as a full agonist for the human adenosine A_1 receptor with a very high affinity, comparable to the $K_{i,\ high}$-value of CPA, and as a partial agonist for the A_{2B} receptor with high affinity[3]. For these reasons, LUF5834 was chosen as the non-ribose ligand to radioactively label with ^{3}H.

In this study, we describe the characterization of the new, non-adenosine agonist radioligand for the human adenosine A_1 receptor. Displacement experiments were performed, and the effect of PD81,723 and GTP on the displacement of a non-adenosine-like agonist (LUF5834), adenosine-like agonist (CPA), and an inverse agonist (DPCPX) was studied (see Figure 7.1 for the chemical structures of the compounds). Surprisingly, [^{3}H]LUF5834 revealed three binding sites in the displacement with DPCPX. Finally, the influence of G proteins on the different affinity states of the receptor was studied using Sf9 Insect cell homogenates, expressing the human adenosine A_1his_{10} receptor but lacking functionally active endogenous G_i proteins.



LUF5834 CPA DPCPX

Figure 7.1. Structures of LUF5834, CPA and DPCPX.

Methods

Cell culture

Chinese hamster ovary (CHO) cells stably expressing the human adenosine A_1 receptor were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium containing 10% newborn calf serum, 50 µg/mL streptomycin, 50 IU/mL penicillin and 0.2 mg/mL neomycin (G418), respectively. The cells were maintained in a humidified atmosphere at 37 ºC and 5% CO_2, and subcultured twice weekly (ratio 1:20).

Spodoptera frugiperda (Sf9) cells were cultured in Insect Xpress medium containing 50 µg/mL streptomycin and 50 IU/mL penicillin at 27 ºC. The cells were subcultured twice weekly (1:5). Confluent Sf9 Insect cells were infected with baculovirus, containing the human adenosine A_1his_{10} construct. Six to seven days post infection, the infected Sf9 Insect cells expressing the human adenosine A_1his_{10} receptor were harvested by centrifugation (5 min, 1300 rpm) and the cell pellets were stored at – 80 ºC.

Membrane preparation

Membranes of CHO cells stably expressing the human adenosine A_1 receptor were prepared as previously described[7]. Membrane protein concentrations were measured using the BCA (bicinchonic acid) method with BSA as a standard[8].

Radioligand-binding assays

Membrane aliquots containing 10 µg ($CHOhA_1$-wt) protein were incubated in a total volume of 400 µl of 50 mM Tris-HCl, 0.1% CHAPS, ADA (1U/mL) pH 7.4, at 25 ºC

for 60 min in the absence or presence of 1 mM GTP or 10 μM PD81,723. Displacement experiments were performed using 24 concentrations of cold ligand in the presence of 2.6 nM [^{3}H]LUF5834. Nonspecific binding was determined in the presence of 10 μM CPA and represented approximately 10% of the total binding. Saturation experiments were carried out using nine to 21 different concentrations of [^{3}H]LUF5834, [^{3}H]DPCPX or [^{3}H]LUF5834/[^{3}H]DPCPX ranging from 0.1 to 12 nM. In kinetic studies, the association of the radioligand [^{3}H]LUF5834 (2.6 nM) was initiated by addition of the membrane preparation (10 μg) to the radioligand. To study the dissociation of [^{3}H]LUF5834, membranes were preincubated with [^{3}H]LUF5834 (2.6 nM) at 25 ºC for 60 min. Dissociation of [^{3}H]LUF5834 was then initiated by the addition of LUF5834 (1 μM), DPCPX (1 μM) or CPA (10 μM). Incubations were terminated by dilution with ice-cold 50 mM Tris-HCl buffer. Separation of bound from free radioligand was performed by rapid filtration through Whatman GF/C filters using a Brandel harvester. Filters where subsequently washed three times with ice-cold buffer, or six times for saturation experiments. Filter-bound radioactivity was measured by scintillation spectrometry (Tri-Carb 2900TR, Perkin Elmer) after addition of 3.5 mL Packard Emulsifier Safe. Experiments were performed at least in triplicate, unless otherwise stated.

Data analysis

Data of radioligand binding experiments were analyzed using the non-linear regression curve fitting program Prism v. 4.0.2 (GraphPad, San Diego, CA, USA). k_{on} and k_{off} values were obtained by computer analysis of the association and dissociation data. Both association and dissociation experiments showed a two site binding profile for [^{3}H]LUF5834.

Saturation curves were fitted to one and two state/site binding models. For [^{3}H]DPCPX and [^{3}H]DPCPX/[^{3}H]LUF5834, a one site binding curve was favoured. For [^{3}H]LUF5834 saturation however, a two site binding hyperbola was preferred. Radioligand displacement curves were fitted to one, two and three state/site binding models. The LUF5834 displacement curve was best fitted to a one state/site binding model, CPA data were best fitted to a two state/site binding model. For DPCPX however, a three state/site binding model was preferred. IC_{50}-values were taken instead of calculated K_i-values, since we did not attempt to attribute K_d-values to each affinity state, especially in the case of the three site binding model.

Three site equation

Since the datapoints of the DPCPX displacement curves did not fit a two site displacement curve very well, a three site competition equation was incorporated in

Prism to check whether the datapoints would fit better to a three site model. Analogous to two site competition, a three site competition equation was written:

$$Y = Bottom + PART1 + PART2 + PART3$$
$$PART1 = Span \times \frac{Fraction1}{1+10^{(X-LogIC_{50-1})}}$$
$$PART2 = Span \times \frac{Fraction2}{1+10^{(X-LogIC_{50-2})}}$$
$$PART3 = Span \times \frac{1-(Fraction1+Fraction2)}{1+10^{(X-LogIC_{50-3})}}$$
$$Span = Top - Bottom$$

with Y: the binding of the radioligand, and X: the log concentration of unlabeled (competing) ligand. Fraction 1 is the fraction of all sites that have affinity 1, and the same applies to fractions 2 and 3. Log $IC_{50,1}$, Log $IC_{50,2}$ and Log $IC_{50,3}$ are the Log IC_{50}-values for the three sites.

Materials

N^6-cyclopentyladenosine (CPA) was obtained from Research Biochemicals Inc. (Natick, MA, U.S.A.). 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), bovine serum albumin (BSA) and 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) were from Sigma (St. Louis MO, U.S.A.). Adenosine deaminase (ADA) was purchased from Roche Biochemicals (Mannheim, Germany) and Bicinchonic acid (BCA) protein assay reagent was obtained from Pierce Chemical Company (Rockford, IL, U.S.A.). [^{3}H] 1,3-dipropyl-8-cyclopentylxanthine ([^{3}H]DPCPX -specific activity 124 Ci/mmol) was purchased from NEN (Du Pont Nemours, 's Hertogenbosch, The Netherlands). [^{3}H] 2-amino-4-(4-hydroxyphenyl)-6-(1*H*-imidazol-2ylmethylsulfanyl)-pyridine-3,5-dicarbonitrile ([^{3}H]LUF5834 – specific activity 25 Ci/mmol) was labeled by Sibtech, Inc. (Newington, CT, U.S.A.). The radiolabeled ligand was purified over a Kromasil C18 column with a gradient of 10% to 50% MeCN/H_2O/0.1% TFA. TLC on SiO_2 was developed with CH_2Cl_2:MeOH:NH_4OH (10:1.5:0.25). TLC on C18 was developed with MeOH:0.1M NH_4OAc pH 3.5 (3:1). G418 (neomycin) was obtained from Stratagene (Cedar Creek, U.S.A.). Guanosine triphosphate (GTP) was purchased from Acros Organics (Geel, Belgium). (2-Amino-4,5-dimethyl-3-thienyl)-[3-(trifluoromethyl)phenyl]methanone (PD81,723) and LUF5834 were synthesized in our laboratory as described by Van der Klein et al.[9] and Chang et al.[4], respectively. Chinese hamster ovary (CHO) cells stably expressing the human adenosine A_1 receptor were obtained from A. Townsend – Nicholson[10]. Sf9 insect cells were obtained from BD-Biosciences (Alphen aan den Rijn, the Netherlands). Insect Xpress Protein Free Insect Cell Growth Media were

obtained from Cambrex (Verviers, Belgium), Dulbecco's Modified Eagle's Medium and F-12 Ham medium were purchased from Sigma Aldrich (Zwijndrecht, the Netherlands). All other chemicals were of analytical grade and obtained from standard commercial sources.

Results

Radioligand-binding assay

As a starting point for the set up of a radioligand binding assay for [^{3}H]LUF5834, the radioligand-binding assay for [^{3}H]DPCPX was taken. CHOhA_1-wt membrane protein (10 μg) was incubated in a total volume of 400 μl Tris-HCl buffer, 50 mM, pH 7.4 as described in the Methods section. On control CHO-K1 cell membranes, [^{3}H]LUF5834 did not display any specific binding. To maximize the window of specific binding to the CHOhA_1-wt membranes, the following parameters were varied: GF/B vs GF/C glass fiber filters, with or without pretreatment of the filters with 0.25% polyethylene imine (pei) for 1 hour. The effect of addition of 0.1% CHPAS or 0.1% BSA to the assay buffer was also verified. It appeared that GF/B and GF/C filters performed equally well; only the addition of 0.1% CHAPS increased the window of specific binding significantly (results not shown). It was therefore decided to use the radioligand-binding assay of [^{3}H]DPCPX plus 0.1% CHAPS added to the assay buffer. The samples were harvested over GF/C filters.

Kinetics of [^{3}H]LUF5834

To characterize the new, non-adenosine radioligand [^{3}H]LUF5834, kinetic association and dissociation experiments on membranes of CHO cells stably expressing the wt human adenosine A_1 receptor were performed.

Association of [^{3}H]LUF5834 reached a plateau in 30 min and appeared to be biphasic (Figure 7.2A). From these experiments, the k_{on} values and half lives were determined (Table 7.1). The $k_{on,1}$ value was 2.01 ± 0.26 nM^{-1} min^{-1} and $k_{on,2}$ value was 0.12 ± 0.03 nM^{-1} min^{-1}. The corresponding half lives were 0.35 ± 0.04 min and 6.01 ± 1.33 min.

Dissociation of [^{3}H]LUF5834 was achieved by adding CPA (10 μM) or LUF5834 (1 μM), see Figure 7.2B. In both cases, the dissociation of [^{3}H]LUF5834 appeared to be biphasic. Full dissociation, i.e. to levels of non-specific binding, was reached within 60 minutes. In table 7.1, the radioligand half-life and k_{off} values are represented.

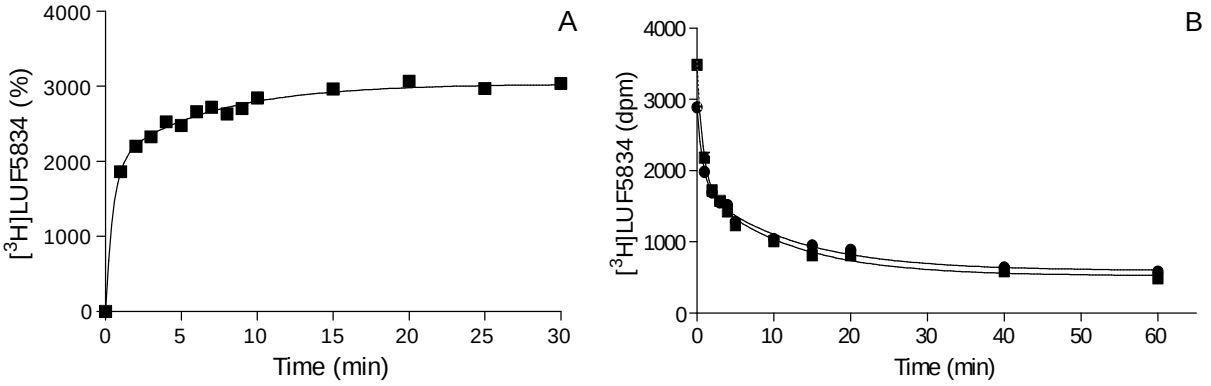


Figure 7.2. Association of [^{3}H]LUF5834 (A) to and dissociation of [^{3}H]LUF5834 (B) from the wt human adenosine A_1 receptor stably expressed on CHO cell membranes. Dissociation of [^{3}H]LUF5834 was achieved by adding CPA (10 μM,●) or LUF5834 (1 μM,■). Experiments were performed three times, curves from a representative experiment performed in duplicate are shown.

Table 7.1. Association and dissociation kinetic parameters (± s.e.m.) of [^{3}H]LUF5834 binding to wt human adenosine A_1 receptors. Dissociation was induced with either CPA (10 μM) or LUF5834 (1 μM). Significant differences between ligands are marked with an asterisk. Values are means (± s.e.m.) of three separate assays each performed in duplicate.

Association

	$t_{1/2,1}$ (min)	$t_{1/2,2}$ (min)	$k_{on,1}$ (nM^{-1} min^{-1})	$k_{on,2}$ (nM^{-1} min^{-1})
[^{3}H]LUF5834	0.35 ± 0.04	6.01 ± 1.33	2.01 ± 0.26	0.12 ± 0.03

Dissociation

	$t_{1/2,1}$ (min)	$t_{1/2,2}$ (min)	$k_{off,1}$ (min^{-1})	$k_{off,2}$ (min^{-1})
CPA	0.35 ± 0.21	8.5 ± 0.60	2.60 ± 1.64	0.088 ± 0.006
LUF5834	0.50 ± 0.13	11.8 ± 5.55	1.43 ± 0.37	0.066 ± 0.031

As expected, the half lives for the two states were the same independent of the use of CPA or LUF5834 as the displacing ligand. Since these studies provide insight in ligand binding rather than activation, we cannot specify whether the two sites represent two states i.e. G protein dependent affinities or two different binding sites. The half lives for the high affinity states/sites, were 0.35 ± 0.21 min and 0.50 ± 0.13 min (CPA and LUF5834, respectively). The half lives for the low affinity states/sites, were 8.5 ± 0.60 min vs 11.8 ± 5.55 min. From the k_{on}- and k_{off} values, kinetic K_d values were calculated according to the formula: $K_d = k_{off} / k_{on.}$ This yielded a kinetic $K_{d,1}$ value of 0.71 nM and $K_{d,2}$ value of 0.55 nM.

Saturation

Saturation experiments were performed with [^{3}H]LUF5834 on membranes of CHO cells stably expressing the wt adenosine A_1 receptor (Figure 7.3). The binding of [^{3}H]LUF5834 was saturable and best characterized by a two-site model with a $K_{d,1}$ value of 0.16 ± 0.07 nM and a $K_{d,2}$ value of 1.69 ± 0.05 nM. Given the large s.e.m. of the $k_{off,2}$ value of LUF5834, the calculated kinetic K_d values are in the same range as the K_d values determined in the equilibrium saturation studies.

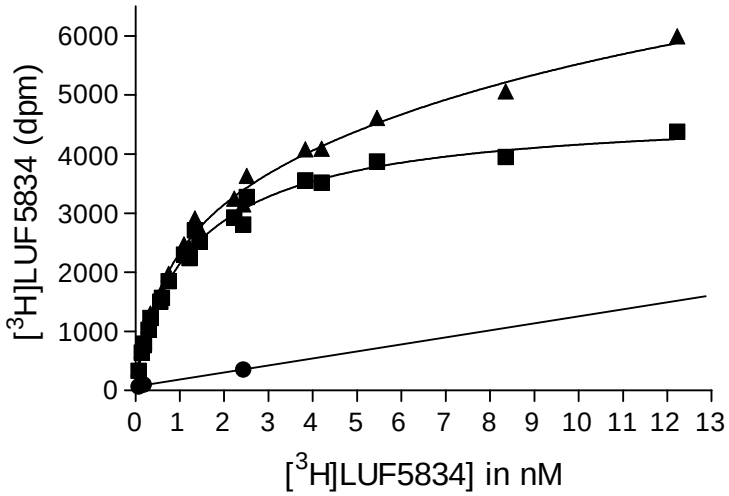


Figure 7.3. Equilibrium saturation curve of [^{3}H]LUF5834 on wt human adenosine A_1 receptor stably expressed on CHO cell membranes, total binding (▲), specific binding (■), non-specific binding (●). Experiments were performed three times, curves from a representative experiment performed in duplicate are shown.

The corresponding B_{max} values were 1.68 ± 0.05 pmol/mg ($B_{max,1}$) and 7.82 ± 0.84 pmol/mg ($B_{max,2}$), respectively, yielding a total receptor density of 9.50 pmol/mg (Table 7.2). The non-specific binding accounted for approximately 10% of the total binding. In addition, saturation experiments with [^{3}H]DPCPX and a combination of [^{3}H]LUF5834/[^{3}H]DPCPX were performed to show that both radioligands share the same binding site on the receptor (Figure 7.4). The differences in dpm values can be explained by the fact that [^{3}H]DPCPX has a five-fold higher specific activity than [^{3}H]LUF5834. These saturation curves fitted best to a one-site model, with K_d values of 2.87 ± 1.03 nM and an apparent "mixed" K_d value of 2.30 ± 0.48 nM for the combined ([^{3}H]LUF5834/[^{3}H]DPCPX) saturation, respectively. The calculated B_{max} values were 12.8 ± 1.7 pmol/mg and 13.2 ± 1.1 pmol/mg respectively, comparable to the B_{max} value found with [^{3}H]LUF5834 saturation alone.

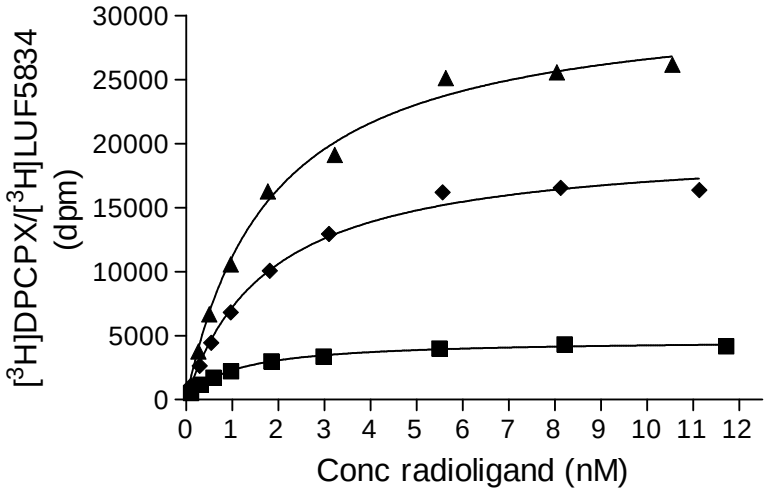


Figure 7.4. Saturation curves of [^{3}H]LUF5834 (■), [^{3}H]DPCPX (▲), or a combination of [^{3}H]LUF5834/[^{3}H]DPCPX (♦) on wt human adenosine A_1 receptor stably expressed on CHO cell membranes. Experiments were performed three times, curves from a representative experiment performed in duplicate are shown.

Table 7.2. K_d-values (± s.e.m.) of [^{3}H]LUF5834, [^{3}H]DPCPX and an apparent 'mixed' K_d-value for the combination of [^{3}H]LUF5834/ [^{3}H]DPCPX for the wt human adenosine A_1 receptor, stably expressed in CHO cell membranes. Corresponding B_{max}-values are also presented. Values are means (± s.e.m.) of three separate assays each performed in duplicate.

	K_d (nM)	$K_{d,1}$ (nM)	$K_{d,2}$ (nM)	B_{max} (pmol/mg)	$B_{max,1}$ (pmol/mg)	$B_{max,2}$ (pmol/mg)
[^{3}H]LUF5834		0.16 ± 0.07	1.69 ± 0.05		1.68±0.05	7.82±0.84
[^{3}H]DPCPX	2.87 ± 1.03			12.8±1.7		
[^{3}H]LUF5834/ [^{3}H]DPCPX	2.30 ± 0.48			13.2±1.1		

Displacement of [^{3}H]LUF5834

To determine the affinity of CPA, DPCPX and LUF5834, displacement experiments were performed with the new radioligand [^{3}H]LUF5834 on membranes of CHO cells stably expressing the wt human adenosine A_1 receptor (see Figure 7.5). LUF5834 recognized only one binding state/site with an IC_{50}-value of 5.4 ± 1.3 nM. CPA identified two binding states/sites with IC_{50}-values of 4.8 ± 1.9 nM and 339 ± 150 nM for the high and low affinity sites, respectively (see Table 7.3). DPCPX on the other hand recognized three binding states/sites, one with picomolar affinity (1.2 ± 1.4 pM), and the other two with nanomolar affinity (0.26 ± 0.24 nM and 127 ± 44 nM), respectively.

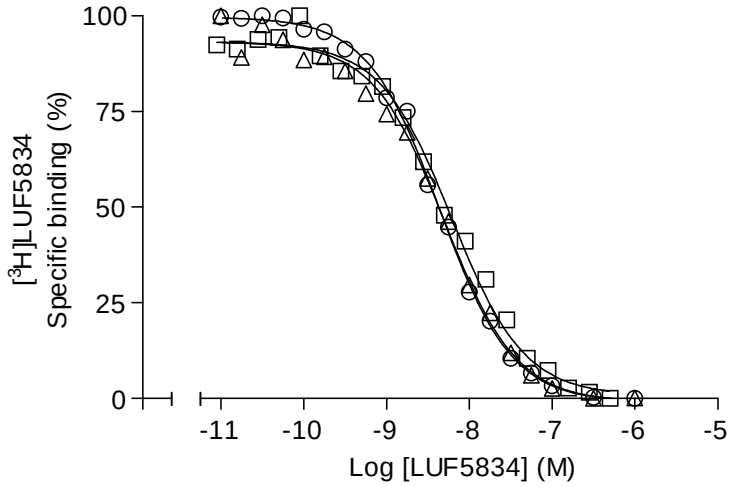


Figure 7.5. Displacement of [^{3}H]LUF5834 by LUF5834 from the wt human adenosine A_1 receptor stably expressed on CHO cell membranes, in the absence (○) or presence (Δ) of 1 mM GTP, or in the presence of 10 μM PD81,723 (□). Experiments were performed three times, curves from a representative experiment performed in duplicate are shown.

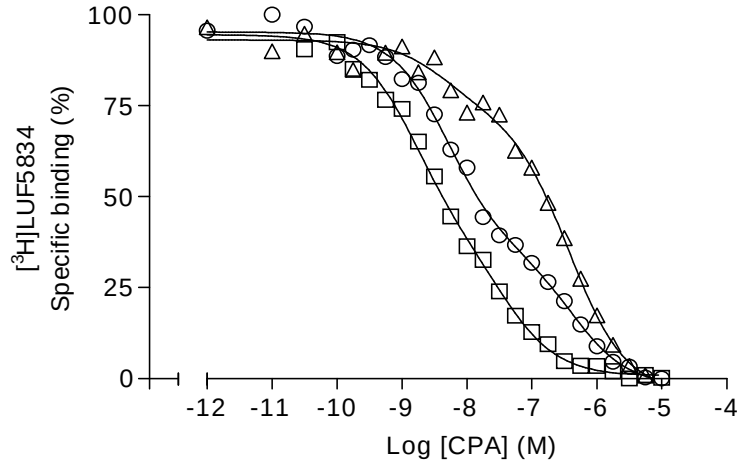


Figure 7.6. Displacement of [^{3}H]LUF5834 by CPA from the wt human adenosine A_1 receptor stably expressed on CHO cell membranes, in the absence (○) or presence (Δ) of 1 mM GTP, or in the presence of 10 μM PD81,723 (□). Experiments were performed three times, curves from a representative experiment performed in duplicate are shown.

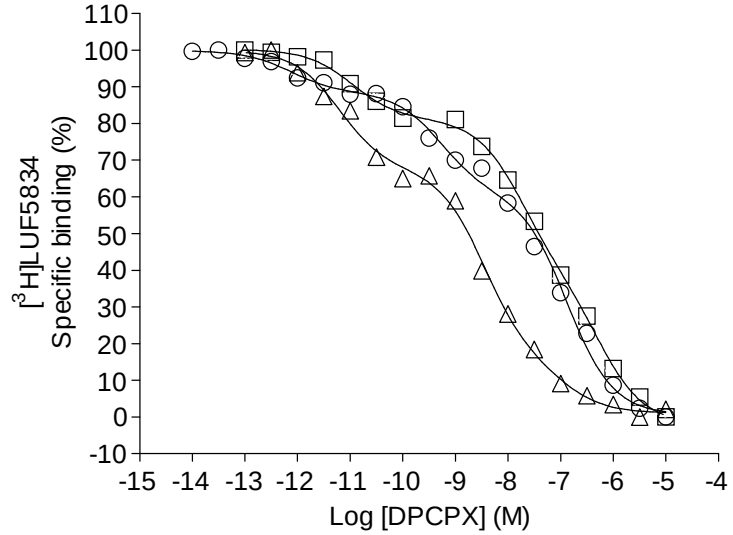


Figure 7.7. Displacement of [^{3}H]LUF5834 by DPCPX from the wt human adenosine A_1 receptor stably expressed on CHO cell membranes, in the absence (○) or presence (Δ) of 1 mM GTP, or in the presence of 10 μM PD81,723 (□). Experiments were performed three times, curves from a representative experiment performed in duplicate are shown.

Table 7.3. Affinities of LUF5834, CPA and DPCPX for the wt human adenosine A_1 receptor in the absence or presence of either 10 μM PD81,723 or 1 mM GTP, expressed as IC_{50}-values (± s.e.m.). Significant differences with respect to the control values are marked with an asterisk. Values are means (± s.e.m.) of three separate assays each performed in duplicate, unless indicated otherwise.

	$IC_{50,high\,2}$ (pM)	$IC_{50,high\,1}$ (nM)	$IC_{50,low}$ (nM)	Fraction in%	Fraction in%	Fraction in%
LUF5834		5.4 ± 1.3				
LUF5834 + GTP		7.2 ± 0.8				
LUF5834 + PD		6.3 / 4.5[a]				
CPA		4.8 ± 1.9	339 ± 150		56 ± 6	44 ± 6
CPA + GTP		7.7 ± 2.9	440 ± 67		22 ± 5***	78 ± 5
CPA + PD		2.5 ± 0.8	36 ± 15*		51 ± 14	49 ± 6
DPCPX	1.2 ± 1.4	0.26 ± 0.24	127 ± 44	23 ± 13	29 ± 8	48 ± 11
DPCPX + GTP	2.6 ± 2.2	3.7 ± 1.4 *	120 ± 22	38 ± 4	51 ± 4*	11 ± 3**
DPCPX + PD	9.2 ± 3.4*	8.9 ± 7.2	573 ± 91**	22 ± 10	29 ± 11	49 ± 8

***: P < 0.0001, **: P < 0.005, *: P < 0.05, [a] n=2

Effects of the allosteric modulator PD81,723 and GTP

Radioligand binding experiments were performed in the absence and presence of 1 mM GTP or 10 μM of the allosteric modulator PD81,723 (see Figures 7.5-7.7 and Table 7.3). In contrast to CPA and DPCPX, LUF5834 was virtually insensitive to both GTP and PD81,723. The IC_{50}-values of LUF5834 in the presence of GTP and PD81,723 were 7.2 ± 0.8 nM and 6.3/4.5 nM, respectively, almost identical to the control value of 5.4 ± 1.3 nM.

Addition of 1 mM GTP did not affect the affinity of CPA for either the high or low affinity states/sites. The $IC_{50,high}$ value was 4.8 ± 1.9 nM vs 7.7 ± 2.9 nM in the absence and presence of GTP, whereas the $IC_{50,low}$ value was 339 ± 150 nM vs 440 ± 67 nM, respectively. In contrast, the percentage receptors in the high affinity state decreased from 56 ± 6% to 22 ± 5% in the presence of 1 mM GTP.

Addition of 10 μM PD81,723 significantly lowered the $IC_{50,low}$ value to 36 ± 15 nM, whereas the $IC_{50,high}$ value remained unaltered, 2.5 ± 0.8 nM. The percentage receptors in the high affinity state was not affected by PD81,723 (51 ± 14%).

Like CPA, the affinities of DPCPX were not much affected by the addition of GTP. The $IC_{50,low}$-value and $IC_{50,high\,2}$ values remained unaltered 127 ± 44 nM vs 120 ± 22 nM and 1.2 ± 1.4 nM to 2.6 ± 2.2 nM, respectively and the $IC_{50,high1}$ value increased from 0.26 ± 0.24 nM to 3.7 ± 1.4 nM (P<0.05). In contrast, GTP did increase the

percentage receptors in the two high affinity states from 23 ± 13% to 38 ± 4% and from 29 ± 8% to 51 ± 4%, respectively.

Whereas addition of GTP resulted in a shift from low to high affinity states, PD81,723 affected only the IC_{50}-values. PD81,723 increased the $IC_{50,high}$ values to 9.2 ± 3.4 pM ($P < 0.05$) and 8.9 ± 7.2 nM, respectively, and significantly increased the $IC_{50,low}$ value to 573 ± 91 nM ($P<0.005$). The percentage receptors in the high affinity states was unaffected, 22 ± 10% vs 23 ± 13% and 29 ± 11% vs 29 ± 8%, respectively.

Sf9 insect cell preparation

Displacement studies on a cell preparation of Sf9 insect cells expressing the human adenosine A_1his_{10} receptor were also performed. [^{3}H]LUF5834 was displaced by CPA or DPCPX, in the absence or presence of 1 mM GTP (Figure 7.8). In both cases, the addition of GTP did not shift the displacement curve. The CPA displacement curve showed only one binding site, with an affinity comparable to the $IC_{50,low}$-value found in the displacement experiment performed on wt hA_1-CHO membranes (360 nM vs 339 ± 150 nM, table 7.4). The DPCPX displacement curve showed two binding sites, with affinities of 36 pM and 21 nM respectively.

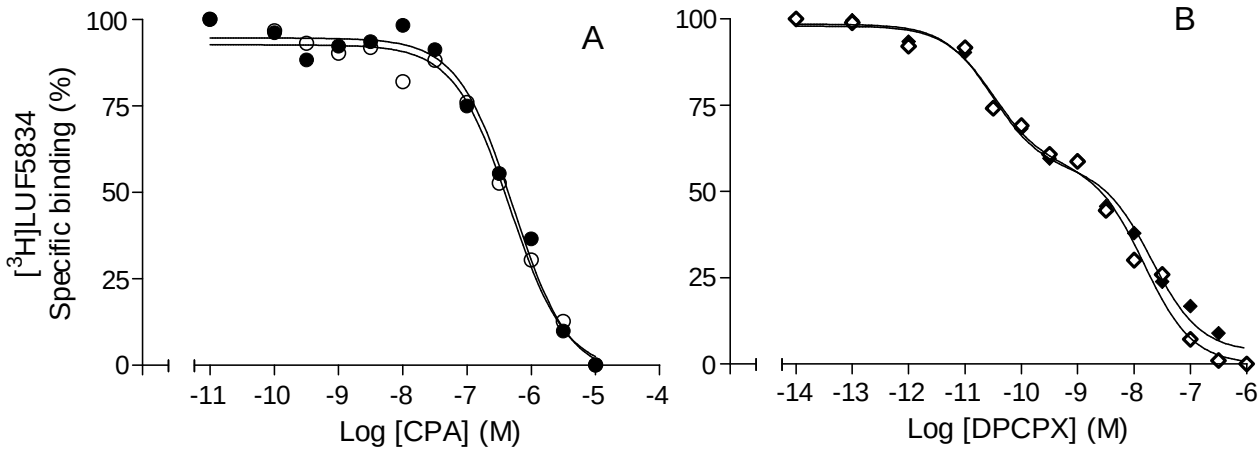


Figure 7.8. Displacement of [^{3}H]LUF5834 by CPA (A, ●) or DPCPX (B, ♦) from the human adenosine A_1his_{10} receptor expressed on Sf9 membranes, in the absence or presence of 1 mM GTP (○,◊). Experiments were performed three times, curves from a representative experiment performed in duplicate are shown.

Table 7.4. Affinities of CPA and DPCPX in the absence or presence of 1 mM GTP for the human adenosine A_1His_{10} receptor expressed in Sf9 Insect cells, expressed as IC_{50}-values. Two experiments (unless indicated otherwise) were performed, each in duplicate, yielding average IC_{50} values.

	IC_{50} (nM)	$IC_{50,high}$ (pM)	$IC_{50,low}$ (nM)	Fraction 1 (%)	Fraction 2 (%)
CPA	360				
CPA + GTP	504				
DPCPX		31*	21*	48	52
DPCPX + GTP		30	31	50	50

* n=1

Discussion

In this study, the new, non-ribose radioligand [^{3}H]LUF5834 was characterized for its kinetic, saturation and displacement properties on the human adenosine A_1 receptor. Also the effects of the allosteric modulators PD81,723 and GTP on the displacement of [^{3}H]LUF5834 were investigated. Finally, displacement studies of [^{3}H]LUF5834 on Sf9 Insect cell homogenates containing the human adenosine A_1 receptor were performed.

Kinetics of [^{3}H]LUF5834

Since LUF5834 had been shown to be a full agonist in cAMP experiments[3], the association and dissociation kinetics of [^{3}H]LUF5834 are best compared with other radiolabeled agonists. Characterization of ^{125}I-*N*^{6}-aminobenzyladenosine ([^{125}I]ABA) revealed a two site association. A $t_{1/2,1}$ of 1.85 min and a $t_{1/2,2}$ of 12.8 min were reported, five and two times slower, respectively than measured for [^{3}H]LUF5834[11]. [^{3}H]CCPA and [^{3}H]CHA, on the other hand, showed a one site association only, with a $t_{1/2}$ of 2.6 $\pm$ 0.3 min[12] and a $t_{1/2}$ value of 57.5 $\pm$ 14.4 min[13], respectively.

The $t_{1/2}$ for the dissociation of [^{3}H]CCPA was reported to be 10.8 $\pm$ 2.8 min[12], similar to the $t_{1/2,2}$ for the dissociation of [^{3}H]LUF5834. This is much faster than the k_{off} values reported for [^{3}H]CHA, 4.76 $\pm$ 0.26 min[13]. In conclusion, [^{3}H]LUF5834 has relatively fast kinetics among the agonist radioligands, and showed a two site association and dissociation profile.

Saturation

In line with the kinetic experiments, [^{3}H]LUF5834 also obeyed a two-site binding model in the saturation experiments. The $K_{d,1}$ value was 0.16 $\pm$ 0.07 nM and the $K_{d,2}$ value was 1.69 $\pm$ 0.05 nM. Other useful radioligands for the adenosine A_1 receptor also display affinities in the low nanomolar range, such as [^{3}H]DPCPX (2.87 $\pm$ 1.03 nM, (this study); 3.86 nM[14]; 1.6 $\pm$ 0.1 nM[6,13,15]; [^{3}H]CCPA (1.8 $\pm$ 0.2 nM)[15], [^{125}I]ABA, ($K_{d,1}$-value of 0.33 $\pm$ 0.16 nM and $K_{d,2}$-value of 10 $\pm$ 25 nM)[11]. The K_d-value of the

new, non-adenosine agonist radioligand [^{3}H]LUF5834 is thus in the same range of the K_d-values of the traditional agonist and inverse agonist radioligands for the human adenosine A_1 receptor. Additionally, the low non-specific binding of [^{3}H]LUF5834 makes it a suitable radioligand for binding studies.

The calculated B_{max}-values from the saturation curves (Figure 7.4) showed that both [^{3}H]DPCPX (12.8 ± 1.7 pmol/mg) and [^{3}H]LUF5834 (9.5 ± 0.9 pmol/mg) label approximately the same number of binding sites, despite the fact that LUF5834 is an agonist. The calculated B_{max} value from the combined [^{3}H]LUF5834/[^{3}H]DPCPX saturation experiment was 13.2 ± 1.1 pmol/mg, thus, no additional sites appeared occupied by [^{3}H]LUF5834 in the combined [^{3}H]LUF5834/[^{3}H]DPCPX saturation experiments. To compare, Kourounakis *et al.*[15] found that [^{3}H]CCPA when compared to [^{3}H]DPCPX labeled only 20% of the receptor binding sites. A putative explanation is that agonist radioligands such as [^{3}H]CCPA in low concentrations only label the receptors in the active, high affinity R* conformation. In contrast, the very high affinities of [^{3}H]LUF5834 (0.16 nM and 1.69 nM) allow full receptor occupancy in the concentration range studied (0-12 nM).

Displacement of [^{3}H]LUF5834

The displacement of [^{3}H]LUF5834 by LUF5834 followed a one site model with an IC_{50}-value of 5.4 ± 1.3 nM, equal to the affinity of LUF5834 displacing [^{3}H]DPCPX as the radioligand, 2.6 ± 0.3 nM[3]. The affinity of LUF5834 for the human adenosine A_1 receptor is slightly higher, but in the same range as the affinity of its close analogue LUF5831, 18 ± 1 nM[6]. The reason that only one K_i value is observed for LUF5834 in the displacement experiments versus two K_d values in the saturation experiments may be that the percentage receptors in the high affinity state was rather low (<20%). In addition, there were many more data points in the saturation curve around the $K_{d,1}$ value (10 points between 0 – 1 nM) than in the displacement curve (4 points). The displacement of [^{3}H]LUF5834 by CPA yielded a two site binding curve with IC_{50}-values of 4.8 ± 1.9 nM and 339 ± 150 nM, similar to the affinities found in displacement studies with [^{3}H]DPCPX as a radioligand, 2.2 ± 0.9 nM and 338 ± 24 nM[6]. LUF5834 thus has an affinity for the human adenosine A_1 receptor comparable to the $K_{i,high}$–value of CPA (5.4 ± 1.3 nM vs 2.2 ± 0.9 nM).

The displacement curve of DPCPX showed the most striking features. Not two, but three sites were observed. An $IC_{50,low}$- value of 127 ± 44 nM, and two $IC_{50,high}$-values were determined, one with nanomolar affinity (0.26 ± 0.24 nM) and the other one in the picomolar range (1.2 ± 1.4 pM). So far, a $K_{i,\ high}$ value of DPCPX with an affinity in the picomolar range, has never been observed before. Chang *et al.*[4] reported a K_i-value of 6.1 ± 1.6 nM and de Ligt *et al.*[16] reported a K_i-value of 2.4 ± 0.1 nM for DPCPX, which is in the same range as the $IC_{50,high\ 1}$-value. The percentage receptors

in the two high affinity states were 23 ± 13% and 29 ± 8% respectively, and 48 ± 11% of the receptors were in the low affinity state.

Effect of the allosteric modulator PD81,723 and GTP

Addition of 10 μM PD81,723, an allosteric enhancer[17,18], resulted in an increase in the affinity of CPA for the human adenosine A_1 receptor[6,7,15,19].

On the contrary, PD81,723 did not have an allosteric effect on the displacement of [^{3}H]LUF5834 by LUF5834. Similar results were previously reported for the displacement of [^{3}H]DPCPX by a structural analogue of LUF5834, LUF5831[6]. The affinities of LUF5834 and LUF5831 were apparently not affected by the presence of PD81,723, indicating that the change in receptor conformation induced by PD81,723 is unable to enhance the binding of these non-adenosine compounds to the human adenosine A_1 receptor. A possible explanation could be that non-adenosine ligands bind to a different binding site, insensitive to PD81,723. However, the ability of the non-adenosine ligands to displace [^{3}H]DPCPX binding, just like the adenosine derivatives, suggests that these ligands share – at least partly – the same binding site. The presence of 1 mM GTP did not shift the displacement curve of LUF5834 either. Apparently, the affinity of LUF5834 was not affected by the addition of GTP, which had also been observed for LUF5831[6]. Probably, the non-ribose agonists LUF5834 and LUF5831 have no prevalence for one of the two receptor conformations, R or R*.

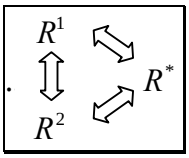
The addition of 10 μM PD81,723 in the [^{3}H]LUF5834/CPA displacement experiments only affected the $IC_{50,low}$-value of CPA; a ten-fold leftward shift was observed (339 ± 150 nM vs 36 ± 15 nM). The percentage receptors in the high affinity state was not affected by the presence of PD81,723. Heitman *et al.*[6] reported comparable data i.e. a 3.5-fold leftward shift of the low affinity site for CPA and no effect on the percentage receptors in the high affinity state. The addition of 1 mM GTP did not alter the affinities of CPA for the receptor, it only decreased the percentage receptors in the high affinity state from 56 ± 6% to 22 ± 5%. Heitman *et al.*[6] observed an even more pronounced effect in the experiments with [^{3}H]DPCPX; the receptors in the high affinity state were completely shifted to the low affinity state in the presence of 1 mM GTP, resulting in a one site displacement curve. As can be seen in the CPA curve, GTP decouples the receptor from its G protein, thereby decreasing the amount of receptors in the high affinity state (R*).

For all three affinities of DPCPX, the addition of 10 μM PD81,723 resulted in a decrease of affinity; for the $IC_{50,\ high\ 2}$-value and the $IC_{50,low}$-value this decrease was significant. However, the percentage receptors in the different affinity states remained unaltered.

In concert with the results obtained for CPA, the affinity of DPCPX for the different receptor states was not influenced by the addition of 1 mM GTP. The percentage receptors in the high affinity states, however, were significantly increased.

PD81,723 is usually viewed upon as an allosteric enhancer of agonist binding. However, our data in concert with the findings of Heitman et al.[6] show that PD81,723 is an allosteric enhancer of CPA binding, but not of LUF5834 or LUF5831 binding, despite the agonistic nature of both CPA and the non-ribose LUF compounds. In addition, PD81,723 lowered the affinity of the inverse agonist DPCPX for the receptor. Hence, it is not justified to coin PD81,723 as an allosteric enhancer per se, as is generally done.

GTP on the other hand, did not affect the affinities of the ligands for the receptor in any of the cases. It only shifted the equilibrium between the active (R*) and the inactive (R) state of the receptors. Moreover, according to the results obtained with the DPCPX displacement experiments, the R-state of the receptor seems to be divided in two high affinity sites for DPCPX, R^1 and R^2. The ratio between the two high affinity sites R^1 and R^2 is almost 1:1 (23 $\pm$ 13% vs 29 $\pm$ 8%), and increase both with more or less the same percentage (ca. 20%) upon addition of 1 mM GTP to the system. These observations point to the fact that these high affinity states may not be induced by differences in receptor conformation caused by e.g. accessory proteins or allosteric modulators which would be expected to affect the affinity, but are much more determined by the interactions between radioligand and/or displacing ligand and the uncoupled receptor, which may favour or induce certain receptor conformations. Following these observations, we propose an equilibrium of three receptor states, differing from the two state (R $\leftrightarrow$ R*) model, whereby the inactive, R state, of the receptor is subdivided in two states, R^1 and R^2.



Effect of G protein

All three states are recognized by the inverse agonist DPCPX, but only two of these by the agonist ligands. It has been shown before that the G_i-protein is involved in the active conformation, R*, of the adenosine A_1 receptor[6,20]. Interestingly, Sf9 insect cell membranes hardly contain G_i-protein. Figler ***et al.***[21] showed that the addition of both $G_i\alpha$ and $G_i\beta\gamma$ subunits was necessary to regain the high affinity binding of recombinant bovine adenosine A_1 receptor in these cell membranes. We therefore

performed displacement experiments on an Sf9 insect cell membrane preparation expressing the his-tagged human adenosine A_1 receptor. The ligand pharmacology for the tagged receptor is identical to the wt human adenosine A_1 receptor (data not shown).

CPA's displacement curve was monophasic, and addition of 1 mM GTP had no effect on CPA's affinity (504 nM). Obviously, no receptors in the active conformation (R*) were present in this Sf9 insect cell preparation.

For DPCPX, two rather than three binding sites were found. The R*-conformation, the low affinity state of the receptor for DPCPX, did not seem to be present in the Sf9 Insect cell preparation. The addition of 1 mM GTP had no effect on the affinity of DPCPX, again proof of two receptor conformations despite the absence of G proteins.

Conclusion

In conclusion, a new, non-adenosine, agonist radioligand has been characterized for the human adenosine A_1 receptor. This radioligand, [^{3}H]LUF5834, recognized two high-affinity binding sites with a mere 10-fold difference in K_d values. Therefore it occupied all the receptor binding sites present on the cell membrane in saturation studies, comparable to the high affinity inverse agonist radioligand [^{3}H]DPCPX. Displacement experiments revealed that LUF5834 is not sensitive to PD81,723 or GTP addition. In addition, an 'extra' third site was revealed in displacement experiments with the inverse agonist DPCPX. This extra third site represents most probably a subdivision of the R conformation of the receptor into two additional states, R^1 and R^2. Based on these results, an extended receptor equilibrium was proposed for the different receptor states.

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CHAPTER 8

General Discussion,

Conclusions and Perspectives

General Discussion, Conclusions and Perspectives

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.

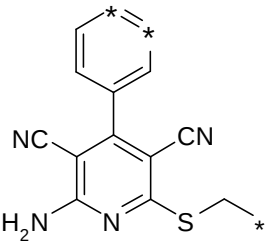

Figure 8.1. Core structure of 2-amino-4-(3 and/or 4-disubstituted phenyl)-6-(substituted)sulfanyl-pyridine-3,5-dicarbonitriles. The * represent the substitution positions.

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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Summary

The effect of adenosine on heartbeat and arterial pressure was first described in 1929. Much later, almost 30 years ago, it became clear that certain seven transmembrane proteins were responsible for this effect of adenosine. These proteins belong to the large class of G protein-coupled receptors (GPCRs), a protein class which has proven itself to be very amenable as drug targets. Four GPCRs have been identified that recognise adenosine. All are expressed throughout the body and each contains its own specific characteristics concerning ligand binding and signal transduction. An introduction to the four adenosine receptors subtypes, their history and cloning, occurrence, functioning, trafficking and therapeutic potential is given in **Chapter 1.**

The process of desensitization and internalization of adenosine receptors in cell systems, tissues and *in vivo* studies is described in **Chapter 2.** An overview of the current literature concerning desensitization and internalization of the adenosine receptors is given and the regulation of the different subtypes upon agonist binding is discussed. For instance, the A_1R internalizes slowly, showing a typical half-life of several hours, while the other G_i-coupled adenosine receptor (A_3R) internalizes in a matter of minutes. In addition, molecular mechanisms involved in adenosine receptor desensitization are discussed.

In **Chapter 3**, human adenosine A_1 receptors fused to ^{351}Cys mutated $G_{i\alpha}$-subunits are used as tools to study inverse agonism. In addition, the enhancing effect of the allosteric modulator PD81,723 on agonist affinity is shown. It appeared that the physical linking of the $G_{i1\alpha}$ subunit to the adenosine A_1R in a 1:1 ratio, resulted in an eightfold higher affinity of the reference agonist CPA. However, the affinity of the antagonist DPCPX for the fusion protein was not affected, indicating that G protein-coupling does not affect antagonist affinity. Although the A_1R was precoupled to the $G_{i\alpha}$-subunit, thereby shifting the receptor equilibrium to the high affinity state, PD81,723 was still able to increase the affinity of CPA for the fusion proteins. Again, the affinity of DPCPX for the fusion proteins was hardly affected. Na^+-ions, acting as allosteric inhibitors, were unable to decrease agonist binding to the A_1-$G_{i1\alpha}$ fusion proteins, presumably because they exhibit their effect through uncoupling of the R-G complex. Exceptions were the ^{351}Pro and ^{351}Arg A_1-$G_{i\alpha}$ fusion proteins for which Na^+-ions did decrease the affinity of CPA. One may speculate that the side-chain characteristics of these amino acids (ring structure and positive charge respectively) may alter the conformation of the $G_{i\alpha}$ protein and with it the conformation of the fusion protein as a whole. [^{35}S]GTPγS binding experiments showed that all A_1-$G_{i1\alpha}$ fusion proteins tested had a higher basal receptor activity, thereby providing improved conditions to observe inverse agonism. It was also found that maximal receptor (de)activation depended on the amino acid at position 351 of the $G_{i1\alpha}$–

subunit. Especially the ^{351}Cys and ^{351}Ile mutants can be used as research tools to investigate inverse agonism, due to their increased readout window in [^{35}S]GTPγS binding experiments.

The influence of allosteric modulators on the internalization of the A_1R is detailed in **Chapter 4**. For that purpose, the adenosine A_1R was equipped with a C-terminal yellow fluorescent protein tag, resulting in an A_1YFP receptor. The adenosine A_1R appears to internalize very slowly, a matter of hours. Upon long-term exposure (16 h), CPA was able to internalize 25% and 40% of the receptors at a concentration of 400 nM or 4 µM, respectively. However, in the presence of PD81,723 a slight amount of internalization was already obtained at 40 nM of CPA, and at 400 nM CPA 59% of the receptors internalized, an increase of 34%. SCH-202676 on the other hand effectively prevented CPA-induced internalization of the receptor. Recent investigations, however, have shown that the action of SCH-202676 is irreversible and the result of a reaction with among others cysteine residues in a protein. These properties render SCH-202676 unsuitable as an allosteric inhibitor. Addition of either PD81,723 or SCH-202676 alone had no effect on internalization. PD81,723 was not able to accelerate the internalization process it was, however, able to reduce the threshold agonist concentration at which internalization takes place.

Recently, a series of selective agonists for human adenosine A_1 receptors with an unusual structure lacking the ribose moiety of traditional adenosine-like agonists were synthesized. The synthesis, affinity and activity of twelve of these non-ribose ligands, all 2-amino-4-(3 and/or 4-disubstituted phenyl)-6-(substituted)sulfanyl-pyridine-3,5-dicarbonitriles, are described in **Chapter 5**. Substitution at the phenyl ring mostly dictated affinity, whereas the substitution pattern at the sulfanyl position appeared to rather govern intrinsic activity. cAMP studies revealed that this series of compounds contains inverse agonists, partial agonists and very potent full agonists. It is quite remarkable to find such a great variety in potency in such a small series of compounds. From these series, the 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 *N*^{6}-cyclopentyladenosine (CPA) in affinity and potency.

Chapter 6 evaluates the characteristics of LUF6037 a representative of these 3,4-methylenedioxyphenyl substituted compounds. LUF6037 recognized two binding states/sites on the human adenosine A_1 receptor with picomolar and nanomolar affinity, both much higher than the reference agonist CPA. Just like CPA, LUF6037 was able to inhibit the cAMP production, although again with a much lower EC_{50} value (picomolar range) than CPA (nanomolar range). In addition, thermodynamic radioligand binding experiments also classified LUF6037 as a full agonist. However, the allosteric enhancer PD81,723 did not influence the interaction of LUF6037 with

the receptor whereas the interaction of CPA with the receptor was affected. Furthermore, LUF6037 did not induce internalization whereas CPA was able to induce dose-dependent internalization of the adenosine A_1 receptor. Taking these results together, LUF6037 is a new, high affinity agonist that due the absence of internalization inducing properties can be preferred over classical agonists like CPA in drug treatment.

One of the other non-ribose agonists with high affinity for the A_1R, LUF5834, was chosen to be radioactively labelled. **Chapter 7** describes the evaluation of [^{3}H]LUF5834 as a new agonist radioligand. It appeared that [^{3}H]LUF5834 recognizes two high-affinity binding sites with only a small difference in K_d values, occupying all the receptor binding sites present on the cell membrane. This is a remarkable feature for an agonist radioligand, resembling the properties of the inverse agonist/antagonist [^{3}H]DPCPX. Displacement experiments revealed that LUF5834 is insensitive to PD81,723 or GTP. In addition, an 'extra' third site was revealed in displacement experiments with the inverse agonist DPCPX. This extra third site represents most probably a subdivision of the R conformation of the receptor, here named R^1 and R^2. Based on these results, an extended receptor equilibrium was proposed for the different receptor states.

Finally, in **Chapter 8,** general conclusions about the research described in this thesis are drawn. These are followed by some future perspectives and suggestions for research to be pursued, based on the interesting results obtained from this work.

Samenvatting

Het effect van adenosine op de hartslag en bloeddruk werd voor het eerst beschreven in 1929. Veel later, bijna 30 jaar geleden, werd duidelijk dat bepaalde eiwitten die bestaan uit zeven transmembraan domeinen verantwoordelijk waren voor dit effect van adenosine. Deze eiwitten behoren tot de G eiwit-gekoppelde receptoren (GPCRs), een klasse eiwitten die zichzelf bewezen heeft als zeer geschikte aangrijpingspunten voor geneesmiddelen. Vier GPCRs die adenosine herkennen, zijn geïdentificeerd. Zij worden alle verspreid in het lichaam tot expressie gebracht, en elk subtype bezit zijn eigen karakteristieken met betrekking tot ligand binding en signaal transductie. Een introductie tot de vier adenosine receptor subtypes, hun geschiedenis en klonering, hun voorkomen, functioneren, beweging door de cel en therapeutische mogelijkheden wordt beschreven **in Hoofdstuk 1.**

Het desensitisatie- en internalisatieproces van adenosinereceptoren in celsystemen, weefsels en *in vivo* studies wordt beschreven in **Hoofdstuk 2.** Een overzicht van de huidige literatuur met betrekking tot desensitisatie en internalisatie van adenosinereceptoren wordt gegeven, en de regulatie van de verschillende subtypes na agonist binding wordt besproken. De adenosine A_1 receptor (A_1R) internaliseert bijvoorbeeld langzaam, met een halfwaarde tijd van een paar uur, terwijl de andere G_i-gekoppelde receptor (A_3R) internaliseert in een tijdsbestek van een paar minuten. Tevens worden de moleculaire mechanismen die betrokken zijn bij de desensitisatie van adenosinereceptoren besproken.

In **Hoofdstuk 3** worden fusie-eiwitten tussen de adenosine A_1 receptor van de mens en ^{351}Cys gemuteerde $G_{i\alpha}$-subunits gebruikt om invers agonisme te bestuderen. Tevens wordt het faciliterende effect van de allostere modulator PD81,723 op de affiniteit van de agonist aangetoond. Het bleek dat de fysieke koppeling van de $G_{i1\alpha}$ subunit aan de adenosine A_1R in een 1:1 verhouding resulteert in een 8 maal hogere affiniteit van de referentie agonist CPA. De affiniteit van de antagonist DPCPX voor het fusie-eiwit werd echter niet beïnvloed, wat aangeeft dat G-eiwit koppeling geen invloed heeft op de affiniteit van antagonisten. Hoewel de A_1R al gekoppeld was aan de $G_i\alpha$-subunit waardoor het evenwicht verschoven wordt naar de hoge affiniteitstoestand, was PD81,723 nog steeds in staat om de affiniteit van CPA voor de fusie-eiwitten te verhogen. Wederom werd de affiniteit van DPCPX voor de fusie-eiwitten nauwelijks beïnvloed. Na^+-ionen die zich gedragen als allostere remmers, waren niet in staat om de agonist binding aan de A_1-$G_{i1\alpha}$ fusie-eiwitten te verminderen, waarschijnlijk omdat zij hun effect via ontkoppeling van het R-G complex bewerkstelligen. Uitzonderingen waren de ^{351}Pro en ^{351}Arg A_1-$G_i\alpha$ fusie-eiwitten waar de Na^+-ionen de affiniteit van CPA verminderden. Men zou kunnen speculeren dat de karakteristieken van de zijketens van deze aminozuren (respectievelijk ringstructuur en positieve lading) de conformatie van het $G_i\alpha$ eiwit, en

daarmee de conformatie van het fusie-eiwit als geheel zou kunnen veranderen. [^{35}S]GTPγS bindingsexperimenten lieten zien dat alle geteste A_1-$G_{i1\alpha}$ fusie-eiwitten een hogere basale receptor activiteit hadden, waardoor invers agonisme nauwkeuriger gemeten kan worden. Ook werd gevonden dat de maximale receptor deactivatie afhing van het aminozuur op positie 351 van het $G_{i1\alpha}$–subunit. Vooral de ^{351}Cys en ^{351}Ile mutanten zijn geschikt om invers agonisme te onderzoeken dankzij hun vergrote uitleesvenster in [^{35}S]GTPγS bindingsexperimenten.

De invloed van allostere modulatoren op de internalisatie van de A_1R is uitgewerkt in **Hoofdstuk 4.** Voor dat doeleinde werd de adenosine A_1R voorzien van een geel fluorescerende eiwit aan de C-terminus, resulterend in een A_1YFP receptor. De adenosine A_1R blijkt heel langzaam te internaliseren, een kwestie van uren. Gedurende lange termijn blootstelling (16 uur), was CPA in staat om 25% en 40% van de receptoren te internaliseren bij een concentratie van respectievelijk 400 nM of 4 μM. Echter, in de aanwezigheid van PD81,723, werd al enige internalisatie verkregen bij een concentratie van 40 nM CPA, en bij een concentratie van 400 nM CPA internaliseerde 59% van de receptoren, een toename van 34%. SCH-202676 daarentegen voorkwam op een effectieve manier de door CPA geïnduceerde internalisatie van de receptor, geïnduceerd door CPA. Recente onderzoeken hebben aangetoond dat de werking van SCH-202676 onomkeerbaar is en het resultaat is van een reactie met onder andere cysteine residuen in een eiwit. Deze eigenschappen maken SCH-202676 ongeschikt als een allostere remmer. Toevoeging van enkel PD81,723 of SCH-202676 had geen effect op internalisatie. PD81,723 was niet in staat om het internalisatie proces te versnellen; maar kon wel de kritische agonist concentratie waarbij internalisatie plaats vindt, verlagen.

Onlangs is een serie selectieve agonisten voor de adenosine A_1 receptor van de mens gesynthetiseerd met een ongewone structuur waarin de ribose groep van de traditionele adenosine-achtige liganden ontbreekt. De synthese, affiniteit en activiteit van twaalf van deze non-ribose liganden, alle behorende tot de 2-amino-4-(3 en/of 4-gesubstitueerde fenyl)-6-(gesubstitueerde)sulfanyl-pyridine-3,5-dicarbonitrillen, worden beschreven in **Hoofdstuk 5.**

Substitutie aan de fenylring dicteert voornamelijk de affiniteit, terwijl het substitutiepatroon op de sulfanyl positie de intrinsieke activiteit lijkt te bepalen. cAMP studies brachten aan het licht dat deze serie verbindingen zowel inverse agonisten, partiële agonisten en zeer potente volle agonisten omvat. Het is nogal opmerkelijk om zo'n grote variëteit in potentie te vinden in zo'n kleine serie verbindingen. De 3,4-methyleendioxyfenyl gesubstitueerde verbindingen uit deze serie herkennen twee bindingstoestanden of –plaatsen met een picomolaire affiniteit voor de hoge affiniteitsplaats. Deze verbindingen waren volle agonisten die de klassieke agonist *N*^{6}-cyclopentyladenosine (CPA) veruit overtreffen in affiniteit en potentie.

Hoofdstuk 6 evalueert de karakteristieken van LUF6037, een voorbeeld van een 3,4-methyleendioxyfenyl gesubstitueerde verbinding. LUF6037 herkende 2 bindingstoestanden of –plaatsen op de adenosine A_1 receptor van de mens met picomolaire en nanomolaire affiniteiten, beiden veel hoger dan de referentie agonist CPA. Net als CPA was LUF6037 in staat om de cAMP productie te remmen, en opnieuw met een veel lagere EC_{50}-waarde (picomolaire schaal) dan CPA (nanomolaire schaal). Tevens werd LUF6037 in thermodynamische radioligand bindings experimenten gekarakteriseerd als een volle agonist. De allostere enhancer PD81,723 beïnvloedde echter de interactie tussen LUF6037 en de receptor niet, terwijl dat wel het geval was bij de interactie van CPA met de receptor. Verder induceerde LUF6037 geen internalisatie terwijl CPA wel dosis-afhankelijke internalisatie van de adenosine A_1-receptor veroorzaakte. Samengevat laten deze resultaten zien dat LUF6037 een nieuwe agonist is met een hoge affiniteit, die door de afwezigheid van internalisatie-inducerende eigenschappen de voorkeur kan verdienen ten opzichte van klassieke agonisten zoals CPA.

Een van de andere non-ribose agonisten met een hoge affiniteit voor de A_1R, LUF5834, werd uitgekozen om radioactief te labelen. **Hoofdstuk 7** beschrijft de evaluatie van [^{3}H]LUF5834 als een nieuw agonistisch radioligand. Het bleek dat [^{3}H]LUF5834 twee hoge-affiniteits bindingsplaatsen herkent, met slechts een klein verschil in K_d-waarden, waardoor alle receptor bindingsplaatsen op het celmembraan bezet kunnen worden. Dit is een opmerkelijke eigenschap voor een agonistisch radioligand, wat overeenkomt met de eigenschappen van het inverse agonistische/antagonistische radioligand [^{3}H]DPCPX.

Verdringingsexperimenten lieten zien dat LUF5834 ongevoelig is voor de toevoeging van zowel PD81,723 als GTP. Bovendien werd in verdringingsexperimenten met de inverse agonist DPCPX een ‘extra’ derde bindingsplaats aangetoond. Deze extra bindingsplaats vertegenwoordigt hoogstwaarschijnlijk een onderverdeling van de R conformatie van de receptor, hier R^1 en R^2 genoemd. Op basis van deze resultaten is een uitgebreider evenwicht tussen verschillende receptor toestanden gepostuleerd.

Tenslotte worden in **Hoofdstuk 8** algemene conclusies getrokken over het onderzoek dat in dit proefschrift beschreven is. Deze worden gevolgd door toekomstperspectieven en suggesties voor vervolgonderzoek, die gebaseerd zijn op de interessante resultaten verkregen uit dit werk.

List of Publications

Beukers MW, Wanner MJ, Von Frijtag Drabbe Künzel JK, **Klaasse EC**, IJzerman AP, Koomen GJ. N^6-cyclopentyl-2-(3-phenylaminocarbonyltriazene-1-yl)adenosine (TCPA), a very selective agonist with high affinity for the human adenosine A_1 receptor. *J Med Chem.* **2003**, 46:1492-1503.

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Klaasse EC, Roerink SF, van Veldhoven JPD, von Frijtag Drabbe Künzel JK, de Grip WJ, Brussee J, IJzerman AP, Beukers MW. Structure-activity relationships of 2-amino-4-(substituted)phenyl-6-(substituted)sulfanyl-pyridine-3,5-dicarbonitriles reveal full agonists with picomolar affinity for the human adenosine A_1 receptor. *Manuscript in preparation.*

Klaasse EC, Roerink SF, van Veldhoven JPD, von Frijtag Drabbe Künzel JK, de Grip WJ, IJzerman AP, Beukers MW. LUF6037, a non-adenosine agonist with picomolar potency for the adenosine A_1 receptor is unable to internalize the receptor. *Manuscript in preparation.*

Klaasse EC, Chang LCW, de Vries H, de Grip WJ, IJzerman AP, Beukers MW. [^{3}H]LUF5834, a new non-adenosine radioligand for the adenosine A_1 receptor, revealing 3 binding sites for DPCPX on the A_1 receptor. *Manuscript in preparation.*

Curriculum Vitae

Elisabeth Klaasse werd geboren op 22 april 1978 te Katwijk aan zee. Na het behalen van het VWO-diploma aan het Pieter Groen College te Katwijk aan zee, is zij in september 1996 begonnen met de studie Bio-Farmaceutische Wetenschappen aan de Universiteit Leiden. In augustus 1997 haalde zij het propedeutisch examen, gevolgd door het doctoraal examen in september 2001. Tijdens haar eerste stage van juni 1999 tot en met januari 2000 op de afdeling Medische Farmacologie heeft zij onder begeleiding van Dr. J. de Koning gewerkt aan "The Fine-regulation of the Onset of the Mid-cyclic LH surge". De tweede stage van februari tot september 2000 op de afdeling Farmacochemie heeft zij gewerkt aan "Allosteric Modulation of fusion proteins between the human adenosine A_1 receptors and different mutated (C351X) rat $G_{i1}\alpha$ subunits" onder leiding van Rianne de Ligt en Prof. dr. A.P. IJzerman. Aansluitend heeft zij een buitenlandstage gedaan bij GlaxoSmithKline (Harlow, UK) alwaar zij gedurende 10 maanden gewerkt heeft aan het maken van induceerbare knock out muismodellen voor verschillende genen, m.b.v. moleculair biologische technieken onder leiding van Dr. H. Prosser.
Vanaf oktober 2001 tot oktober 2006 is zij werkzaam geweest als promovenda bij de Leidse vakgroep Farmacochemie van het Leiden/Amsterdam Center for Drug Research (LACDR) onder begeleiding van Prof. dr. A.P. IJzerman, Prof. dr. W.J. de Grip en Dr. M.W. Beukers. Gedurende deze periode werd het onderzoek verricht dat in dit proefschrift staat beschreven. Tijdens haar AIO-schap heeft zij verschillende (inter)nationale symposia en congressen bezocht alwaar zij haar onderzoek gepresenteerd heeft. Tevens ontving zij tijdens de FIGON Dutch Medicines Days 2004 de eerste prijs voor de poster getiteld: "Internalization of the adenosine A_1 Receptor. The influence of Allosteric Modulators."

Elisabeth Klaasse was born on the 22th of April 1978 in Katwijk aan zee. After graduating from secondary school at the "Pieter Groen College" in Katwijk aan zee in 1996, she started studying Bio Pharmaceutical Sciences at Leiden University. In August 1997, she passed the propaedeutic exam, followed by the Masters degree in Bio Pharmaceutical Sciences in September 2001. From June 1999 until January 2000, she worked at the department of Medicinal Pharmacology under supervision of Dr. J. de Koning on the research subject "The Fine-regulation of the Onset of the Mid-cyclic LH surge". A second internship was done from February until September 2000 at the department of Medicinal Chemistry under supervision of Rianne de Ligt and Prof. dr. A.P. IJzerman, where she participated in the study towards "Allosteric Modulation of fusion proteins between the human adenosine A_1 receptors and different mutated (C351X) rat $G_{i1}\alpha$ subunits". Subsequently, a third internship was performed for 10 months at GlaxoSmithKline (Harlow, UK), where she worked on inducible knock out mice models for different genes, under supervision of Dr. H. Prosser.
From October 2001 until October 2006, she was employed as a PhD-student at the department of Medicinal Chemistry, Leiden/Amsterdam Center for Drug Research (LACDR) under supervision of Prof. dr. A.P. IJzerman, Prof. dr. W.J. de Grip and Dr. M.W. Beukers. During this time, the research described in this thesis was conducted. Several (inter)national symposia and congresses were attended during this period, where she presented her research results. During the FIGON Dutch Medicines Days 2004, she received the 1st prize for the poster entitled: "Internalization of the adenosine A_1 Receptor. The influence of Allosteric Modulators."

Nawoord

Aan het einde van dit proefschrift rest mij nog om een heel aantal mensen te bedanken zonder wie dit proefschrift niet tot stand was gekomen. Als eerste de mensen van de afdeling Moleculaire Genetica: Hans, Tineke en Riekje, jullie stonden altijd voor mij klaar als ik met technische of praktische vragen kwam, ik heb veel van jullie geleerd op het moleculair biologische vlak. Op de afdeling Toxicology was Hans de Bont altijd stand-by als ik achter de confocale fluorescentie microscoop zat. Dank voor de introductie in de fluorescentiemicroscopie en de bijbehorende software. Dank ook aan Gerrit Lodder en zijn medewerkers voor de gastvrijheid op jullie lab waar ik menig radioligand bindingsexperiment heb gedaan. Prasad Ratnala is thanked for providing me with Sf9 insect cells, and teaching me how to culture them. De mensen van het Nijmegen Centre of Molecular life Sciences, UMC Nijmegen, hebben me de eerste beginselen van het maken van FastBac constructen, baculovirussen, het infecteren van Sf9 insecten cellen en het opzetten van grote kweken bijgebracht. Giel en Jenny, bedankt voor jullie advies en praktische hulp wanneer dat nodig was! Henk, jouw jarenlange lab-ervaring, technisch inzicht en creatieve oplossingen hebben een grote bijdrage geleverd aan mijn onderzoek. Zonder jouw goedgevulde gereedschapskist hadden we vast niet zo gemakkelijk de bioreactoren in elkaar geschroefd. Alle foto's en handleidingen die je voor de nieuwe apparatuur gemaakt hebt zijn van onschatbare waarde voor a-technische gebruikers zoals ik! Dat we nog steeds niet weten waarom de A_1-receptor niet functioneel gereconstitueerd kan worden, ligt zeker niet aan jouw inzet. Verder wil ik graag alle voorgaande en huidige collega's van de afdeling Farmacochemie bedanken voor de zeer prettige samenwerking, collegialiteit en lol die we samen gehad hebben. Niet alleen elkaars cellen doorzetten, de wekelijkse 'biomeetings' en gezamenlijk congresbezoek horen daarbij, maar ook (strand)lablunches, sinterklaasvieringen en labweekenden vallen wat mij betreft daaronder. Ik heb ervan genoten!
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De laatste paragraaf is gereserveerd voor de mensen die mijn promovendus-periode van een wat grotere afstand gevolgd hebben, maar die zeker niet minder geïnteresseerd waren. Vrienden van de dinnerclub, CJV-vrienden, al 5 jaar onafgebroken elke woensdag dinnerclub met een mannetje of 12, supergeslaagde vakanties, tripjes, lol en gezelligheid, een lach en een traan, jullie zorgden voor tijdige ontspanning. Agatha, Astrid, Anneke, Corine, Daphne, Gijs A., Hendrine, Jan-Willem, Johan-Krijn, Mark, Martin, Peet H., Peet alias 'VanderO', Pieter en Vera, a big Thank You is hier wel op zijn plaats! Ienske en Miranda, al vanaf respectievelijk de kleuterschool en middelbare school bevriend, *keep in touch*!
Corine en Johan-Krijn, als zusje en broertje en als mede 'Leiden University students' kunnen jullie je wellicht nog het beste een voorstelling maken van de wetenschappelijke wereld waarin ik me bevind, en alles wat daarbij komt kijken. Dank voor de broeder- en zusterband, die is heel bijzonder! Pa en Ma, jullie hebben altijd onvoorwaardelijk in me geloofd, me gestimuleerd en gesteund in school en studie. Ziehier het resultaat. Jullie zijn niet alleen 'Gouden Ouders', maar zeker ook 'Gouden Grootouders'! Daniël en Ruben mogen zich bevoorrecht voelen met zulke 'oppas-opa's en oma'. En natuurlijk Houtzelf, de andere oppas-opa. Jouw wekelijkse oppasdag (om half 6 opstaan, de files trotserend) heeft het mede mogelijk gemaakt dat ik dit werk heb kunnen afronden, dank daarvoor!
Tenslotte, Gijs, waar een 10-weekse stage niet allemaal toe kan leiden…

Elisabeth

List of Abbreviations

β_1-AR	β_1 adrenergic receptor
[^{3}H]cAMP	[^{3}H] cyclic adenosine monophosphate
[^{3}H]CHA	[^{3}H] *N*^{6}-cyclohexyladenosine
[^{3}H]DPCPX	[^{3}H] 1,3-dipropyl-8-cyclopentylxanthine
[^{3}H]LUF5834	[^{3}H] 2-amino-4-(4-hydroxyphenyl)-6-(1*H*-imidazol-2ylmethylsulfanyl)-pyridine-3,5-dicarbonitrile
2HE-NECA	2-hexynyl-5'-*N*-ethylcarboxamidoadenosine
5-HT	Serotonin, 5-hydroxytryptamine
A_1R	Adenosine A_1 receptor
A_{2A}R	Adenosine A_{2A} receptor
A_{2B}R	Adenosine A_{2B} receptor
A_3R	Adenosine A_3 receptor
ADA	Adenosine deaminase
ARNO	Arf nucleotide site opener
BCA	Bicinchonic acid
BSA	Bovine serum albumin
CADO	2-chloroadenosine
CGS21680	2-[4-(2-carboxyethyl)phenethylamino]-5'-*N*-ethylcarboxamidoadenosine
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHO cells	Chinese hamster ovary cells
Cl-IB-MECA	2-chloro-*N*^{6}-(3-iodobenzyl)adenosine-5'-*N*-methyluronamide
CPA	*N*^{6}-cyclopentyladenosine
CSC	8-(3-chlorostyryl)caffeine
D_1R	Dopamine D_1 receptor
DEAE	Diethylaminoethyl
DMEM	Dulbecco's modified Eagle's medium
DPCPX	1,3-dipropyl-8-cyclopentylxanthine
GIRK-channels	G protein-activated inwardly rectifying K^+-channels
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
IB-MECA	*N*^{6}-(3-iodobenzyl)adenosine-5'-*N*-methyluronamide
$K_{i,H}$	affinity for the high affinity state/site of the receptor
$K_{i,L}$	affinity for the low affinity state/site of the receptor
k_{off}	Dissociation rate constant (min^{-1})
k_{on}	Association rate constant ($nM^{-1}\ min^{-1}$)
LUF5834	2-amino-4-(4-hydroxyphenyl)-6-(1*H*-imidazol-2ylmethylsulfanyl)-pyridine-3,5-dicarbonitrile
LUF6037	2-amino-4-(benzo-[1,3]-dioxol-5-yl)-6-(2-hydroxy-propylsulfanyl)-pyridin-3,5-dicarbonitrile
MRS 1523	3-propyl-6-ethyl-5-[(ethylthio)carbonyl]-2 phenyl-4-propyl-3-pyridine carboxylate
MRS 1706	*N*-(4-acetylphenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)phenoxy]acetamide
NECA	*5'-N*-ethylcarboxamidoadenosine
PD81,723	(2-Amino-4,5-dimethyl-3-thienyl)-[3-(trifluoromethyl)phenyl]methanone
PKA	Protein kinase A
R-PIA	(*R*)-*N*^{6}-(2-Phenylisopropyl)adenosine

Sf9 cells	*Spodoptera frugiperda* cells
YFP	yellow fluorescent protein
ZM 241385	(4-(2-[7-amino-2-(2-furyl {1,2,4}-triazolo {2,3-a {1,3,5}triazin-5-yl-aminoethyl)phenol