Constitutive receptor activation and pharmacological modeling : the adenosine A2b receptor as a prototype

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Constitutive receptor activation and pharmacological modeling

The adenosine A_{2B} receptor as a prototype

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Chapter 1

General introduction

G protein-coupled receptors (GPCRs)

G protein-coupled receptors (GPCRs) make up a large family of cell surface receptors. So far, more than a thousand GPCR family members have been identified, including the receptors for many neurotransmitters, neuropeptides, peptide hormones and small molecules such as ions and protons (Bartfai et al., 2004). These proteins derive their name from their interaction with guanine nucleotide-binding proteins (G proteins).

The members of GPCRs share two major structural and functional similarities. Firstly, they are transmembrane proteins characterized by seven membrane-spanning α helices connected by intra- and extracellular loops. Secondly, the binding of agonistic ligands to the receptors elicits conformational changes of the receptor and activates the G protein. In this manner the receptors transfer extracellular signals to intracellular targets.

G proteins consist of three subunits: α, β, and γ. The binding of guanine nucleotides to the α subunit regulates G protein activity. In the resting state, GDP is bound to the α subunit which forms a complex with the β and γ subunit. Agonist binding to a GPCR induces a conformational change in the receptor, enabling the cytosolic domain of the receptor to interact with the G protein and stimulate the exchange of bound GDP for GTP. Next, the β and γ subunits, which remain together and function as a $\beta\gamma$ complex, dissociate from the activated GTP-bound α subunit. Subsequently, both the active GTP-bound α subunit and the $\beta\gamma$ complex interact with their targets to activate downstream responses. The activity of the α subunit is terminated by the hydrolysis of the bound GTP, and the inactive α subunit (now with GDP bound) reassociates with the $\beta\gamma$ complex, making the G protein ready for a new activation cycle (Patrick, 2001).

G proteins have been classified into four protein families based on their α-subunit composition: G_s, G_i, $G_{q/11}$ and $G_{12/13}$. The major effectors regulated by Gα include adenylyl cyclase (stimulated by G_s and inhibited by G_i), phospholipase C (PLC) (stimulated by $G_{q/11}$) and K^+ channels (stimulated by G_i). The second messengers produced by these enzymes trigger the complex downstream signaling cascades. So far, 16 α, 5 β and 14 γ isoforms have been identified which implies the potential to create many different G protein complexes (Milligan and Kostenis, 2006).

Classification of GPCR Ligands

Two classic GPCR ligands are well known: ligands that produce physiological responses through activation of receptors are referred to as agonist while molecules which interfere with the interaction between agonists and the receptors are denoted as antagonists.

From a more recent pharmacological point of view, it is accepted that antagonists can be further classified as neutral antagonists and inverse agonists based on their ability to reduce the agonist-independent activity of receptors (Neubig et al., 2003; Milligan, 2003). Antagonists that reduce the level of agonist-independent functional responses are called inverse agonists or ligands with negative intrinsic activity whereas antagonists that do not reduce agonist-independent activity are referred to as neutral antagonists. Most endogenous ligands are agonists, but on a few constitutively active receptors endogenous inverse agonists have been identified such as retinal for rhodopsin and agouti-related protein for the melanocortin-4 receptor (Fishkin et al., 2004; Adan et al., 2003). Moreover, chemokines may be agonist ligands for one subtype of receptors while interacting as inverse agonists with other chemokine receptors (Petkovic et al., 2004).

Constitutive activity or the spontaneous activation of receptors is a feature of quite a few GPCRs and was initially reported for the δ opioid receptor (Koski et al., 1982) and the β_2-adrenoceptor (Cerione et al., 1984). Increased constitutive GPCR activity is one of the causes of GPCR-related diseases, a prominent example being the increased constitutive activity of rhodopsin resulting in night blindness (Rao et al, 1994). Since inverse agonists are the only ligands which can reduce the constitutive activity of GPCRs, the therapeutic relevance of these ligands is implied in diseases stemming from increased constitutive activity. So far, the majority of clinically used compounds, which were originally reported to be antagonists, have been reclassified as inverse agonists (Bond and IJzerman, 2006; Milligan, 2003).

Negative intrinsic efficacy is a property of the ligand but its magnitude depends on receptor test systems (Kenakin, 2004b). In theory, inverse agonism or positive partial agonism should predominate in the world of antagonists and true neutral antagonists should be rare (Kenakin, 2004b). However, as will be described in **Chapter 3**, the experimental window determines whether partial (inverse) agonism can be detected.

The site where these endogenous ligands bind is referred to as the orthosteric binding site. Allosteric ligands or allosteric modulators on the other hand bind to a site different from this orthosteric site, the so-called allosteric site, to modulate the binding and/or signaling properties of the endogenous ligand (May et al., 2007). (Kinetic) radioligand binding assays are powerful tools to identify allosteric ligands.

Receptor models

Mathematical models have proven instrumental to link experimental observations to theoretical predictions of receptor-ligand interactions at the molecular level (Kenakin, 2003). To understand the relationship between receptor-ligand interactions and physiological responses, various receptor models have been developed and tested. The most important models are discussed below.

Two-state receptor model

The two-state receptor model is a receptor activation model which originated from a model explaining ion channel activation and which was adapted for receptors (Kenakin, 2003). The two-state model assumes that the receptors exist in equilibrium between two states, the active R* and inactive R states. The isomerization constant (L) determines the ratio between the two receptor populations and the intrinsic efficacy α determines the affinity of a ligand to R* and R state receptors (see Figure 1). This model assumes that ligands have biased affinity for

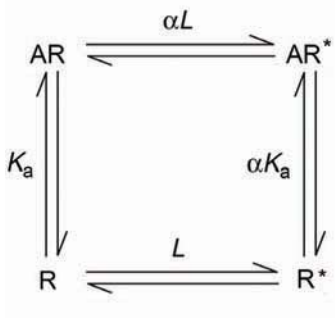


Figure 1. Two-state receptor model.

either one of the two receptor states. It provides a useful tool to explain receptor activation/inactivation in the presence or absence of a ligand. This model will be discussed in detail in **Chapter 3 and Chapter 4.**

Ternary complex model

The ternary complex model was introduced to recognize the ability of guanine nucleotides to affect the affinities of agonists. This model assumes the existence of two stages in ligand binding: firstly ligands bind to receptors, and then the receptor-ligand complex binds to a G protein (De Lean et al., 1980). This model accounts for a novel concept in receptor activation, which defines that the responsivity of a system is subject to the availability of G proteins (Kenakin, 2004a). The ternary complex model is rather simple but illustrates that a signalling molecule functions via a receptor to activate a G-protein (see Figure 2). The limitation of this model lies in the fact that a receptor is allowed to exist in only one of two ligand-dependent states, an inactive (agonist free) and an active (agonist bound) state.

Figure 2. Ternary complex model.

Extended ternary complex model

To explain experimental observations such as ligand independent G protein activation or constitutive activation (Costa and Herz, 1989), the extended ternary complex model, or ETC model was developed (Samama et al., 1993). The ETC model incorporates an important concept from the two-state receptor model, namely the assumption that ligands have biased affinity for different receptor species. This model is therefore able to explain how a receptor functions in both an agonist-dependent and an agonist-independent manner and is also able to explain antagonist-mediated and inverse agonist-mediated effects (see Figure 3). On the other hand, in contrast to the two state receptor model and the ternary complex model, the ETC model assumes the existence of an infinite number of receptor states rather than just two states. Parameter γ in the ETC model confers different affinities for G proteins interacting with ligand-receptor complexes than for G proteins interacting with unliganded receptors (Kenakin, 2004a). This model, however, does not consider an association of G-proteins with receptors in the inactive state.

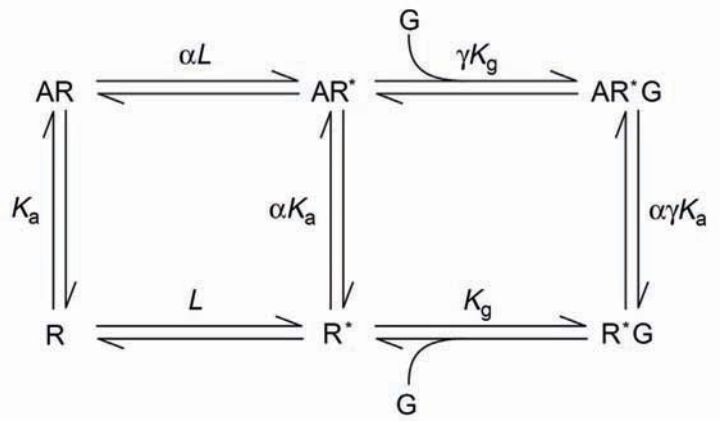


Figure 3. Extended ternary complex model.

Cubic ternary complex model

To account for an association of G-proteins with inactive state receptors, the cubic ternary complex model (CTC model) was created (Weiss et al., 1996). This model allows both ligands and G-proteins to interact independently with either the active or inactive state of a receptor. As a result this model can explain both ligand-independent signalling, as well as the influence of G-proteins on ligand binding and vice versa the influence of ligands on G-protein interactions (see Figure 4).

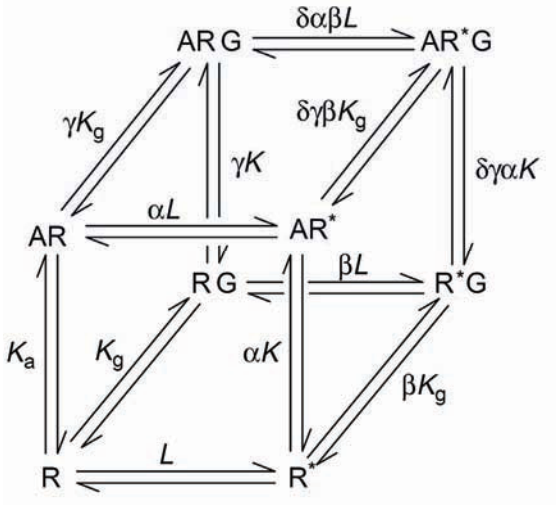


Figure 4. Cubic ternary complex model.

Allosteric two-state model

Accommodation of allosteric ligand interactions into the two-state model resulted in yet another cubic model known as the allosteric two-state model (Hall, 2000). In this model, effects of the allosteric ligand on orthosteric ligand binding as well as effects of allosteric ligands on the receptor by themselves are considered (see Figure 5). This allosteric two-state model successfully explained the allosteric effects of PD 81,723 on the adenosine A_1 receptor (Hall, 2000). The parameters of this model will be discussed in **Chapter 6**.

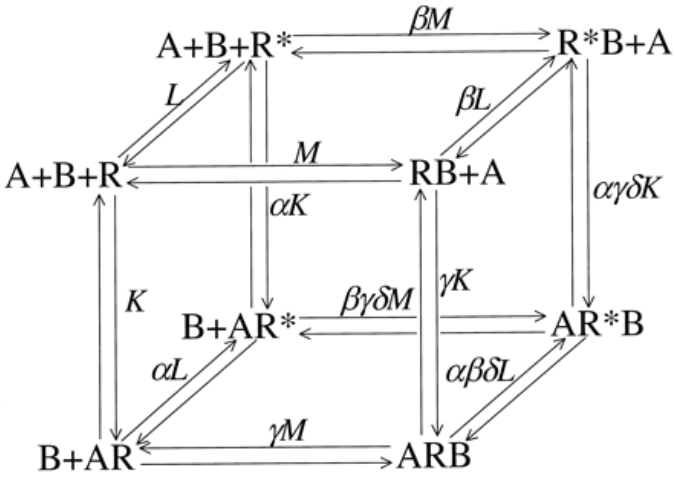


Figure 5. Two-state allosteric receptor model.

Classification of GPCRs

GPCRs have been classified into three major subfamilies on the basis of their similarity to rhodopsin (class A), secretin receptors (class B) and glutamate receptors (class C) (Probst *et al.*, 1992). This classification is based on the chemical nature of their natural ligands (Morris and Malbon , 1999). Class A (rhodopsin-like) GPCRs are by far the largest group, and include besides rhodopsin e.g. the olfactory receptors, amine receptors and nucleotide-like receptors. Class B GPCRs consist of only 25 members, including the receptors for the gastrointestinal peptide hormone family. Class C is also relatively small, and contains the metabotropic glutamate receptor family, the $GABA_B$ receptor, and the calcium-sensing receptor, as well as some taste receptors. All Class C members are characterized by a very large extracellular amino terminus which seems to be crucial for ligand binding and activation. There are two minor subfamilies of GPCRs as well. Yeast pheromone receptor and cAMP receptor have been classified as Class D and Class E GPCRs, respectively. So far, the existence of frizzled/smoothened family is still debated.

Adenosine receptors and their subtypes

Adenosine receptors were named after their endogenous ligand adenosine, which is a purine produced in our body with a short half life (Moser et al., 1989). Adenosine receptors belong to Class A GPCRs and can be divided into four subtypes: A_1, A_{2A}, A_{2B} and A_3. This nomenclature is based on several discoveries: Van Calker found different effects of adenosine on cAMP production, and discriminated between inhibitory A_1 (or R_i) and stimulatory A_2 (or Rs) receptors (Van Calker et al., 1979). Daly and Bruns further subdivided the A_2 receptors into two groups based on the identification of high (A_{2A}) and low (A_{2B}) affinity binding sites (Daly et al., 1983; Bruns et al., 1986). In contrast to these adenosine receptors the A_3 receptor was first cloned and then pharmacologically characterized (Meyerhof et al. 1991; Zhou, et al., 1992).

Adenosine A_{2B} receptor

Cloning

Adenosine A_{2B} receptors were first cloned from rat hypothalamus (Rivkees and Reppert, 1992) and human hippocampus (Pierce et al., 1992). Two years later, in 1994, this receptor was cloned from mouse mast cells (Marquardt et al., 1994). In 1997, the adenosine A_{2B} receptor was identified and cloned from chicken cell lines and tissues (Worpenberg et al., 1997). It is noticeable that A_{2B} receptors are rather conserved among mammalian species: A_{2B} receptors from closely related species rat and mouse share 96% amino acid sequence homology; and human A_{2B} receptors share 86 to 87% amino acid sequence homology with the rat and mouse A_{2B} receptors individually (Feoktistov and Biaggioni, 1997).

Distribution

The mRNA of the adenosine A_{2B} receptor was originally detected in a limited number of rat tissues by Northern blot, such as cecum, bowel, bladder, brain, spinal cord, lung, epididymis, vas deferens, and pituitary (Stehle et al., 1992). Later, a more sensitive reverse transcriptase-polymerase chain reaction (RT-PCR) technique revealed a ubiquitous distribution of adenosine A_{2B} receptors, with the highest levels found in the proximal colon and the lowest levels in rat liver (Dixon et al., 1996). The wide-spread distribution of adenosine A_{2B}

receptors was further confirmed by western blotting and immuno-staining with an anti-A_{2B} receptor antibody in human and murine tissues (Puffinbarger et al., 1995).

Structure

Being a member of the GPCR family, adenosine A_{2B} receptors consist of 7 transmembrane regions linked by intra- and extracellular loops and flanked by an N terminal and a C-terminal. Two potential N-glycosylation sites were found in the 2nd extracellular loop of the A_{2B} receptors (Rivkees and Reppert, 1992; Pierce et al., 1992; Marquardt et al., 1994), which accounts for the 50-52 kD protein bands on western blot observed for a 36-37 kD receptor (Feoktistov et al., 2003a). However, the functional role of glycosylation is still unknown.

So far, the crystal structure of the A_{2B} receptor has not yet been experimentally elucidated, however a number of homology models according to the crystal structure of bovine rhodopsin and the human β_2-adrenoceptor have been built (Ivanov et al., 2005; Chapter 5).

Signaling pathway

It is well known that A_{2B} receptors are coupled to G_s proteins and activate adenylyl cyclase (Linden, 2001). Activation of this pathway results in accumulation of cAMP and stimulation of protein kinase A (PKA); the latter in turn phosphorylates other proteins in the cells. Another important signalling pathway of A_{2B} receptors is the phospholipase C (PLC) pathway via $G_{q/11}$, which was found in mast cells and HEK293A_{2B} cells (Marquardt et al., 1994; Feoktistov and Biaggioni, 1995; Auchampach et al., 1997; Linden 1999). Activation of this pathway results in an increased production of diacylglycerol (DAG) and inositol trisphosphate (IP_3). Both molecules activate downstream signaling cascades. DAG activates protein kinase C (PKC), which phosphorylates other cellular proteins and modulates cellular Ca^{2+} concentrations (Feoktistov et al., 1997). IP_3 activates the mobilization of calcium from intracellular stores. Except for modulating cellular Ca^{2+} concentrations via G_q, A_{2B} receptors were also suggested to increase intracellular calcium by directly activating ion channels in human erythroleukemia (HEL) cells via G_s (Feoktistov et al., 1994). In addition, recombinant rat A_{2B} receptors were reported to increase a calcium-dependent chloride conductance in *Xenopus* oocytes presumably via the PLC pathway (Yakel et al., 1993). A few studies revealed the ability of adenosine A_{2B} receptors to regulate guanylate cyclase in various tissues. Shin indicated that adenosine A_{2B} receptors induced vasodilation through cGMP in the pial artery (Shin et al., 2000). Kang suggested that stimulation of the A_{2B} receptor plays an inhibitory role in central cardiovascular regulation via the cGMP pathway (Kang et al., 2007). cGMP-mediated signaling via the adenosine A_{2B} receptor was also reported by Olanrewaju and Mustafa in porcine coronary artery endothelial cells resulting in NO release (Olanrewaju and Mustafa, 2000).

To study desensitisation of the A_{2B} receptor, several heterologous expression systems have been used (Mundell et al., 1997; Peters et al., 1998; Sitaraman et al., 2000; Haynes et al., 1999). Experiments in COS-7 cells showed the A_{2B} receptor to be subject to agonist-induced desensitization. In addition, restoration of activity was observed after recovery of COS-7 cells in growth medium for 24 hr (Peters et al., 1998). Desensitisation of endogenously expressed A_{2B} receptors has been investigated as well. In rat lung microcirculation preconstricted with a hypoxic gas, initial administration of NECA caused a normal vasodilatory response after 3-4 min while readministration of NECA after 45 min resulted in minimal vasodilation, which is caused by the internalization of A_{2B} receptor (Haynes et al., 1999). In a further study, G

protein-coupled receptor kinase 2 (GRK2) was suggested to be involved in A_{2B} receptor phosphorylation and internalization in NG108-15 mouse neuroblastoma X rat glioma cells. In these experiments the NG108-15 cells transfected with the inactive K220R mutant GRK2, demonstrated significantly reduced NECA-induced A_{2B} receptor desensitization compared with control (Mundell et al., 1997).

More recent mutation studies revealed involvement of arrestin-2 and showed that the C terminus of A_{2B} receptor is critical for the receptor desensitization and internalization (Matharu et al., 2001). F328stop and the Q325stop mutant A_{2B} receptors were resistant to rapid agonist-induced desensitization and internalization although both mutants were able to induce cAMP accumulation. Fluorescently labeled arrestin-2-GFP showed a rapid translocation when co-expressed with WT A_{2B} receptor in the presence of agonist. However, when coexpressed with these two truncated mutants, no translocation was observed. Within the C-terminus, S329 is a critical residue for A_{2B} receptor internalization, as this mutant could not undergo rapid agonist-induced desensitization and internalization.

Physiological effects and therapeutic relevance

Due to the fact that selective agonists for the A_{2B} adenosine receptors were lacking in the past decades, the functional significance of this receptor is not fully understood despite intensive experimental efforts. However, by using combinatorial pharmacological approaches with nonselective A_{2B} agonists and selective receptor antagonists, the involvement of the adenosine A_{2B} receptor in several biological systems has been revealed. The emergence of more selective A_{2B} agonists in latest years provided useful tools to further study physiologic roles of A_{2B} receptors (Kuno et al., 2007; Eckle et al., 2007).

Vascular and cardiac function

Adenosine elicits relaxation in smooth muscle cells in the cardiovascular system via adenosine A_{2A} and/or A_{2B} receptors, which results in vasodilation (Feoktistov et al., 1997). The involvement of either A_{2A} or A_{2B} receptors in vasodilation is species-dependent. The role of A_{2B} receptors in vasodilation in the vascular beds of guinea pig aorta, dog saphenous vein and coronary arteries was proven quite inconclusively by the fact that this effect was mediated by the nonselective agonist NECA rather than the selective A_{2A} receptor agonist CGS 21680 (Martin, 1992; Balwierczak et al., 1991).

In addition, several studies suggest a vascular and cardioprotective role of adenosine A_{2B} receptors. The adenosine A_{2B} receptor was suggested to play a critical role in regulating vascular remodelling associated with endothelial cell proliferation in angiogenesis, collateral vessel development, and recovery after vascular injury since activation of A_{2B} receptors was observed to induce endothelial cell growth (Dubey et al., 2002). In addition, long-term stimulation of adenosine A_{2B} receptors after myocardial infarction was shown to attenuate cardiac fibrosis in the non-infarcted myocardium and to improve cardiac function (Wakeno et al., 2006). Moreover, a recently published study reveals a cardioprotective function of adenosine A_{2B} receptors during myocardial ischemia based on two observations: 1) mice with deficiencies in adenosine A_{2B} receptors showed increased susceptibility to acute myocardial ischemia; 2) treatment with the selective A_{2B} receptor agonist BAY 60–6583 significantly attenuated infarct sizes after ischemia (Eckle et al., 2007). This protective effect was confirmed upon reperfusion of rabbit heart by application of BAY 60-6583 (Kuno et al.,

2007). Finally, a study in A_{2B} receptor knockout (KO) mice model, suggests a mechanism of action as the A_{2B} receptor was found to regulate CXCR4 expression *in vivo* thereby protecting against vascular lesion formation (Yang et al., 2008).

Next to this interaction with a chemokine receptor, the adenosine A_{2B} receptors have also been reported to act in a functionally cooperative fashion with other adenosine receptor subtypes in the cardiovascular system. For example, A_{2B} receptors cooperatively act with A_3 receptors to promote angiogenesis by stimulating human umbilical vein endothelial cell proliferation and migration, and to induce capillary tube formation (Feoktistov et al., 2003b).

Role in inflammation and lung diseases

Due to low affinity to adenosine, A_{2B} receptors are assumed to remain silent under normal physiological conditions, and become important only during conditions such as inflammation when concentrations of adenosine increase (Fredholm et al., 2001).

Evidence for a potential role of adenosine in the pathogenesis of asthma has been growing steadily since the early observation of its bronchoconstrictor effect in human asthmatics. In the early 1980s, it was shown that adenosine or AMP induces bronchoconstriction in asthmatics but not in normal subjects (Cushley et al., 1983).

Activation of the adenosine A_{2B} receptor has been shown to result in degranulation in canine mastocytoma mast cells (BR line) and to increase the release of inflammatory cytokines IL-3, IL-4, IL-8 and IL-13 in human leukemia mast cells (HMC-1) (Feoktistov and Biaggioni, 1995; Auchampach et al., 1997; Feoktistov et al., 2003b; Ryzhov et al., 2004). The release of these cytokines can induce IgE synthesis by B lymphocytes (Ryzhov et al., 2004). Likewise, adenosine-mediated activation of A_{2B} receptors increases the release of inflammatory cytokines from human bronchial smooth muscle cells, human lung fibroblasts, and human airway epithelial cells (Zhong et al., 2004; Zhong et al., 2005). These cytokines, in turn, induce differentiation of lung fibroblasts into myofibroblasts (Zhong et al., 2005) and increase the release of tumor necrosis factor α (TNFα) from monocytes (Zhong et al., 2006). These effects of adenosine have been shown to be inhibited by selective antagonists of the A_{2B} receptors (Feoktistov and Biaggioni, 1995; Feoktistov et al., 2001; Ryzhov et al., 2004; Zhong et al., 2004; Zhong et al., 2005; Zhong et al., 2006).

In a more recent study, an allergic mouse model was set up and used to study the role of A_{2B} receptors on airway reactivity and inflammation in asthma (Mustafa et al., 2007). In this study, the A_{2B} selective antagonist CVT-6883 significantly inhibited airway inflammation. In another study with adenosine deaminase-deficient (ADA-deficient) mice, which develop pulmonary inflammation and injury due to increased lung adenosine levels, the A_{2B} selective antagonist CVT-6883 was found to prevent the development of pulmonary inflammation, airspace enlargement, and airway fibrosis in the lungs (Sun et al., 2006). All this evidence points to an important role for adenosine A_{2B} receptors in the pathophysiology of asthma and suggests that this receptor is a key player in lung diseases.

Neurosecretion and Neurotransmission

Adenosine inhibits norepinephrine release from peripheral noradrenergic nerve terminals (Wakade and Wakade, 1978). According to the rank order of potencies of agonists, the inhibition of norepinephrine release in isolated canine pulmonary arteries was attributed to A_{2B} receptors (Tamaoki et al., 1997). In a similar way, adenosine-induced inhibition of

neurotransmission in rabbit corpus cavernosum is also mediated via A_{2B} receptors (Chiang et al., 1994).

Various

Next to the above-mentioned physiological and therapeutic applications of the adenosine A_{2B} receptor, this receptor has been shown to affect the expression of a series of genes and even other receptors to modulate other cellular effects. For example, stimulation of A_{2B} receptors elevated cAMP levels, which in turn decreased collagenase gene expression in interleukin-1-stimulated cultured fibroblast-like synoviocytes (Boyle et al., 1996). On the contrary, fibroblast growth factor-7 (FGF-7) was observed to be upregulated by A_{2B} receptors (Iino et al., 2007). In a recent report, A_{2B} receptors were observed to up-regulate the cell surface expression of CXCR4 receptor (yang et al., 2008) and to down-regulate the netrin UNC5A receptor (McKenna, et al., 2008). In addition to receptor expression levels, the adenosine A_{2B} receptor was also demonstrated to recruit apoptosis-inducing DCC (deleted in colon cancer) receptors from an intracellular pool to the cell surface (Bouchard et al., 2004).

Other physiological role of A_{2B} receptors includes being implied in epithelial chloride secretion (Strohmeier et al., 1995), to prevent loss of the endothelial barrier's integrity in corneal endothelial cells (D'hondt et al., 2007).

Therapeutic application of agonists and antagonists

A_{2B} receptor is able to induce angiogenesis, to reduce vascular permeabilization and to increase anti-inflammatory cytokine (Volpini et al., 2003; Clancy et al., 1999; Dubey et al., 2005; Mohsenin and Blackburn, 2006). Thus A_{2B} receptor selective agonists were proposed for the treatment of septic shock, cystic fibrosis, and cardiac, kidney and pulmonary diseases associated with remodeling and hyperplasia.

Adenosine A_{2B} receptor antagonists, on the other hand, may play an important role in the treatment of inflammatory disorders and lung diseases (Feoktistov et al., 1998; Rosi et al., 2003; Mustafa et al., 2007; Sun et al., 2006), The therapeutic benefit of A_{2B} antagonists includes the treatment of asthma (Landells et al., 2000), type-II diabetes (Volpini et al., 2003), and Alzheimer's disease (Rosi et al., 2003).

Pharmacology of adenosine A_{2B} Receptors

Due to the therapeutic relevance, intensive synthesis efforts have been devoted in the past decades to identify selective, high affinity adenosine A_{2B} receptors ligands. The search for antagonists was very successful and led to the identification of selective compounds belonging to various chemical classes. Just recently also selective agonists have been identified. A brief overview of the identification of selective ligands is presented below, more details are to be found in a recently published review (Beukers et al., 2006).

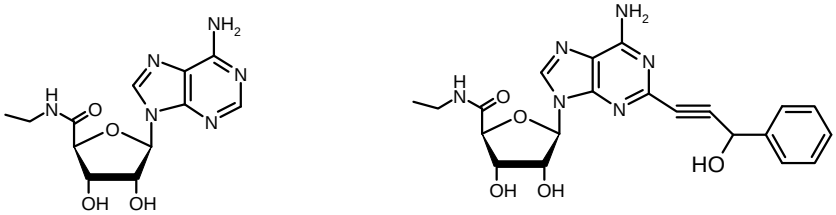
Table 1. EC_{50} of A_{2B} agonists and K_i of A_{2B} antagonists commonly used to study the A_{2B} receptor

agonists	EC_{50} (nM)				references
	hA_{2B}	hA_1	hA_{2A}	hA_3	
NECA	104 - 1140	26	26.1	129	Varani et al., 2005 Beukers et al., 2004 Schulte and Fredholm, 2000
(S)-PHPNECA	220				Volpini et al., 2002
LUF5835	10				Beukers et al., 2006
BAY 60-6583	3-10	>10,000	>10,000	>10,000	Eckle et al., 2007
antagonists	K_i (nM)				
DPCPX	18.4	3.9*	129	3960	Klotz et al., 1998
ZM241385	50	255	0.8	>10,000	De Zwart et al., 1999
CGS15943	65.8	3.5	4.18	50.8	Klotz et al., 1998
MRE2029F20	5.5	200	>1000	>1000	Baraldi et al., 2004
MRS1754	2	403	503	570	Kim et al., 2000
MRS1706	1.4	157	112	230	Kim et al., 2000
OSIP339391	0.5	37	328	450	Stewart, 2004

* K_D instead of K_i

Agonists

For several decades, NECA was considered to be the most potent known agonist at the A_{2B} receptor, with an EC_{50} of around 104 nM to 1140 µM (Hide et al., 1992; Klotz et al., 1998; Varani et al., 2005; Beukers et al., 2004; Schulte and Fredholm, 2000). However, NECA is by no means selective, in fact it acts as a universal agonist to all adenosine receptor subtypes.



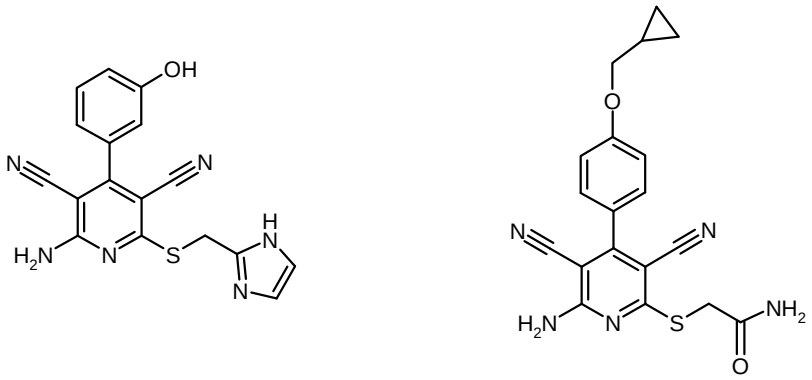
NECA (*S*) PHPNECA

Figure 6. Chemical structure of two adenosine derived A_{2B} agonists: NECA and (*S*) PHPNECA.

In order to identify selective and high affinity agonists for the adenosine A_{2B} receptor, laborious synthesis efforts have been devoted to modify the purine ring and ribose moiety of adenosine (De Zwart et al., 1998). Nevertheless, NECA still remained the most potent agonist for A_{2B} receptors in this class of compounds. Finally, an equally potent, but more selective C2-substituted analog of NECA was obtained. This compound, (S)-PHPNECA has an EC_{50} value for the adenosine A_{2B} receptor of 0.22 µM and showed 3- to 10-fold selectivity towards the other adenosine receptors (Volpini et al., 2002). The structures of both compounds are presented in figure 6.

A major breakthrough resulting in an improved affinity and selectivity for the A_{2B} receptors was achieved with the discovery of a new series of non-ribose compounds, the

dicarbonitrilepyridines (Rosentreter et al., 2001; Rosentreter et al., 2003; Beukers et al., 2004a). Several ligands belonging to this class displayed low nanomolar affinity for adenosine A_{2B} receptors expressed in CHO cells (Beukers et al., 2004a; Beukers et al., 2006). The most potent and selective ligand among this series was LUF5835 (see Figure 7). These compounds, however, still lacked selectivity with respect to the adenosine A_1 receptor. Recently, a new adenosine A_{2B} receptor agonist BAY 60-6583 was patented by Bayer HealthCare and was used to study the cardioprotective function of A_{2B} receptors (Kuno et al., 2007; Eckle et al., 2007). This compound is very selective for the A_{2B} receptor with an EC_{50} value of 3–10 nM for the human A_{2B} receptor and EC_{50} values > 10 μM for the A_1, A_{2A} and A_3 receptors (Kuno et al., 2007; Eckle et al., 2007). The structure of BAY 60-6583 is shown in figure 7.

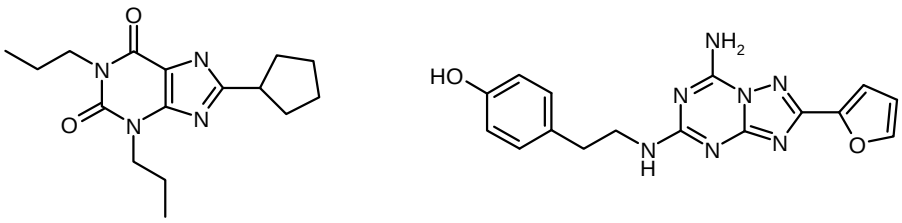


LUF5835 BAY60-6583

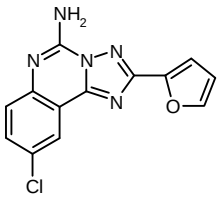
Figure 7. Chemical structure of two non-ribose adenosine A_{2B} receptor agonists: LUF5835 and BAY 60-6583.

Antagonists

The prototypic high affinity, but not selective, adenosine A_{2B} receptor antagonists are DPCPX,



DPCPX ZM241385



CGS15943

Figure 8. Structure of four prototypic adenosine receptor antagonists: DPCPX, ZM241385, and CGS15943. used as lead compounds to synthesize antagonists with even higher affinity (Kim et al., 1998; De Zwart et al., 1999).

ZM241385 and CGS15943 (see Figure 8). They have nanomolar affinities for the A_{2B} receptors and are frequently used to study adenosine A_{2B} receptors (Klotz et al., 1998; Ongini et al., 1999; Beukers et al., 2000). Moreover, DPCPX, ZM241385, and CGS15943 have been Intensive synthesis efforts have been devoted to five different classes of antagonists: xanthines, pyrrolopyrimidines, triazoles, aminothiazoles, and quinazolines (Moro et al., 2006; Beukers et al., 2006). Among these five classes of compounds, the best results were achieved with the xanthine and pyrrolopyrimidine scaffolds. Substitutions at the 1, 3, and 8 positions of the xanthine core yielded quite a few highly selective and potent antagonists. For example, MRE2029F20, MRS1754 and MRS1706 have affinities of 5.5, 2 and 1.4 nM for the human adenosine A_{2B} receptor, respectively. Moreover, these compounds were over 180, 200 and 78 fold selective on the human A_1, A_{2A} and A_3 receptors (Baraldi et al., 2004; Kim et al., 2000). Modifying pyrrolopyrimidines resulted in an even more potent antagonist with a decent selectivity, OSIP339391. It has an affinity of 0.5 nM toward the human adenosine A_{2B} receptor, which is more than 70-fold selective with respect to the human A_1, A_{2A} and A_3 receptors (Stewart, 2004). Such antagonists with both high affinity and selectivity have not been identified using triazoles, aminothiazoles, or quinazolines as a scaffold (Beukers et al., 2006).

MRE2029F20, MRS1754 and OSIP339391 have been tritiated to perform radioligand binding studies (Baraldi et al., 2004a; Ji, 2001). [^{3}H]-MRE 2029F20 showed a K_d value of 1.65 nM to human A_{2B} receptors expressed in CHO cells and [^{3}H]MRS 1754 showed a K_d value of 1.13 nM to human A_{2B} receptors expressed in HEK293 cells. Among these three antagonists, [^{3}H] OSIP339391 is the most potent with a K_d value of 0.41 nM for the human A_{2B} receptor as present on HEK293 cell membranes (Stewart, 2004).

Inverse agonists

Constitutive receptor activity is a prerequisite to discriminate inverse agonists from antagonists. Since wild-type (wt) adenosine A_{2B} receptors lack constitutive activity no inverse agonists were known and all ligands were classified as antagonists. The construction of constitutively active mutant adenosine A_{2B} receptors enabled us to determine the intrinsic efficacy of these compounds. In fact, ZM241385, DPCPX and MRS1706 were shown to possess inverse agonistic properties, with a rank order of potency of ZM241385 > DPCPC > MRS1706 (Li et al., 2007).

Progress on A_{2B} receptor research and aim of the present thesis

Much effort is put on investigating the physiological function of A_{2B} receptors and on the identification of selective, high affinity ligands. In the mean time, studies were performed to elucidate the activation mechanism and receptor-ligand interactions of A_{2B} receptors through the use of mutant A_{2B} receptors. In our group, the human adenosine A_{2B} receptor has been studied in both CHO cells and yeast cells (Beukers et al., 2000; Beukers et al., 2004b; Li et al., 2007). Compared to mammalian cells, yeast cells are more convenient in both random and site-directed mutagenesis studies as these cells take up a single plasmid. When such a mutagenesis approach is combined with a robust screening assay, a great tool arises to investigate receptor activation (a review is presented in Chapter 2). This yeast screening method has been applied to identify inverse agonists (Chapter 3), leading to the conclusion that the antagonists ZM241385, DPCPX and MRS1706 are inverse agonists for human adenosine A_{2B} receptors. In Chapter 4 we quantified the relationship between the intrinsic efficacy of an inverse agonist and the constitutive activity level of a receptor. Rather than

random mutagenesis we applied site-directed approaches in Chapter 5, particularly focussing on the role of conserved receptor motifs such as the NxxxNPxxY motif and the adenosine receptor specific salt bridges in receptor activation. We learned that both motifs are critical in receptor activation due to the fact that mutation of both the NxxxNPxxY motif and the salt bridge generally results in loss-of–function receptors. Finally, in Chapter 6 we summarized evidence for the advantage of allosteric modulators over inverse agonists to treat disease-related receptor mutations causing constitutive activity.

In conclusion, in this thesis the concepts of constitutive activity and inverse agonism are applied to the otherwise silent adenosine A_{2B} receptor. The results of our investigations on the A_{2B} receptor are extended to somatic mutations within the entire superfamily of GPCRs that cause unwanted constitutive activity, emphasizing that allosteric modulators may be more privileged as future therapeutics than inverse agonists .

References

Adan RA, Hillebrand JJ, De Rijke C, Nijenhuis W, Vink T, Garner KM and Kas MJ (2003) Melanocortin system and eating disorders. *Ann N Y Acad Sci* 994: 267-274.

Auchampach JA, Jin X, Wan TC, Caughey GH and Linden J (1997) Canine mast cell adenosine receptors: cloning and expression of the A3 receptor and evidence that degranulation is mediated by the A_{2B} receptor. *Mol Pharmacol* 52:846-860.

Balwierczak JL, Sharif R, Krulan CM, Field FP, Weiss GB and Miller MJ (1991) Comparative effects of a selective adenosine A_2 receptor agonist, CGS21680, and nitroprusside in vascular smooth muscle. *Eur J Pharmacol* 196: 117-123.

Baraldi PG, Tabrizi MA, Preti D, Bovero A, Fruttarolo F, Romagnoli R, Moorman AR, Gessi S, Merighi S, Varani K and Borea PA (2004a) [^{3}H]-MRE 2029-F20, a selective antagonist radioligand for the human A_{2B} adenosine receptors. *Bioorg Med Chem Lett* 14: 3607-3610.

Baraldi PG, Tabrizi MA, Preti D, Bovero A, Romagnoli R, Fruttarolo F, Zaid NA, Moorman AR, Varani K, Gessi S, Merighi S and Borea PA (2004b) Design, synthesis, and biological evaluation of new 8-heterocyclic xanthine derivatives as highly potent and selective human A_{2B} adenosine receptor antagonists. *J Med Chem* 47: 1434-1447.

Bartfai T, Benovic JL, Bockaert J, Bond RA, Bouvier M, Christopoulos A, Civelli O, Devi LA, George SR, Inui A, Kobilka B, Leurs R, Neubig R, Pin JP, Quirion R, Roques BP, Sakmar TP, Siefert R, Stenkamp RE, Strange PG (2004) *Nature Rev Drug Discov* 3: 574-626.

Beukers MW, Chang LC, von Frijtag Drabbe Künzel JK, Mulder-Krieger T, Spanjersberg RF, Brussee J and AP IJzerman (2004a) New, non-adenosine, high-potency agonists for the human adenosine A_{2B} receptor with an improved selectivity profile compared to the reference agonist N-ethylcarboxamidoadenosine. *J Med Chem* 47: 3707-3709.

Beukers MW, den Dulk H, van Tilburg EW, Brouwer J and IJzerman AP (2000) Why are A_{2B} receptors low-affinity adenosine receptors? Mutation of Asn273 to Tyr increases affinity of human A_{2B} receptor for 2-(1-Hexynyl) adenosine. *Mol Pharmacol* 58: 1349-1356.

Beukers MW, Meurs I and IJzerman AP (2006) Structure-affinity relationships of adenosine A_{2B} receptor ligands. *Med Res Rev* 26: 667-698.

Beukers MW, van Oppenraaij J, van der Hoorn PP, Blad CC, den Dulk H, Brouwer J and IJzerman AP (2004b) Random mutagenesis of the human adenosine A_{2B} receptor followed by growth selection in yeast. Identification of constitutively active and gain of function mutations. *Mol Pharmacol* 65: 702-710.

Bond RA and IJzerman AP (2006) Recent developments in constitutive receptor activity and inverse agonism, and their potential for GPCR drug discovery. *Trends Pharmacol Sci* 27: 92-96.

Bouchard JF, Moore SW, Tritsch NX, Roux PP, Shekarabi M, Barker PA, Kennedy TE (2004) Protein kinase A activation promotes plasma membrane insertion of DCC from an intracellular pool: A novel mechanism regulating commissural axon extension. *J Neurosci* 24: 3040-3050.

Boyle DL, Sajjadi FG, Firestein GS (1996) Inhibition of synoviocyte collagenase gene expression by adenosine receptor stimulation. *Arthritis Rheum* 39: 923-930.

Bruns RF, Lu GH and Pugsley TA (1986) Characterization of the A_2 adenosine receptor labeled by [^{3}H]NECA in rat striatal membranes. *Mol Pharmacol* 29: 331-346.

Cerione RA, Sibley DR, Codina J, Benovic JL, Winslow J, Neer EJ, Birnbaumer L, Caron MG and Lefkowitz RJ (1984) Reconstitution of a hormone-sensitive adenylate cyclase system. The pure beta-adrenergic receptor and guanine nucleotide regulatory protein confer hormone responsiveness on the resolved catalytic unit. *J Biol Chem* 259: 9979-9982.

Chiang PH, Wu SN, Tsai EM, Wu CC, Shen MR, Huang CH and Chiang CP (1994) Adenosine modulation of neurotransmission in penile erection. *Br J Clin Pharmacol* 38: 357-362.

Clancy JP, Ruiz FE and Sorscher EJ (1999) Adenosine and its nucleotides activate wild-type and R117H CFTR through an A_{2B} receptor-coupled pathway. *Am J Physiol* 276: C361-369.

Costa T and Herz A (1989) Antagonists with negative intrinsic activity at delta opioid receptors coupled to GTP-binding proteins. *Proc Natl Acad Sci U S A* 86: 7321-7325.

Cushley MJ, Tattersfield AE and Holgate ST (1983) Inhaled adenosine and guanosine on airway resistance in normal and asthmatic subjects. *Br J Clin Pharmacol* 15: 161-165.

D'Hondt C, Srinivas SP, Vereecke J and Himpens B (2007) Adenosine opposes thrombin-induced inhibition of intercellular calcium wave in corneal endothelial cells. *Invest Ophthalmol Vis Sci* 48: 1518-1527.

Daly JW, Butts-Lamb P and Padgett W (1983) Subclasses of adenosine receptors in the central nervous system: interaction with caffeine and related methylxanthines. *Cell Mol Neurobiol* 3: 69-80.

De Lean A, Stadel JM and Lefkowitz RJ (1980) A ternary complex model explains the agonist-specific binding properties of the adenylate cyclase-coupled beta-adrenergic receptor. *J Biol Chem* 255: 7108-7117.

de Zwart M, Link R, von Frijtag Drabbe Künzel JK, Cristalli G, Jacobson KA, Townsend-Nicholson A and IJzerman AP (1998) A functional screening of adenosine analogues at the adenosine A_{2B} receptor: a search for potent agonists. *Nucleosides Nucleotides* 17: 969-985.

de Zwart M, Vollinga RC, Beukers MW, Sleegers DF, Von Frijtag Drabbe Künzel J, Groote de M and IJzerman AP (1999) Potent antagonists for the human adenosine A_{2B} receptor. Derivatives of the triazolotriazine adenosine receptor antagonist ZM241385 with high affinity. *Drug Dev Res* 48: 95-103.

Dixon AK, Gubitz AK, Sirinathsinghji DJ, Richardson PJ and Freeman TC (1996) Tissue distribution of adenosine receptor mRNAs in the rat. *Br J Pharmacol* 118: 1461-1468.

Dubey RK, Gillespie DG and Jackson EK (2002) A_{2B} adenosine receptors stimulate growth of porcine and rat arterial endothelial cells. *Hypertension* 39: 530-535.

Dubey RK, Gillespie DG, Mi Z, Jackson EK (2005) Adenosine inhibits PDGF-induced growth of human glomerular mesangial cells via A_{2B} receptors. *Hypertension* 46: 628-634.

Eckle T, Krahn T, Grenz A, Kohler D, Mittelbronn M, Ledent C, Jacobson MA, Osswald, H, Thompson LF, Unertl K and Eltzschig HK (2007) Cardioprotection by ecto-5'-nucleotidase (CD73) and A_{2B} adenosine receptors. *Circulation* 115: 1581-1590.

Feoktistov I and Biaggioni I (1995) Adenosine A_{2B} receptors evoke interleukin-8 secretion in human mast cells. An enprofylline-sensitive mechanism with implications for asthma. *J Clin Invest* 96: 1979-1986.

Feoktistov I and Biaggioni I (1997) Adenosine A_{2B} receptors. *Pharmacol Rev* 49: 381-402.

Feoktistov I, Goldstein A, Sheller JR, Schwartz LB and Biaggioni I (2003a) Immunological characterization of A_{2B} adenosine receptors in human mast cells. *Drug Dev Res* 58: 461–471.

Feoktistov I, Murray JJ, Biaggioni I (1994) Positive modulation of intracellular Ca^{2+} levels by adenosine A_{2B} receptors, prostacyclin, and prostaglandin E1 via a cholera toxin-sensitive mechanism in human erythroleukemia cells. *Mol Pharmacol* 45: 1160-1167.

Feoktistov I, Ryzhov S, Goldstein AE and Biaggioni I (2003b) Mast cell-mediated stimulation of angiogenesis: cooperative interaction between A_{2B} and A_3 adenosine receptors. *Circ Res* 92: 485-492.

Feoktistov I, Wells JN and Biaggioni I (1998) Adenosine A_{2B} receptors as therapeutic targets. *Drug Dev Res* 45: 198–206.

Fishkin N, Berova N and Nakanishi K (2004) Primary events in dim light vision: a chemical and spectroscopic approach toward understanding protein/chromophore interactions in rhodopsin. *Chem Rec* 4: 120-135.

Fredholm BB, Irenius E, Kull B, Schulte G. (2001) Comparison of the potency of adenosine as an agonist at human adenosine receptors expressed in Chinese hamster ovary cells. *Biochem Pharmacol*. 61: 443-448.

Hall DA (2000) Modeling the functional effects of allosteric modulators at pharmacological receptors: an extension of the two-state model of receptor activation. *Mol Pharmacol* 58: 1412-1423.

Haynes J Obiako B Babal P and Stevens T (1999) 5-(N-ethylcarboxamido)adenosine desensitizes the A_{2B} -adenosine receptor in lung circulation. *Am J Physiol* 276: H1877–H1883

Hide I, Padgett WL, Jacobson KA and Daly JW (1992) A_{2A} adenosine receptors from rat striatum and rat pheochromocytoma PC12 cells: characterization with radioligand binding and by activation of adenylate cyclise. *Mol Pharmacol* 41: 352-359.

Iino M, Ehama R, Nakazawa Y, Iwabuchi T, Ogo M, Tajima M and Arase S (2007) Adenosine stimulates fibroblast growth factor-7 gene expression via adenosine A_{2B} receptor signaling in dermal papilla cells. *J Invest Dermatol* 127: 1318-1325.

Ivanov AA, Baskin II, Palyulin VA, Piccagli L, Baraldi PG and Zefirov NS (2005) Molecular modeling and molecular dynamics simulation of the human A_{2B} adenosine receptor. The study of the possible binding modes of the A_{2B} receptor antagonists. *J Med Chem* 48: 6813-6820.

Ji X, Kim YC, Ahern DG, Linden J and Jacobson KA (2001) [^{3}H]MRS 1754, a selective antagonist radioligand for A_{2B} adenosine receptors. *Biochem Pharmacol* 61: 657-663.

Kang MJ, Koh HC. Involvement of guanylate cyclase in the cardiovascular response induced by adenosine A_{2B} receptor stimulation in the posterior hypothalamus of the anesthetized rats (2007) *Auton Neurosci* 134: 55-60.

Kenakin T (2003) *A Pharmacology Primer*. Elsevier Academic Press, California and London.

Kenakin T (2004a) Principles: receptor theory in pharmacology *Trends Pharmacol Sci* 25: 186-192.

Kenakin T (2004b) Efficacy as a vector: the relative prevalence and paucity of inverse agonism. *Trends Pharmacol Sci* 65: 2-11.

Kim YC, de Zwart M, Chang L, Moro S, von Frijtag Drabbe Künzel JK, Melman N, IJzerman AP and Jacobson KA (1998) Derivatives of the triazoloquinazoline adenosine antagonist (CGS15943) having high potency at the human A_{2B} and A_3 receptor subtypes. *J Med Chem* 41: 2835-2845.

Kim YC, Ji X, Melman N, Linden J and Jacobson KA (2000) Anilide derivatives of an 8-phenylxanthine carboxylic congener are highly potent and selective antagonists at human A_{2B} adenosine receptors. *J Med Chem* 43: 1165-1172.

Klotz KN, Hessling J, Hegler J, Owman C, Kull B, Fredholm BB and Lohse MJ (1998) Comparative pharmacology of human adenosine receptor subtypes - characterization of stably transfected receptors in CHO cells. *Naunyn Schmiedebergs Arch Pharmacol* 357: 1-9.

Koski G, Streaty RA and Klee WA (1982) Modulation of sodium-sensitive GTPase by partial opiate agonists. An explanation for the dual requirement for Na^+ and GTP in inhibitory regulation of adenylate cyclase. *J Biol Chem* 257: 14035-14040.

Kuno A, Critz SD, Cui L, Solodushko V, Yang XM, Krahn T, Albrecht B, Philipp S, Cohen MV and Downey JM (2007) Protein kinase C protects preconditioned rabbit hearts by increasing sensitivity of adenosine A_{2B}-dependent signaling during early reperfusion. *J Mol Cell Cardiol* 43: 262-271.

Landells LJ, Jensen MW, Orr LM, Spina D, O'Connor BJ, Page CP (2000) The role of adenosine receptors in the action of theophylline on human peripheral blood mononuclear cells from healthy and asthmatic subjects. *Br J Pharmacol* 129: 1140-1144.

Li Q, Ye K, Blad CC, den Dulk H, Brouwer J, IJzerman AP and Beukers, MW (2007) ZM241385, DPCPX, MRS1706 are inverse agonists with different relative intrinsic efficacies on constitutively active mutants of the human adenosine A_{2B} receptor. *J Pharmacol Exp Ther* 320: 637-645.

Linden J (2001) Molecular approach to adenosine receptors: Receptor-Mediated Mechanisms of tissue protection. *Annu Rev Pharmacol Toxicol* 41: 775–787.

Linden J, Thai T, Figler H, Jin X and Robeva AS (1999) Characterization of human A_{2B} adenosine receptors: radioligand binding, western blotting, and coupling to G_q in human embryonic kidney 293 cells and HMC-1 mast cells. *Mol Pharmacol* 56: 705-713.

Marquardt DL, Walker LL and Heinemann S (1994) Cloning of two adenosine receptor subtypes from mouse bone marrow-derived mast cells. *J Immunol* 152: 4508-4515.

Martin, P.L. (1992) Evidence that adenosine receptors in the dog left atrium are not of the typical A_1 or A_2 adnosine receptor subtypes. *Eur J Pharmacol* 214: 199-205.

Matharu AL, Mundell SJ, Benovic JL, Kelly E. (2001) Rapid agonist-induced desensitization and internalization of the A_{2B} adenosine receptor is mediated by a serine residue close to the COOH terminus. *J Biol Chem* 276: 30199-30207

May LT, Leach K, Sexton PM and Christopoulos A (2007)_Allosteric modulation of G protein-coupled receptors. *Annu Rev Pharmacol Toxicol* 47: 1-51.

McKenna WL, Wong-Staal C, Kim GC, Macias H, Hinck L and Bartoe JL (2008) Netrin-1-independent adenosine A_{2B} receptor activation regulates the response of axons to netrin-1 by controlling cell surface levels of UNC5A receptors, *J Neurochem* 104: 1080-1091.

Meyerhof W, Muller-Brechlin R and Richter D (1991) Molecular cloning of a novel putative G-protein coupled receptor expressed during rat spermiogenesis. *FEBS Lett* 284: 155-160.

Milligan G (2003) Constitutive activity and inverse agonists of G protein-coupled receptors: a current perspective. *Mol Pharmacol* 64: 1271-1276.

Milligan G and Kostenis E (2006) Heterotrimeric G-proteins: a short history. *Br J Pharmacol* 147: 46-55.

Mohsenin A and Blackburn MR (2006) Adenosine signaling in asthma and chronic obstructive pulmonary disease. *Curr Opin Pulm Med* 12: 54-59.

Moro S, Gao ZG, Jacobson KA and Spalluto G (2006) Progress in the pursuit of therapeutic adenosine receptor antagonists. *Med Res Rev* 26: 131-159.

Morris AJ and Malbon CC (1999) Physiological regulation of G protein-linked signalling. *Physiol Rev* 79: 1373-1430.

Moser GH, Schrader J and Deussen A (1989) Turnover of adenosine in plasma of human and dog blood. *Am J Physiol* 256: C799-806.

Mundell SJ, Benovic JL, and Kelly E (1997) A dominant negative mutant of the G protein-coupled receptor kinase 2 selectively attenuates adenosine A_2 receptor desensitization. *Mol Pharmacol* 51*:* 991–998*.*

Mustafa SJ, Nadeem A, Fan M, Zhong H, Belardinelli L and Zeng D (2007) Effect of a specific and selective A_{2B} adenosine receptor antagonist on adenosine agonist AMP and allergen-induced airway responsiveness and cellular influx in a mouse model of asthma. *J Pharmacol Exp Ther* 320: 1246-1251.

Neubig RR, Spedding M, Kenakin T and Christopoulos A (2003) International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification. XXXVIII. Update on terms and symbols in quantitative pharmacology. *Pharmacol Rev* 55: 597-606.

Olanrewaju HA and Mustafa SJ (2000) Adenosine A_{2A} and A_{2B} receptors mediated nitric oxide production in coronary artery endothelial cells. *Gen Pharmacol* 35: 171-177.

Ongini E, Dionisotti S, Gessi S, Irenius E and Fredholm BB (1999) Comparison of CGS15943, ZM 241385 and SCH 58261 as antagonists at human adenosine receptors. *Naunyn Schmiedebergs Arch Pharmacol* 359: 7-10.

Patrick GL (2001) An introduction to Medicinal chemistry, 2nd ed., Oxford University Press 2001

Peters DM, Gies EK, Gelb CR, and Peterfreund RA (1998) Agonist-induced desensitization of A_{2B} adenosine receptors. *Biochem Pharmacol* 55: 873–882.

Petkovic V, Moghini C, Paoletti S, Uguccioni M, and Gerber B (2004) Eotaxin-3/CCL26 Is a Natural Antagonist for CC Chemokine Receptors 1 and 5. *J Biol Chem* 279: 23357–23363.

Pierce KD, Furlong TJ, Selbie LA and Shine J (1992) Molecular cloning and expression of an adenosine A_{2B} receptor from human brain. *Biochem Biophys Res Commun* 187: 86-93.

Probst WC, Snyder LA, Schuster DI, Brosius J, Sealfon SC (1992) Sequence alignment of the G-protein coupled receptor superfamily. *DNA Cell Biol* 11:1-20.

Puffinbarger NK, Hansen KR, Resta R, Laurent AB, Knudsen TB, Madara JL and Thompson LF (1995) Production and characterization of multiple antigenic peptide antibodies to the adenosine A_{2B} receptor. *Mol Pharmacol* 47: 1126-1132.

Rao VR, Cohen, GB and Oprian DD (1994) Rhodopsin mutation G90D and a molecular mechanism for congenital night blindness. *Nature* 367: 639-642.

Rivkees, S.A. and Reppert, S.M. (1992) RFL9 encodes an A_{2B} -adenosine receptor. *Mol Endocrinol* 6: 1598-1604.

Rosentreter U, Henning R, Bauser M, Kraemer T, Vaupel A, Huebsch W, Dembowsky K, Salcher-Schraufstaetter O, Stasch J-P, Krahn T, Perzborn E. Substituted 2-thio-3,5-dicyano-4-aryl-6-aminopyridines and the use thereof. WO patent 001/0125210.

Rosentreter U, Kraemer T, Shimada M, Huebsch W, Diedrichs N, Krahn T, Henninger K, Stasch J-P. Substituted 2-thio-3,5-dicyano-4-phenyl-6-aminopyridines and their use as adenosine receptor-selective ligands. WO patent 2003/03008384.

Rosi S, McGann K, Hauss-Wegrzyniak B and Wenk GL (2003) The influence of brain inflammation upon neuronal adenosine A_{2B} receptors. *J Neurochem* 86: 220-227.

Ryzhov S, Goldstein AE, Matafonov A, Zeng D, Biaggioni I and Feoktistov I (2004) Adenosine-activated mast cells induce IgE synthesis by B lymphocytes: an A_{2B}-mediated process involving Th2 cytokines IL-4 and IL-13 with implications for asthma. *J Immunol* 172: 7726-7733.

Samama P, Cotecchia S, Costa T and Lefkowitz RJ (1993) A mutation-induced activated state of the beta 2-adrenergic receptor. Extending the ternary complex model. *J Biol Chem* 268: 4625-4636.

Schulte G and Fredholm BB (2000) Human adenosine A_1, A_{2A}, A_{2B} and A_3 receptors expressed in Chinese hamster ovary cells all mediate the phosphorylation of extracellular-regulated kinase 1/2. *Mol Pharmacol.* 58:477-82.

Shin HK, Shin YW, Hong KW (2000) Role of adenosine A_{2B} receptors in vasodilation of rat pial artery and cerebral blood flow autoregulation. *Am J Physiol Heart Circ Physiol* 278: H339-344.

Sitaraman SV, Si-Tahar M, Merlin D, Strohmeier GR and Madara JL (2000) Polarity of A_{2B} adenosine receptor expression determines characteristics of receptor desensitization. *Am J Physiol* 278: C1230–C1236.

Stehle JH, Rivkees SA, Lee JJ, Weaver DR, Deeds JD and Reppert SM (1992) Molecular cloning and expression of the cDNA for a novel A_2-adenosine receptor subtype. *Mol Endocrinol* 6: 384-393.

Stewart M, Steinig AG, Ma C, Song JP, McKibben B, Castelhano AL and MacLennan SJ (2004) [^{3}H]OSIP339391, a selective, novel, and high affinity antagonist radioligand for adenosine A_{2B} receptors. *Biochem Pharmacol* 68: 305-312.

Sun CX, Zhong H, Mohsenin A, Morschl E, Chunn JL, Molina JG, Belardinelli L, Zeng D, Blackburn MR (2006) Role of A_{2B} adenosine receptor signaling in adenosine-dependent pulmonary inflammation and injury. *J Clin Invest* 116: 2173-2182.

Tamaoki J, Tagaya E, Chiyotani A, Takemura H, Nagai A, Konno K, Onuki T and Nitta S (1997) Effect of adenosine on adrenergic neurotransmission and modulation by endothelium in canine pulmonary artery. *Am J Physiol* 272: 1100-1105.

van Calker D, Muller M and Hamprecht B (1979) Adenosine regulates via two different types of receptors, the accumulation of cyclic AMP in cultured brain cells. *J Neurochem* 33: 999-1005.

Varani K, Gessi S, Merighi S, Vincenzi F, Cattabriga E, Benini A, Klotz KN, Baraldi PG, Tabrizi MA, Lennan SM, Leung E and Borea PA (2005) Pharmacological characterization of novel adenosine ligands in recombinant and native human A_{2B} receptors. *Biochem Pharmacol* 70: 1601-1612.

Volpini R, Costanzi S, Lambertucci C, Taffi S, Vittori S, Klotz KN, Cristalli G (2002) N(6)-alkyl-2-alkynyl derivatives of adenosine as potent and selective agonists at the human adenosine A_3 receptor and a starting point for searching A_{2B} ligands. *J Med Chem* 45: 3271-3279.

Volpini R, Costanzi S, Vittori S, Cristalli G and Klotz KN (2003) Medicinal chemistry and pharmacology of A_{2B} adenosine receptors. *Curr Top Med Chem* 3: 427-443.

Wakade AR and Wakade TD (1978) Inhibition of noradrenaline release by adenosine. *J Physiol* 282: 35-49.

Wakeno M, Minamino T, Seguchi O, Okazaki H, Tsukamoto O, Okada K, Hirata A, Fujita M, Asanuma H, Kim J, Komamura K, Takashima S, Mochizuki N, Kitakaze M (2006) Long-term stimulation of adenosine A_{2B} receptors begun after myocardial infarction prevents cardiac remodeling in rats. *Circulation* 114: 1923-1932.

Weiss JM, Morgan PH, Lutz MW and Kenakin TP (1996) The cubic ternary complex receptor-occupancy model. III. resurrecting efficacy. *J Theor Biol* 181: 381-397.

Worpenberg S, Burk O and Klempnauer KH (1997) The chicken adenosine receptor A_{2B} gene is regulated by v-myb. *Oncogene* 15: 213-221.

Yakel JL, Warren RA, Reppert SM and North RA (1993) Functional expression of adenosine A_{2B} receptor in Xenopus oocytes. *Mol Pharmacol* 43: 277-280.

Yang D, Koupenova M, McCrann DJ, Kopeikina KJ, Kagan HM, Schreiber BM, Ravid K (2008) The A_{2B} adenosine receptor protects against vascular injury. *Proc Natl Acad Sci U S A*. 105: 792-796.

Zhong H, Belardinelli L, Maa T, Feoktistov I, Biaggioni I and Zeng D (2004) A_{2B} adenosine receptors increase cytokine release by bronchial smooth muscle cells. *Am J Respir Cell Mol Biol* 30: 118-125.

Zhong H, Belardinelli L, Maa T and Zeng D (2005) Synergy between A_{2B} adenosine receptors and hypoxia in activating human lung fibroblasts. *Am J Respir Cell Mol Biol* 32: 2-8.

Zhong H, Wu Y, Belardinelli L, Zeng D. (2006) A_{2B} adenosine receptors induce IL-19 from bronchial epithelial cells, resulting in TNF-alpha increase. *Am J Respir Cell Mol Biol* 35: 587-592.

Zhou QY, Li C, Olah ME, Johnson RA, Stiles GL, Civelli O (1992) Molecular cloning and characterization of an adenosine receptor: the A_3 adenosine receptor. *Proc Natl Acad Sci U S A*. 89: 7432-7436.

Chapter

Application of mutagenesis techni ues to study the acti ation of GPCRs functionally expressed in yeasts

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ummar

G protein-coupled receptors (GPCRs) are the major targets of today's medicines. To elucidate the mechanism of activation and the interaction of these receptors with their G proteins, mutagenesis studies have proven to be a powerful tool and have provided insight in their structure and function. Random mutagenesis is very useful in this respect especially when it is combined with a robust screening assay that is based on the functional properties of the mutants. In this chapter, the use of random mutagenesis combined with a functional screening assay in yeast will be described and compared with alternative approaches such as site-directed mutagenesis *per se* and alanine/cysteine scanning. In addition, applications of the screening assay in yeast will be discussed.

G protein-coupled receptors

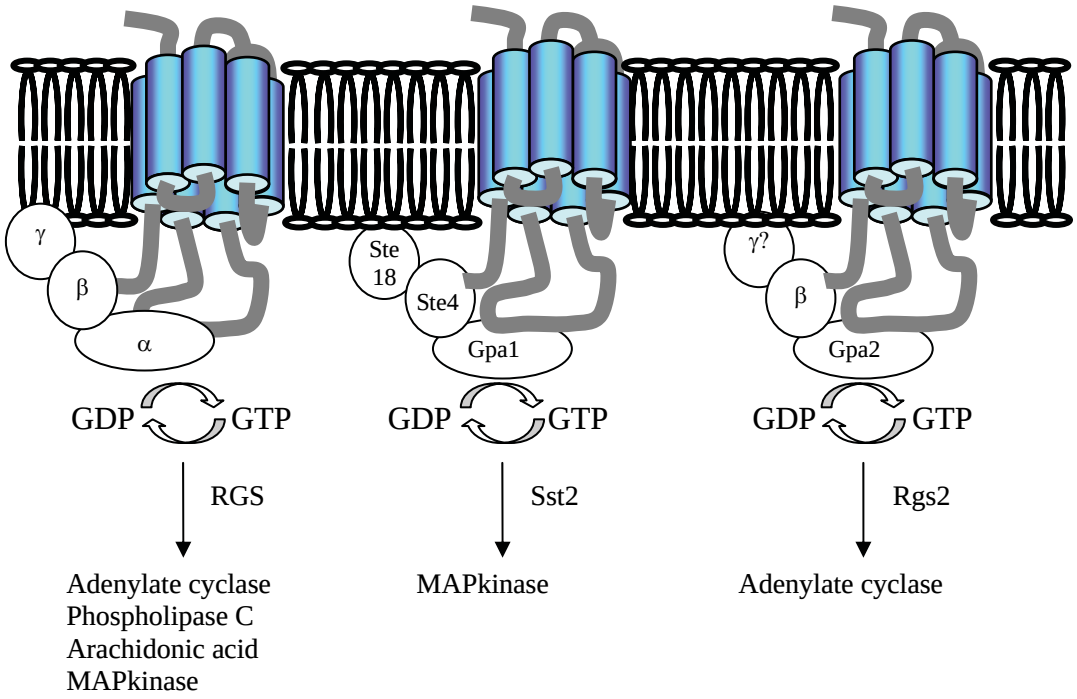
G protein-coupled receptors, GPCRs, constitute one of the larger classes of proteins. Information on these proteins is collected in a target specific database, the GPCRDB (www.gpcr.org/7tm). In humans approximately 650 members of this protein class have been identified (Lander et al., 2001; Venter et al., 2001). In addition also other eukaryotes, such as the model organisms *C. Elegans*, *Drosophila*, Zebrafish and even the unicellular yeast *Saccharomyces cerevisiae*, express GPCRs (Inoue et al., 2004). In all these organisms, GPCRs are involved in crucial signal transduction pathways including such vital processes as reproduction. Among them, yeast cells in particular provide a very convenient model system due to their short generation time. In addition, they are cheap to culture, their physiology is well-known and they are amenable to rapid molecular genetic manipulation. The functional similarity between the yeast pheromone pathway and the mammalian signaling cascade allows signaling of mammalian GPCRs via the yeast pathway (Slessareva and Dohlman, 2006). Yeast cells express only two endogenous GPCRs (see Box 1 and Figure 1A).

Box 1: GPCR signaling in yeast:

Two GPCR signaling pathways have been identified in yeast. One is involved in mating, the other in glucose sensing. Mating is activated by the α-factor and a-factor via the structurally unrelated Ste2 and Ste3 pheromone receptors, respectively. Yeast cells expressing the Ste2 receptor secrete the opposite sex hormone, the a-factor and vice versa (Versele et al., 2001). Both receptors activate the MAP kinase pathway via the yeast Gpa1 G protein.

The second signaling pathway is activated through glucose via the Gpr1 receptor and couples to the Gpa2 G protein. Stimulation with glucose activates a yeast adenylate cyclase resulting in the production of intracellular cAMP followed (Eilers et al., 2005) by several phenotypic responses such as a loss of carbohydrate storage, of stress resistance and a reduced life-span as well as an increase in growth and pseudohyphal differentiation (Versele et al., 2001; Lemaire et al., 2004; Tamaki et al., 2005)

A comparison of the GPCR signaling pathways in mammalian and yeast cells is schematically shown (Figure 1A). On a structural level, yeast and mammalian GPCRs share essential proline residues in TM6 and TM7, helix-helix interactions are mediated by polar residues and tight helix packing is mediated by small, weakly polar residues in both receptor classes (Eilers et al., 2005).



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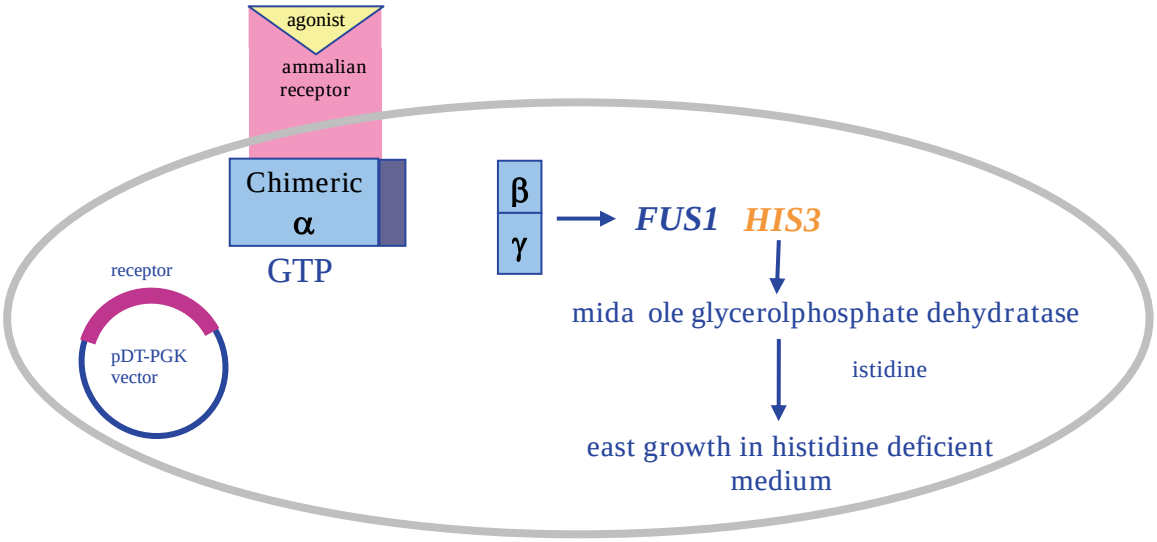

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Figure 1. A) Schematic drawing of GPCR activation in yeast and mammalian cells. B) Schematic drawing of GPCR activation in genetically modified yeasts. When heterologously expressed mammalian receptors were activated by agonists, yeast β and γ subunits disassemble from the α subunit and activate the Fus promoter which in turn activates the transcription of reporter genes, for example imidazole glycerolphosphatedehydratase. This enzyme enables yeast cells to synthesize histidine allowing them to grow in histidine-deficient medium.
Gpa: G protein alpha, Gpr: G protein-coupled receptor, RGS/Rgs: regulators of G protein signaling, Sst2: supersensitivity to pheromone, Ste: sterility

Knocking these genes out in the yeast system provides a null background to study mammalian GPCRs (Minic et al., 2005b). Quite a few yeast vectors have been developed, which enables one to functionally express heterologous GPCRs in yeast (Minic et al., 2005b). So far, more than 20 mammalian receptors have been successfully expressed in the yeast *S. cerevisiae* (for a review, see Minic, 2005). In several reports receptor expression in yeast was compared with expression in mammalian hosts. With respect to ligand binding, the β_2-adrenergic receptor, somatostatin receptor SST2 and adenosine A_{2A} receptor were shown to behave similarly upon expression in mammalian cells or in yeasts (King et al., 1990; Price et al., 1995; Price et al., 1996). In addition, receptor activation of the adenosine A_{2B} receptor was shown to be similar upon expression in mammalian cells or in yeast cells (Li et al., 2007b).

Interest in GPCRs is further substantiated by the fact that they are very successful drug targets. In fact about 50% of today's medicines act via this class of proteins (Drews, 2000). To elucidate the mechanism of activation of the receptors and their interaction with G proteins at a molecular level, mutagenesis studies are a very powerful tool. The GPCRDB provides access to mutation data on GPCRs via the tinyGRAP database and via an automatic tool to extract mutation data from the literature, MuteXt (Horn et al., 1998; Edvardsen et al., 2002; Horn et al., 2004) (www.gpcr.org/7tm). In addition, very recently a database on natural variants of GPCRs has been created (http://nava.liacs.nl/; Kazius et al., 2008).

uta enesis techni ues

To elucidate the mechanism of activation of GPCRs and their interactions with G proteins at a molecular level, mutagenesis studies are a powerful tool.

Available techniques to introduce mutations range from site-directed to random mutagenesis in decreasing order of specificity. Site-directed mutagenesis refers to the site-specific introduction of point mutations. In addition, a less specific mutagenesis approach can be undertaken such as a simultaneous replacement of multiple amino acids e.g. through the construction of chimeras or the use of techniques such as alanine scanning. A keyword search in PubMed reveals that site-directed mutagenesis is the most commonly used tool (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?DB pubmed). More recently, however, the use of random mutagenesis techniques has been gaining popularity.

The latter approach yields large numbers of mutant proteins for which additional techniques are required to select for the mutants of interest (Figure 2).

ite-directed mutagenesis

Selection of an amino acid for mutation assumes a certain perception of its importance and role in the protein. From the triad sequence – structure – function it follows that both structure and sequence might already hint to or even reveal the role of an individual amino acid. The 3D structure of only two GPCRs, rhodopsin and the β_2-adrenoceptor, have been elucidated (Cherezov et al, 2007; Rosenbaum et al., 2007a; Rosenbaum et al., 2007b), and hence structural information is scarce (Palczewski et al., 2000). Fortunately, the large number of GPCR sequences available allows bioinformatics approaches to derive clues for the function of individual amino acids. The importance of bioinformatics in mutagenesis studies has been recently reviewed (Lu et al., 2002; Kristiansen, 2004). In addition, various authors compared the occurrence of residues within GPCR subfamilies versus the GPCR family as a whole

(Oliveira et al., 2003; Ye et al., 2006), these studies provide clues on residues that may be involved in for instance ligand binding.

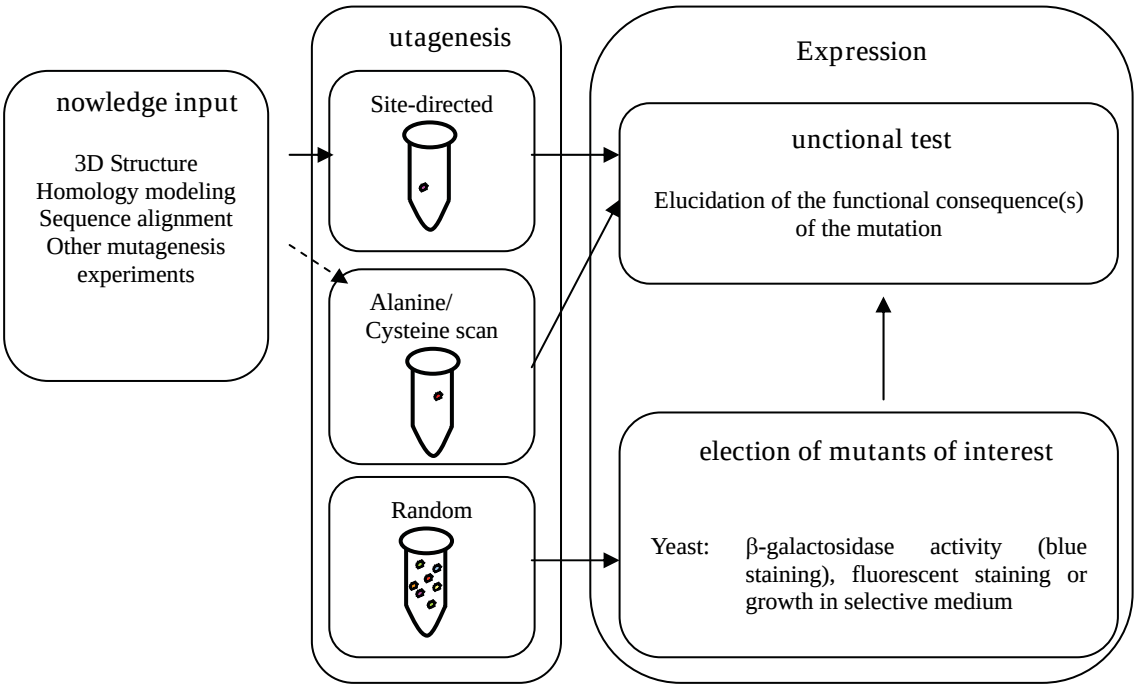


Figure 2. Strategies to generate and subsequently investigate mutated GPCRs.
Techniques to introduce mutations range from site-directed to random mutagenesis. To select amino acids for mutation, prior knowledge on the receptor itself or related receptors is required. Examples include information from an experimentally determined or computer-modeling derived structure, sequence alignments and (other) mutation data. Random mutagenesis generates a large number of mutants, in contrast to site-directed mutagenesis and alanine/cysteine scanning.

Data obtained through site-directed mutagenesis are most easily interpreted. Still, careful analysis of experimental data is required since the replacement of an amino acid can result in a local effect due to a gain or loss of interaction with neighboring residues or in a global effect such as an alteration of protein folding or protein stability.

An extended application of site-directed mutagenesis is to create the reciprocal mutant pair. For instance if an interaction between two residues is considered to be critical for receptor function, mutation of one of the residues must impair receptor function, whereas the reciprocal mutant may display wild-type activity. In addition, a mutation of a critical amino acid may be compensated for by mutating interacting residues as was shown for the yeast Ste2p receptor (Lee et al., 2006). Three approaches have been reported to show that residues are interacting with one another. Through introduction of a histidine pair, pH dependence can be introduced. Alternatively, an ion pair such as lysine-aspartate may be introduced. Finally, the introduction of a pair of cysteines may result in disulfide bond formation provided that the residues are in each other's vicinity (Lee et al., 2006). To aid in the selection of amino acids to be subjected to site-directed mutagenesis a double mutant cycle analysis was developed. This method combines site-directed mutagenesis with free-energy calculations. It enables one

to calculate how a mutation of a selected residue may affect the properties of a second residue (Vogel et al., 1999). In this approach, the binding free energy is calculated for the ligand/wt receptor, for both ligand/single mutant receptors and for the ligand/double mutant receptor. If the effect on the binding free energy of the double mutant is not equal to the sum of that of the two single mutants, then the two residues must affect each other either favourably (the double mutant has the smaller binding free energy) or unfavourably (the sum of the single mutants has the smaller binding free energy). In a study on M_2 muscarinic acetylcholine receptors, the double mutant cycle analysis revealed the absence of an interaction among three asparagine residues in the transmembrane area (N69, N97 and N103) since they showed an independent effect on the free energy of ligand binding as well as independent effects in a phospholipase C stimulation assay (Vogel et al., 1999). Using this approach Naider et al. (2007) proved that for the Ste2p receptor and its α–factor ligand in yeast, N205 and Y266 interact strongly with residues at the amino terminus but not with amino acids at the carboxyl terminus of the receptor.

Alanine cysteine scanning mutagenesis

A less specific mutagenesis strategy is often undertaken when clear indications on exactly which amino acid to mutate are absent. A frequently applied technique is a scanning analysis, e.g. alanine scanning. In this approach, residues are replaced with alanine because of its small size (the side chain is a methyl group) and its relative inertness (it is non-charged). Recently, this method has been applied to two GPCRs, the Ca^{2+} -sensing and the metabotropic glutamate receptor to study ligand binding and G protein-coupling, respectively (Dhami et al., 2005; Hu et al., 2005). Besides alanine also cysteine replacements have been carried out. The advantage of the latter method is that cysteine residues can be modified with sulfhydryl-specific alkylating reagents, such as methanethiosulfonate-ethylammonium. When the chemical modification of native cysteines does not affect ligand binding, cysteine residues can be introduced at the putative ligand binding site and after treatment of the receptor with the reagent, the ability of the ligand to bind to the receptor is determined (Hubbell et al., 2003).

A recent extension of the alanine scanning technique is the shotgun approach (Weiss et al., 2000). This method introduces residues at selected positions within the target protein through the use of a so-called shotgun codon and employs a phage display assay based on protein-protein interactions to analyze the large numbers of mutants generated (Kunkel et al., 1987; Smothers et al., 2002; Szardenings, 2003). The shotgun approach has not yet been applied to GPCRs but might be used to study those receptors that recognize a protein ligand.

Random mutagenesis

Random introduction of mutations can be achieved through the use of spiked oligonucleotides or by manipulating the polymerase chain reaction, PCR. The former method is limited to the introduction of mutations within the synthetic oligonucleotide and is therefore restricted to domains up to approx. 90 basepairs (bp). During synthesis a low percentage of incorrect bases are introduced at each position resulting in mutations. This method has been applied to the yeast α factor receptor (Sommers and Dumont, 1997; Martin et al., 2002; Celic et al., 2003), the cAMP receptor from *Dictyostelium* (Milne et al., 1997) and the human C5a (Baranski et al., 1999; Geva et al., 2000; Klco et al., 2005; Klco et al., 2006) and vasopressin V2 receptors (Erlenbach et al., 2001b), and has been recently reviewed (Celic et al., 2004). The latter,

error-prone PCR method is based on the substitution of Mg^{2+} with Mn^{2+} ions resulting in a compromised fidelity of the DNA polymerase enzyme. Furthermore, the introduction of mutations can be guided by adding excess of one of the nucleotides. The use of this technique to mutate large DNA fragments was initially described and compared to existing methods in 1995, and has been further optimized to introduce mutations in fragments up to 400 bp length (Fromant et al., 1995; Beukers et al., 2004). The use of a combination of polymerases, the so-called genemorph technique has been reported to enable random mutagenesis of fragments up to 6000 bp length (Chao et al., 2004).

Although an error-prone PCR will yield mutated proteins, not every single amino acid substitution can be achieved. The likelihood of a simultaneous substitution of more than one nucleotide within a single codon is very small especially since the PCR conditions are optimized to limit the number of introduced mutations. As a result in practice only one-base substitutions occur, limiting the number of possible amino acid substitutions (see supplementary information). The mutant library should be large enough to contain as many substitutions as possible. As a consequence, analysis of all the mutants is very laborious unless a powerful selection assay is available to identify the mutations of interest.

Combined mutagenesis

To thoroughly investigate the receptor activation/inactivation mechanism, combined mutagenesis methods have been frequently applied. For example, in a study to identify critical amino acids in the function of the yeast Ste2p receptor, N205 and Y266 were found in an alanine mutagenesis assay (Lee et al., 2002). Subsequently, various mutants were made and a double-mutant cycle study was performed to prove the interaction between these two residues (Lee et al., 2006). As mentioned above, the double-mutant cycle approach was used to investigate the interaction between these two residues and the residues in the α-factor peptide (Naider et al., 2007).

In addition, in several reports random and single-point mutagenesis have been combined. In the study of the M_3 muscarinic receptor, random mutagenesis was followed by site-directed mutagenesis to reveal the critical amino acids involved in the stabilization of the active state of the receptor (Scarselli et al., 2007). Another example is the human UDP-glucose (P2Y14) receptor where two rounds of mutagenesis were performed to determine the amino acid residues involved in ligand binding (Ault and Broach, 2006).

To evaluate the effect of the mutagenesis studies on receptor function, engineered yeast cells in which the endogenous pheromone signal transduction route was coupled to mammalian GPCRs have thus been very instrumental. A further coupling of the pheromone signal transduction route to a reporter gene yielded very robust screening assays.

east screenin assa

Endogenous GPCRs of yeast activate the pheromone pathway via the yeast Gpa1 protein. For human GPCRs to interact with this pathway, the yeast G protein needs to be 'humanized'. A major breakthrough in the study of functional expression of human GPCRs in yeast cells was the creation of a chimeric G protein between the yeast Gpa1 and human $G\alpha_{i2}$ proteins (Pausch, 1997; Klein et al., 1998; Baranski et al., 1999). The coupling of human GPCRs to the endogenous yeast G protein or these chimeric G proteins was, however, successful for a

limited number of receptors only. The construction of G protein chimeras of Gpa1 containing the C-terminal five amino acids of mammalian G proteins, the so-called G protein transplants, alleviated this limitation to a large extent (Olesnicky et al., 1999; Brown et al., 2000).

Methods were not only developed to enhance G protein coupling, but also to improve the measurement and read-out of receptor activation. To this end the yeast cell was equipped with a reporter gene.

Read-out

Reporter gene

To assess receptor activity several reporter genes have been used, the most frequently being *LacZ* and *HIS3*. *LacZ* encodes β-galactosidase, the production of which can be monitored through the conversion of a colorless substrate such as X-gal to a colored product. The *HIS3* gene encodes imidazole glycerolphosphate dehydratase, the key enzyme for the production of histidine, an essential amino acid for yeast growth. Whereas some authors based their findings on the β-galactosidase assay only (Sommers et al., 2000), others combined both methods and measured β-galactosidase activity as well as growth (Zhang et al., 2002; Arias et al., 2003). In some cases, however, yeast growth turned out to be a more reproducible measure than the production of β-galactosidase (Beukers et al., 2004).

To measure histidine-dependent growth, 3-aminotriazole (3-AT), an inhibitor of imidazole glycerolphosphate dehydratase, is added to the cells to block receptor-independent growth. To discriminate among mutants of the C5a receptor yeast growth was studied at a fixed concentration of agonist and a range of 3-AT concentrations (Baranski et al., 1999; Geva et al., 2000; Klco et al., 2005). Alternatively, growth upon activation of the adenosine A_{2B} receptor was investigated with different agonist concentrations in the presence of an optimized amount of 3-AT (Beukers et al., 2004; Li et al., 2007b). This method allows the characterization of ligand-dependent as well as constitutive growth.

Whereas the studies mentioned above examined growth at a single, optimized, time-point, others monitored growth in time (Erlenbach et al., 2001a; Erlenbach et al., 2001b; Schmidt et al., 2003). The latter method provides a more detailed insight in growth and allows the determination of the growth rate (see also Figure 3A and 3B with details from our own work).

Interestingly, recent data suggest that the sensitivity of the read-out can be improved by simultaneously using both reporters because the increased yeast growth is able to produce a more extensive color after stimulation. Thus the use of two reporters may result in strong signal amplification. Das et al. reported that stimulation of the genetically modified fission yeast *S. pombe* with 1 µM P factor with a double reporter increased the signal: background ratio to almost 2500:1, while this ratio was 35:1 for a single *LacZ* reporter (Das et al., 2006).

Next to the *LacZ* and the *HIS3* reporter, luc, the gene encoding for luciferase has been placed under the control of an inducible *Fus1* promoter. With this reporter gene assay, receptor activation can be measured upon addition of luciferin and ATP (Minic et al., 2005a). An advantage of the luciferase assay with respect to the *HIS3* gene assay is the amount of time required to perform the assay. Yeast growth assays are time consuming as they include a delay of 2 – 4 days in response (Minic, et al., 2005a). Luciferase assays on the other hand can be performed within hours (Pajot-Augy et al., 2005). If the assay is measured with fluorescence, the ligands for the luciferase assay are cheaper than those for the *LacZ* reporter gene assay.

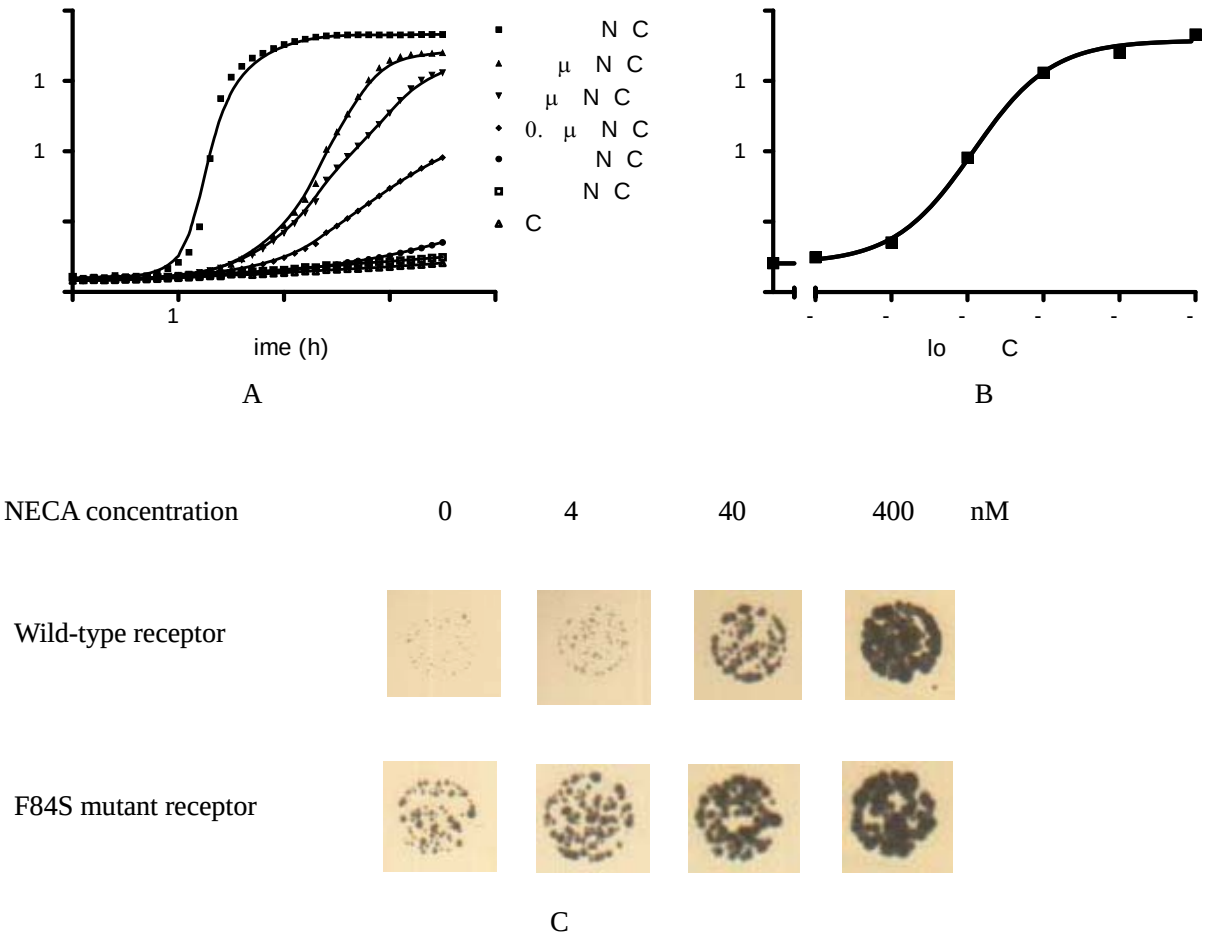


Figure 3. A) Growth curves of yeast cells expressing wild-type human adenosine A_{2B} receptors. Each curve represents the growth of yeast cells as a response to [NECA] in an individual well of a 96-well microtiter plate (liquid medium assay). Based on the yeast growth in various wells with different concentrations of NECA, a dose-response curve B) can be obtained, in this case at 35 h of growth. C) Yeast growth assay (solid medium assay) to detect constitutive and agonist-induced receptor activation. Growth of yeast cells expressing the wild-type and the F84S mutant adenosine A_{2B} receptor is shown. The agonist NECA is required to induce growth of yeast cells expressing the wild-type receptor. The F84S mutant receptor is constitutively active as indicated by the fact that yeast growth occurs in the absence of NECA. Upon addition of NECA the growth of yeast cells expressing this mutant receptor is further enhanced.

Yet another reporter gene is the *Hph* reporter gene. Expression of the *Hph* reporter gene confers the yeast to be resistant to hygromycin. Thus, similar to the *HIS3* reporter gene assay, activation of the *Hph* reporter gene permits yeast growth on a selective hygromycin medium. A word of caution, however, must be mentioned, as tolerance to hygromycin might be dependent on the incubation temperature (Pajot-Augy et al., 2003).

Contrary to the above strategies for positive selection, i.e. gain-of-function', fusing the inducible *Fus1* promoter with *can1* leads to negative selection. The *Can1* gene encodes for arginine permease, which allows the yeast cells to take up a toxic arginine analogue canavanine. Thus in the presence of canavanine, yeast cells expressing functional receptors are unable to grow. This negative selection can be used to screen for inactivating mutants or to screen for antagonists and/or inverse agonists. This method was successfully applied to reveal the importance of the second extracellular loop in the activation of the M_3 muscarinic acetylcholine receptor (Li et al., 2007a; Scarselli et al., 2007).

The reporter gene assays listed above can be performed in liquid media or on agar plates. With respect to the liquid assay, the yeast cells are usually cultured in microtiter dishes for 20 to 35 hours and yeast growth is determined by measuring the optical density at 600 nm. Microtiter cultivation of yeast cells displayed overall growth features and protein expression patterns similar to growth in medium-scale cultures (10 ml) although higher levels of stress-induced proteins were observed in the former culture probably due to a lack of oxygen (Warringer and Blomberg, 2003). Violent shaking at the highest intensity for 60 seconds prior to measurements helps to homogenize cells and improves the reproducibility of the assays. The output from the platereader in Excel files can be directly used to generate yeast growth-time curves or dose-response curves. For the solid medium assay, drops containing the same amount of yeasts are seeded on agar plates. After 24 to 50 hours incubation, the plates are scanned and the growth of yeasts can be quantified by software. Since the outcome is not automatically reported, solid medium assays are more laborious to perform than the liquid ones.

To verify whether abnormal binding or functioning is a result of altered expression levels, folding or localization, GFP has been used as a marker in mammalian cells (Gimpl and Fahrenholz, 2000) and yeast cells (Sarramegna et al., 2002; Butz et al., 2003; Niebauer and Robinson, 2006). Reports in the literature suggest that high quantum GFP fluorescence is able to reveal relatively low expression levels of GPCRs (Niebauer and Robinson, 2006). For a receptor-GFP fusion protein, the expression level can be determined either by comparing the intensity of the receptor-GFP band in a western blot with the expression level of a standard or by comparing whole cell GFP fluorescence with a standard GFP curve. Disadvantages of both methods were reported in a study on the functional expression of human adenosine A_{2A} receptors in yeast (Niebauer and Robinson, 2006). Western blot analysis may induce large standard deviations due to the multiple experimental steps that are involved in the preparation of the sample. GFP fluorescence on the other hand is very sensitive to pH which may affect whole cell GFP fluorescence measurements. Confocal microscopy is able to reveal the cellular localization of receptor-GFP fusion proteins. When a GFP tagged receptor is used together with a vacuolar dye or with a co-expressed subcellular apparatus marker, for example Golgi marker Sec7-DsRed2, confocal microscopy is even able to reveal receptor transportation (Butz et al., 2003). A drawback of these (GFP-) tagged receptors is that these methods reveal the presence of the tag, but do not provide information on functional expression levels.

Application of yeast screening assay

The engineered yeast cells have been successfully used not only to screen for mutants of interest but also in several other applications. Examples of these will be provided below.

High throughput screening (HTS)

To transform the yeast screening assay into a high throughput format a convenient read-out is essential. The coupling of the pheromone pathway to yeast growth via the *HIS-3* gene fuelled the use of engineered yeast cells in high-throughput screening (Pausch, 1997; Dowell and Brown, 2002). Application of this method includes the identification of novel agonists for the yeast α-mating type receptor, of antagonists for heterologously expressed somatostatin receptors and of odorants (Bass et al., 1996; Manfredi et al., 1996; Dowell and Brown, 2002; Pajot-Augy et al., 2003).

Orphan receptors

Orphan receptors are GPCRs for which the endogenous ligand is unknown. The yeast assay has proven successful in the identification of surrogate ligands for these orphan receptors (Klein et al., 1998; Brown et al., 2003). For example, an orphan receptor structurally related to the formyl peptide receptor was successfully expressed in yeast cells together with a library encoding random peptides [Klein et al., 1998]. Growth in the absence of histidine identified six peptides that were able to activate this receptor. Similarly, the GPR41 and GPR43 receptors were expressed in yeast and shown to be activated by short chain carboxylic acids such as propionate [Brown et al., 2003]. The advantage of these yeast cells over other higher eukaryotic expression systems is the zero background they provide. The cells themselves do not release endogenous ligands in contrast to e.g. human embryonic kidney cells. Moreover the yeast cells have been engineered to lack endogenous GPCRs (Wise et al., 2002).

G protein-coupling

Although the involvement of the intracellular domains of GPCRs in G protein-coupling is known, random mutagenesis together with screening in yeast has proven a useful strategy to identify the relevant amino acids. The communication of muscarinic receptors with yeast G proteins (endogenous as well as humanized) was shown to depend on the large third intracellular domain of these receptors (Erlenbach et al., 2001a). Vasopressin V2 receptors were also studied for their interaction with G proteins. Random mutagenesis of the second intracellular loop identified a critical methionine residue in the central part of this domain. Mutation of this amino acid turned out to alter G-protein selectivity as was confirmed through subsequent site-directed mutagenesis experiments (Erlenbach et al., 2001b).

Receptor activation/inactivation

The endogenous yeast GPCR gene, Ste2, encoding the α factor receptor was subjected to random mutagenesis to identify amino acids involved in constitutive activity (Sommers et al., 2000). Within the large library of mutant receptors 14 amino acids were discovered that caused constitutive activity. The endogenous agonist, the α factor, was still able to further activate these mutants. Interestingly, in 10 of these mutants a peptide analog of the α factor that is an antagonist on the wild type receptor behaved as an agonist. Due to the lack of sequence homology between yeast GPCRs and GPCRs of higher eukaryotes, these findings cannot be readily extrapolated to e.g. mammalian GPCRs. Subsequent experiments on human GPCRs have provided insight though in the amino acids involved in receptor activation in higher eukaryotes. An example is the random mutagenesis and screening of the C5a receptor (Baranski et al., 1999; Geva et al., 2000; Klco et al., 2005). Random mutagenesis of the transmembrane domains and the second extracellular loop yielded a large number of receptor mutants that were activated at lower concentrations of C5a than needed for the wild type receptor. In addition, several constitutively active mutants were identified. Although these studies yielded very few point mutants, regions of the receptor involved in ligand-induced activation as well as constitutive activity of the receptor could be identified.

Constitutively active receptor mutants can also be used to discriminate between neutral antagonists and inverse agonists. Analysis of constitutively active mutant CXCR4 receptors revealed that antagonists (for the wild type receptor) possessed intrinsic activities varying from inverse agonism to weak partial agonism (Zhang et al., 2002). A similar study was performed with constitutively active mutant human adenosine A_{2B} receptors and revealed a

parabolic relationship between intrinsic efficacy of inverse agonists and the levels of constitutive activity of the mutant receptor tested (Li et al., 2007b).

Furthermore, constitutively active receptors have been used to identify silencing mutations. Residues reverting the constitutive activity of a mutant muscarinic M_3 receptor were also shown to be involved in receptor/G protein coupling in the wild-type receptor (Schmidt et al., 2003). A similar strategy was applied to compensate for the loss of function of the D113N mutant rat muscarinic M_3 receptor, D113 being the highly conserved aspartate in TM2. Through random mutagenesis three mutations of two highly conserved residues, R165W, R165M and Y250D in TM3 and TM5, respectively, were able to restore the function of the mutated receptor (Li et al., 2005).

The above-mentioned examples to study receptor activation all focused on a rather small amino acid sequence, either a transmembrane domain or an intracellular loop. To increase the domain subjected to mutagenesis without increasing the number of introduced mutations, the random mutagenesis PCR approach needed to be optimized (Beukers et al., 2004). In addition, the screening assay in yeast was adapted to identify and select for constitutively active receptors as well as receptors that are more easily activated than the wild-type receptor. By fine-tuning the assay conditions, such a selection was achieved yielding mutants of the human adenosine A_{2B} receptor with varying degrees of constitutive and agonist-induced activity (Figure 3C).

Most mutagenesis experiments are aimed at the discovery of an activating mutation. However, inactivating mutations can also provide important clues for the mechanism of receptor activation. A screen for loss-of-function mutants of the muscarinic acetylcholine M_3 receptor from a random mutation bank revealed 20 point mutants which had lost their function. Interestingly, these 20 mutated residues were all located in extracellular loop 2 which suggests that extracellular loop 2 plays an important role in agonist-induced activation (Scarselli et al., 2007).

Evaluation of yeast screening assay together with random mutagenesis

The success of a random mutagenesis study depends on the error-prone PCR protocol and on the number of mutants generated. Optimization of the PCR conditions should ideally yield single point mutants or at least a limited number of multiple point mutants. The use of a slight amount of $MnCl_2$ together with excess of one of the nucleotides, the forcing nucleotide, has proven very successful yielding relatively large numbers of single point mutants (Sommers and Dumont, 1997; Sommers et al., 2000; Schmidt et al., 2003; Beukers et al., 2004). Table 1 presents an overview of random mutagenesis studies of GPCRs, through the use of spiked oligonucleotides or error-prone PCR, followed by expression in yeast. As mentioned before, spiked oligonucleotides can be used to mutate domains up to 90 bps, whereas error-prone PCR is applicable to larger sequences too.

Table 1. Evaluation of error-prone PCR and spiked oligonucleotides methods to introduce random mutations.

Investigated domain; receptor	Domain size (amino acids)	Number of mutants	Number of single point mutants of selected mutants of interest	Mutagenesis method	Ref.
TMIII; α-factor	15	9,000-15,000	12 out of 29	Spiked oligonucleotide: 0.7% non wild-type	Sommers and Dumont, 1997
TMI to TMII TMIII to TMIV TMVI to TMVII; all α-factor	69 71 48	5000	2 out of 7	Error-prone PCR: 3 mM $MgCl_2$, 0.6 mM $MnCl_2$ 1 mM forcing NTP, 0.2 mM for either dATP or dGTP	Sommers and Dumont, 1997
TMIII, TMV, TMVI, TMVII; C5a	21-25	50,000-1000,000	TMIII 0 out of 30 TMV: 0 out of 40 TMVI: 0 out of 25 TMVII: 1 out of 28	Spiked oligonucleotide: 20% non wild-type	Baranski et al., 1999
TMI, TMII, TMIV; C5a	19-25	50,000-1000,000	TM1: 0 out of 25 TMII: 0 out of 22 TMIV: 1 out of37	Spiked oligonucleotide: 20% non wild-type	Geva et al., 2000
N-terminus and TM domains; α-factor	14-104	24,000	Number of single point mutants was not specified among 13 selected mutants of interest	Error-prone PCR: 3 mM $MgCl_2$, 0.6 mM $MnCl_2$ 1 mM forcing NTP, 0.2 mM others*	Sommer et al., 2000
IL2; V2 vasopressin	31	30,000	1 out of 11	Spiked oligonucleotide: 10% non wild-type	Erlenbach et al., 2001
Individual TM domains; α-factor	20-25	6,000 to 40,000 per domain	498 out of 576	Spiked oligonucleotide: 20% non wild-type	Martin et al., 2002
full length; CXCR4	352 (human)	>10,000	4 out of 26	Error-prone PCR: not specified, manganese and dITP added	Zhang et al., 2002
TMV-TMVII; M3	four fragments of 16-19	20,000	12 out of around 22 (15% of 150 clones)	Error-prone PCR: 9.5 mM MgCl2, 0.5 mM MnCl2 3.4 mM forcing NTP, 0.2 mM others	Schmidt et al., 2003

Whole receptor except for IL3, M3	87 86 135 107 49	900,000	21.7% of all primary yeast transformants harbored M3 receptor plasmids containing single nucleotide changes	Error-prone PCR: 7 mM MgCl2, 0.5 mM MnCl2 1 mM dCTP and dTTP, 0.2 mM dATP and dGTP	Li et al., 2007
IL3; α-factor	21	20,000	72 out of 83	Spiked oligonucleotide: 20% non wild-type	Celic et al., 2003
full length; CCR5	352	>10,000	1 out of 18	Error-prone PCR: manganese added	Arias et al., 2003
N-terminus to TMIII and TMV to C-terminus; A_{2B}	120 and 128	4,000	6 out of 14	Error-prone PCR: 4.7 mM $MgCl_2$; 0.5 mM $MnCl_2$ 3.4 mM forcing NTP, 0.2 mM others	Beukers et al., 2004
TMII to C-terminus, in IL3 deficient (A274-K469) mutant; M3	277	200,000	3 out of 8	Error-prone PCR: 7 mM MgCl2; 0.5 mM MnCl2 1 mM dCTP, 1mM dTTP, 0.2 mM dATP, 0.2 mM dGTP	Li et al., 2005
N-terminus to TMIII, TMIII-EL2, TMIV-TMVII; α-factor	76, 112 and 146	35,000	18 out of 20 compensate F204S** 24 out of 34 compensate Y266C**	Error-prone PCR: 10-fold lower concentration of dATP or dGTP	Lin et al., 2005
EL2; C5a	24	40,000	0 out of 29	Spiked oligonucleotide: 20% non wild-type	Klco et al., 2005
EL1 and EL3; C5a	16 28	350,000 and 425,000	1 out of 29 0 out of 23	Spiked oligonucleotide: 20% non wild-type	Klco et al., 2006

*: Complementary to the error-prone PCR method, the mutated products obtained were subjected to oligonucleotide-directed mutagenesis through the use of a pool of degenerate oligonucleotides containing 0.7% of an altered nucleotide for each nucleotide position (Kunkel et al., 1987).
**: Mutants were selected for their ability to compensate either the loss of function introduced by a F204S (ligand binding) or Y266C (activation) mutation.
EL: extracellular loop, IL: intracellular loop, NTP: nucleoside triphosphate, TM: transmembrane domain

The number of mutants generated ranged from 4,000 to 2,000,000. These numbers have proven sufficient to generate informative mutants as identified through the screening assay. In several reports, site-directed mutagenesis was used to confirm the role of individual amino acids emerging from a random mutagenesis protocol (Sommers et al. 2000, Erlenbach et al., 2001b; Celic et al., 2003; Klco et al., 2005). In most cases, the random mutagenesis studies yielded single point mutants of which the role could be directly interpreted (Sommers and Dumont, 1997; Baranski et al., 1999; Geva et al., 2000; Sommers et al., 2000; Erlenbach et al., 2001b; Martin et al., 2002; Zhang et al., 2002; Arias et al., 2003; Celic et al., 2003; Schmidt et al., 2003; Beukers et al., 2004; Li et al., 2005; Lin et al., 2005; Scarselli et al., 2007).

Some might doubt whether the heterologous expression of mammalian receptors in yeast can be predictive for the effects obtained when the receptors are expressed in their natural,

mammalian context. To address this issue several groups have compared both expression systems and obtained similar results (Zhang et al., 2002; Arias et al., 2003; Schmidt et al., 2003; Beukers et al., 2004; Klco et al., 2005; Li et al., 2005).

Conclusion

G protein-coupled receptors are the major targets of today's medicines. Mutagenesis techniques are frequently and successfully applied to study their interaction with ligands, their mechanism of activation and their interaction with G proteins. Random mutagenesis is a very powerful tool to gain more insight in these receptor functions. Yeast cells are a very convenient host to express these mutated receptors. Moreover, the lack of endogenous activators makes yeast cells very suitable hosts to study e.g. orphan receptors. In addition, these cells have been engineered to communicate with human GPCRs resulting in the activation of a reporter gene, while at the same time the expression of their endogenous GPCRs has been knocked-out. This combination of random mutagenesis and expression in yeast has proven successful in the identification of ligands for orphan receptors and in high throughput screening, and has provided insight in G-protein coupling and receptor activation.

References

Arias DA, Navenot JM, Zhang WB, Broach J and Peiper SC (2003) Constitutive activation of CCR5 and CCR2 induced by conformational changes in the conserved TXP motif in transmembrane helix 2. *J Biol Chem* 278:36513-36521.

Ault AD and Broach JR (2006) Creation of GPCR-based chemical sensors by directed evolution in yeast. *Protein Eng Des Sel* 19:1-8.

Baranski TJ, Herzmark P, Lichtarge O, Gerber BO, Trueheart J, Meng EC, Iiri T, Sheikh SP and Bourne HR (1999) C5a receptor activation. Genetic identification of critical residues in four transmembrane helices. *J Biol Chem* 274:15757-15765.

Bass RT, Buckwalter BL, Patel BP, Pausch MH, Price LA, Strnad J and Hadcock JR (1996) Identification and characterization of novel somatostatin antagonists. *Mol Pharmacol* 50:709-715.

Beukers MW, van Oppenraaij J, van der Hoorn PP, Blad CC, den Dulk H, Brouwer J and IJzerman AP (2004) Random mutagenesis of the human adenosine A_{2B} receptor followed by growth selection in yeast. Identification of constitutively active and gain of function mutations. *Mol Pharmacol* 65:702-710.

Brown AJ, Dyos SL, Whiteway MS, White JH, Watson MA, Marzioch M, Clare JJ, Cousens DJ, Paddon C, Plumpton C, Romanos MA and Dowell SJ (2000) Functional coupling of mammalian receptors to the yeast mating pathway using novel yeast/mammalian G protein alpha-subunit chimeras. *Yeast* 16:11-22.

Brown AJ, Goldsworthy SM, Barnes AA, Eilert MM, Tcheang L, Daniels D, Muir AI, Wigglesworth MJ, Kinghorn I, Fraser NJ, Pike NB, Strum JC, Steplewski KM, Murdock PR, Holder JC, Marshall FH, Szekeres PG, Wilson S, Ignar DM, Foord SM, Wise A and Dowell SJ (2003) The Orphan G protein-coupled receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids. *J Biol Chem* 278:11312-11319.

Butz JA, Niebauer RT and Robinson AS (2003) Co-expression of molecular chaperones does not improve the heterologous expression of mammalian G-protein coupled receptor expression in yeast. *Biotechnol Bioeng* 84:292-304.

Celic A, Connelly SM, Martin NP and Dumont ME (2004) Intensive mutational analysis of G protein-coupled receptors in yeast. *Methods Mol Biol* 237:105-120.

Celic A, Martin NP, Son CD, Becker JM, Naider F and Dumont ME (2003) Sequences in the intracellular loops of the yeast pheromone receptor Ste2p required for G protein activation. *Biochemistry* 42:3004-3017.

Chao G, Cochran JR and Wittrup KD (2004) Fine epitope mapping of anti-epidermal growth factor receptor antibodies through random mutagenesis and yeast surface display. *J Mol Biol* 342:539-550.

Das A, Forfar R, Ladds G and Davey J (2006) Combined use of two transcriptional reporters improves signalling assays for G protein-coupled receptors in fission yeast. *Yeast* 23:889-897.

Dhami GK, Babwah AV, Sterne-Marr R and Ferguson SS (2005) Phosphorylation-independent regulation of metabotropic glutamate receptor 1 signaling requires g protein-coupled receptor kinase 2 binding to the second intracellular loop. *J Biol Chem* 280:24420-24427.

Dowell SJ and Brown AJ (2002) Yeast assays for G-protein-coupled receptors. *Receptors Channels* 8:343-352.

Drews J (2000) Drug discovery: a historical perspective. *Science* 287:1960-1964.

Edvardsen O, Reiersen AL, Beukers MW and Kristiansen K (2002) tGRAP, the G-protein coupled receptors mutant database. *Nucleic Acids Res* 30:361-363.

Eilers M, Hornak V, Smith SO and Konopka JB (2005) Comparison of class A and D G protein-coupled receptors: common features in structure and activation. *Biochemistry* 44:8959-8975.

Erlenbach I, Kostenis E, Schmidt C, Hamdan FF, Pausch MH and Wess J (2001a) Functional expression of M_1, M_3 and M_5muscarinic acetylcholine receptors in yeast. *J Neurochem* 77:1327-1337.

Erlenbach I, Kostenis E, Schmidt C, Serradeil-Le Gal C, Raufaste D, Dumont ME, Pausch MH and Wess J (2001b) Single amino acid substitutions and deletions that alter the G protein coupling properties of the V2 vasopressin receptor identified in yeast by receptor random mutagenesis. *J Biol Chem* 276:29382-29392.

Fromant M, Blanquet S and Plateau P (1995) Direct random mutagenesis of gene-sized DNA fragments using polymerase chain reaction. *Anal Biochem* 224:347-353.

Geva A, Lassere TB, Lichtarge O, Pollitt SK and Baranski TJ (2000) Genetic mapping of the human C5a receptor. Identification of transmembrane amino acids critical for receptor function. *J Biol Chem* 275:35393-35401.

Gimpl G and Fahrenholz F (2000) Human oxytocin receptors in cholesterol-rich vs. cholesterol-poor microdomains of the plasma membrane. *Eur J Biochem* 267:2483-2497.

Horn F, Lau AL and Cohen FE (2004) Automated extraction of mutation data from the literature: application of MuteXt to G protein-coupled receptors and nuclear hormone receptors. *Bioinformatics* 20:557-568.

Horn F, Weare J, Beukers MW, Horsch S, Bairoch A, Chen W, Edvardsen O, Campagne F and Vriend G (1998) GPCRDB: an information system for G protein-coupled receptors. *Nucleic Acids Res* 26:275-279.

Hu J, McLarnon SJ, Mora S, Jiang J, Thomas C, Jacobson KA and Spiegel AM (2005) A region in the seven-transmembrane domain of the human Ca^{2+} receptor critical for response to Ca^{2+}. *J Biol Chem* 280:5113-5120.

Hubbell WL, Altenbach C, Hubbell CM and Khorana HG (2003) Rhodopsin structure, dynamics, and activation: a perspective from crystallography, site-directed spin labeling, sulfhydryl reactivity, and disulfide cross-linking. *Adv Protein Chem* 63:243-290.

Inoue Y, Ikeda M and Shimizu T (2004) Proteome-wide classification and identification of mammalian-type GPCRs by binary topology pattern. *Comput Biol Chem* 28:39-49.

Kazius J, Wurdinger K, van Iterson M, Kok J, B ck T, IJzerman AP (2008) GPCR NaVa database: natural variants in human G protein-coupled receptors. *Hum Mutat* 2008 29:39-44.

King K, Dohlman HG, Thorner J, Caron MG and Lefkowitz RJ (1990) Control of yeast mating signal transduction by a mammalian β_2-adrenergic receptor and Gs α subunit. *Science* 250:121-123.

Klco JM, Nikiforovich GV and Baranski TJ (2006) Genetic analysis of the first and third extracellular loops of the C5a receptor reveals an essential WXFG motif in the first loop. *J Biol Chem* 281:12010-12019.

Klco JM, Wiegand CB, Narzinski K and Baranski TJ (2005) Essential role for the second extracellular loop in C5a receptor activation. *Nat Struct Mol Biol* 12:320-326.

Klein C, Paul JI, Sauve K, Schmidt MM, Arcangeli L, Ransom J, Trueheart J, Manfredi JP, Broach JR and Murphy AJ (1998) Identification of surrogate agonists for the human FPRL-1 receptor by autocrine selection in yeast. *Nat Biotechnol* 16:1334-1337.

Kristiansen K (2004) Molecular mechanisms of ligand binding, signaling, and regulation within the superfamily of G-protein-coupled receptors: molecular modeling and mutagenesis approaches to receptor structure and function. *Pharmacol Ther* 103:21-80.

Kunkel TA, Roberts JD and Zakour RA (1987) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol* 154:367-382.

Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, Devon K, Dewar K, Doyle M, FitzHugh W, Funke R, Gage D, Harris K, Heaford A, Howland J, Kann L, Lehoczky J, LeVine R, McEwan P, McKernan K, Meldrim J, Mesirov JP, Miranda C, Morris W, Naylor J, Raymond C, Rosetti M, Santos R, Sheridan A, Sougnez C, Stange-Thomann N, Stojanovic N, Subramanian A, Wyman D, Rogers J, Sulston J, Ainscough R, Beck S, Bentley D, Burton J, Clee C, Carter N, Coulson A, Deadman R, Deloukas P, Dunham A, Dunham I, Durbin R, French L, Grafham D, Gregory S, Hubbard T, Humphray S, Hunt A, Jones M, Lloyd C, McMurray A, Matthews L, Mercer S, Milne S, Mullikin JC, Mungall A, Plumb R, Ross M, Shownkeen R, Sims S, Waterston RH, Wilson RK, Hillier LW, McPherson JD, Marra MA, Mardis ER, Fulton LA, Chinwalla AT, Pepin KH, Gish WR, Chissoe SL, Wendl MC, Delehaunty KD, Miner TL, Delehaunty A, Kramer JB, Cook LL, Fulton RS, Johnson DL, Minx PJ, Clifton SW, Hawkins T, Branscomb E, Predki P, Richardson P, Wenning S, Slezak T, Doggett N, Cheng JF, Olsen A, Lucas S, Elkin C, Uberbacher E, Frazier M, et al. (2001) Initial sequencing and analysis of the human genome. *Nature* 409:860-921.

Lee BK, Lee YH, Hauser M, Son CD, Khare S, Naider F and Becker JM (2002) Tyr266 in the sixth transmembrane domain of the yeast alpha-factor receptor plays key roles in receptor activation and ligand specificity. *Biochemistry* 41:13681-13689.

Lee YH, Naider F and Becker JM (2006) Interacting residues in an activated state of a G protein-coupled receptor. *J Biol Chem* 281:2263-2272.

Lemaire K, Van de Velde S, Van Dijck P and Thevelein JM (2004) Glucose and sucrose act as agonist and mannose as antagonist ligands of the G protein-coupled receptor Gpr1 in the yeast Saccharomyces cerevisiae. *Mol Cell* 16:293-299.

Li B, Nowak NM, Kim SK, Jacobson KA, Bagheri A, Schmidt C and Wess J (2005) Random mutagenesis of the M_3 muscarinic acetylcholine receptor expressed in yeast: identification of second-site mutations that restore function to a coupling-deficient mutant M_3 receptor. *J Biol Chem* 280:5664-5675.

Li B, Scarselli M, Knudsen CD, Kim SK, Jacobson KA, McMillin SM and Wess J (2007a) Rapid identification of functionally critical amino acids in a G protein-coupled receptor. *Nat Methods* 4:169-174.

Li Q, Ye K, Blad CC, den Dulk H, Brouwer J, IJzerman AP and Beukers MW (2007b) ZM241385, DPCPX, MRS1706 are inverse agonists with different relative intrinsic efficacies on constitutively active mutants of the human adenosine A_{2B} receptor. *J Pharmacol Exp Ther* 320:637-645.

Lin JC, Duell K, Saracino M and Konopka JB (2005) Identification of residues that contribute to receptor activation through the analysis of compensatory mutations in the G protein-coupled α-factor receptor. *Biochemistry* 44:1278-1287.

Lu ZL, Saldanha JW and Hulme EC (2002) Seven-transmembrane receptors: crystals clarify. *Trends Pharmacol Sci* 23:140-146.

Manfredi JP, Klein C, Herrero JJ, Byrd DR, Trueheart J, Wiesler WT, Fowlkes DM and Broach JR (1996) Yeast alpha mating factor structure-activity relationship derived from genetically selected peptide agonists and antagonists of Ste2p. *Mol Cell Biol* 16:4700-4709.

Martin NP, Celic A and Dumont ME (2002) Mutagenic mapping of helical structures in the transmembrane segments of the yeast α-factor receptor. *J Mol Biol* 317:765-788.

Milne JL, Caterina MJ and Devreotes PN (1997) Random mutagenesis of the cAMP chemoattractant receptor, cAR1, of Dictyostelium. Evidence for multiple states of activation. *J Biol Chem* 272:2069-2076.

Minic J, Persuy MA, Godel E, Aioun J, Connerton I, Salesse R and Pajot-Augy E (2005a) Functional expression of olfactory receptors in yeast and development of a bioassay for odorant screening. *Febs J* 272:524-537.

Minic J, Sautel M, Salesse R and Pajot-Augy E (2005b) Yeast system as a screening tool for pharmacological assessment of g protein coupled receptors. *Curr Med Chem* 12:961-969.

Naider F, Becker JM, Lee YH and Horovitz A (2007) Double-mutant cycle scanning of the interaction of a peptide ligand and its G protein-coupled receptor. *Biochemistry* 46:3476-3481.

Niebauer RT and Robinson AS (2006) Exceptional total and functional yields of the human adenosine (A2a) receptor expressed in the yeast Saccharomyces cerevisiae. *Protein Expr Purif* 46:204-211.

Olesnicky NS, Brown AJ, Dowell SJ and Casselton LA (1999) A constitutively active G-protein-coupled receptor causes mating self-compatibility in the mushroom Coprinus. *Embo J* 18:2756-2763.

Oliveira L, Paiva PB, Paiva AC, Vriend G. (2003) Identification of functionally conserved residues with the use of entropy-variability plots. *Proteins* 52:544-52.

Pajot-Augy E, Crowe M, Levasseur G, Salesse R and Connerton I (2003) Engineered yeasts as reporter systems for odorant detection. *J Recept Signal Transduct Res* 23:155-171.

Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, Yamamoto M and Miyano M (2000) Crystal structure of rhodopsin: A G protein-coupled receptor. *Science* 289: 739-745.

Pausch MH (1997) G-protein-coupled receptors in Saccharomyces cerevisiae: high-throughput screening assays for drug discovery. *Trends Biotechnol* 15:487-494.

Price LA, Kajkowski EM, Hadcock JR, Ozenberger BA and Pausch MH (1995) Functional coupling of a mammalian somatostatin receptor to the yeast pheromone response pathway. *Mol Cell Biol* 15:6188-6195.

Price LA, Strnad J, Pausch MH and Hadcock JR (1996) Pharmacological characterization of the rat A_{2a} adenosine receptor functionally coupled to the yeast pheromone response pathway. *Mol Pharmacol* 50:829-837.

Sarramegna V, Talmont F, Seree de Roch M, Milon A and Demange P (2002) Green fluorescent protein as a reporter of human mu-opioid receptor overexpression and localization in the methylotrophic yeast Pichia pastoris. *J Biotechnol* 99:23-39.

Scarselli M, Li B, Kim SK and Wess J (2007) Multiple residues in the second extracellular loop are critical for M_3 muscarinic acetylcholine receptor activation. *J Biol Chem* 282:7385-7396.

Schmidt C, Li B, Bloodworth L, Erlenbach I, Zeng FY and Wess J (2003) Random mutagenesis of the M_3 muscarinic acetylcholine receptor expressed in yeast. Identification of point mutations that silence a constitutively active mutant M_3 receptor and greatly impair receptor/G protein coupling. *J Biol Chem* 278:30248-30260.

Slessareva JE and Dohlman HG (2006) G protein signaling in yeast: new components, new connections, new compartments. *Science* 314:1412-1413.

Smothers JF, Henikoff S and Carter P (2002) Tech.Sight. Phage display. Affinity selection from biological libraries. *Science* 298:621-622.

Sommers CM and Dumont ME (1997) Genetic interactions among the transmembrane segments of the G protein coupled receptor encoded by the yeast STE2 gene. *J Mol Biol* 266:559-575.

Sommers CM, Martin NP, Akal-Strader A, Becker JM, Naider F and Dumont ME (2000) A limited spectrum of mutations causes constitutive activation of the yeast alpha-factor receptor. *Biochemistry* 39:6898-6909.

Szardenings M (2003) Phage display of random peptide libraries: applications, limits, and potential. *J Recept Signal Transduct Res* 23:307-349.

Tamaki H, Yun CW, Mizutani T, Tsuzuki T, Takagi Y, Shinozaki M, Kodama Y, Shirahige K and Kumagai H (2005) Glucose-dependent cell size is regulated by a G protein-coupled receptor system in yeast Saccharomyces cerevisiae. *Genes Cells* 10:193-206.

Venter JC, Adams MD, Myers EW, Li PW, Mural RJ, Sutton GG, Smith HO, Yandell M, Evans CA, Holt RA, Gocayne JD, Amanatides P, Ballew RM, Huson DH, Wortman JR, Zhang Q, Kodira CD, Zheng XH, Chen L, Skupski M, Subramanian G, Thomas PD, Zhang J, Gabor Miklos GL, Nelson C, Broder S, Clark AG, Nadeau J, McKusick VA, Zinder N, Levine AJ, Roberts RJ, Simon M, Slayman C, Hunkapiller M, Bolanos R, Delcher A, Dew I, Fasulo D, Flanigan M, Florea L, Halpern A, Hannenhalli S, Kravitz S, Levy S, Mobarry C, Reinert K, Remington K, Abu-Threideh J, Beasley E, Biddick K, Bonazzi V, Brandon R, Cargill M, Chandramouliswaran I, Charlab R, Chaturvedi K, Deng Z, Di Francesco V, Dunn P, Eilbeck K, Evangelista C, Gabrielian AE, Gan W, Ge W, Gong F, Gu Z, Guan P, Heiman TJ, Higgins ME, Ji RR, Ke Z, Ketchum KA, Lai Z, Lei Y, Li Z, Li J, Liang Y, Lin X, Lu F, Merkulov GV, Milshina N, Moore HM, Naik AK, Narayan VA, Neelam B, Nusskern D, Rusch DB, Salzberg S, Shao W, Shue B, Sun J, Wang Z, Wang A, Wang X, Wang J, Wei M, Wides R, Xiao C, Yan C, et al. (2001) The sequence of the human genome. *Science* 291:1304-1351.

Versele M, Lemaire K and Thevelein JM (2001) Sex and sugar in yeast: two distinct GPCR systems. *EMBO Rep* 2:574-579.

Vogel WK, Peterson GL, Broderick DJ, Mosser VA and Schimerlik MI (1999) Double mutant cycle analysis of aspartate 69, 97, and 103 to asparagine mutants in the m2 muscarinic acetylcholine receptor. *Arch Biochem Biophys* 361:283-294.

Warringer J and Blomberg A (2003) Automated screening in environmental arrays allows analysis of quantitative phenotypic profiles in Saccharomyces cerevisiae. *Yeast* 20:53-67.

Weiss GA, Watanabe CK, Zhong A, Goddard A and Sidhu SS (2000) Rapid mapping of protein functional epitopes by combinatorial alanine scanning. *Proc Natl Acad Sci U S A* 97:8950-8954.

Wise A, Gearing K and Rees S (2002) Target validation of G-protein coupled receptors. *Drug Discov Today* 7:235-246.

Ye K, Lameijer EW, Beukers MW, IJzerman AP (2006) A two-entropies analysis to identify functional positions in the transmembrane region of class A G protein-coupled receptors. *Proteins*, 63:1018-30.

Zhang WB, Navenot JM, Haribabu B, Tamamura H, Hiramatu K, Omagari A, Pei G, Manfredi JP, Fujii N, Broach JR and Peiper SC (2002) A point mutation that confers constitutive activity to CXCR4 reveals that T140 is an inverse agonist and that AMD3100 and ALX40-4C are weak partial agonists. *J Biol Chem* 277:24515-24521.

Chapter

241385 PCP and R 1706 are in erse agonists with different intrinsic efficacies on constituti ely acti e mutants of the human adenosine A_{2B} receptor

*This chapter is a slightly modified version of Li Q, Ye K, Blad CC, den Dulk H, Brouwer J, IJzerman AP and Beukers, MW (2007) J Pharmacol Exp Ther **32** : 637-645.*

ummar

The human adenosine A_{2B} receptor belongs to class A G protein-coupled receptors (GPCRs). In our previous work, constitutively active mutant (CAM) human adenosine A_{2B} receptors were identified from a random mutation bank based on their ability to grow in histidine-deficient medium. In the current study, three known A_{2B} receptor antagonists, ZM241385, DPCPX and MRS1706 were tested on wild-type and 9 CAM A_{2B} receptors with different levels of constitutive activity in a yeast growth assay. All three compounds turned out to be inverse agonists for the adenosine A_{2B} receptor as they were able to fully reverse the basal activity of 4 low level constitutively active A_{2B} receptor mutants and to partially reverse the basal activity of 3 medium level constitutively active A_{2B} receptor mutants. We also discovered 2 highly constitutively active or locked mutants whose basal activity could not be reversed by any of the three compounds. A two-state receptor model was employed to explain the experimental observations. Varying L, the ratio of active versus inactive receptors in this model yielded simulated dose-response curves that mimicked the experimental ones. This study is the first description of inverse agonists for the human adenosine A_{2B} receptor. Moreover, the use of receptor mutants with varying levels of constitutive activity enabled us to determine the intrinsic efficacy of these inverse agonists.

ntroduction

G-protein-coupled receptors (GPCRs) constitute a large superfamily of transmembrane proteins which represent the target for nearly half of the marketed drugs (Drews, 2000; Hopkins and Groom, 2002). One of the GPCR family members, the adenosine receptor, is a group of widely distributed receptors which is composed of four receptor subtypes: A_1, A_{2A}, A_{2B} and A_3. Of these, the adenosine A_{2B} receptor has been implied in many physiological functions (Ralevic and Burnstock, 1998; Feoktistov et al., 1998; Holgate, 2005; Yaar et al., 2005) and adenosine A_{2B} receptor antagonists may be used in the treatment of asthma, type-II diabetes, Alzheimer's disease and cystic fibrosis (Feoktistov et al. 1998; Volpini et al. 2003).

The adenosine A_{2B} receptor is also referred to as the low affinity receptor due to its very modest affinity for the endogenous ligand adenosine. Despite elaborate synthesis efforts, a long-established reference compound, NECA still has the highest affinity, 360 nM, for the human adenosine A_{2B} receptor expressed in HEK-293 cells, but it lacks selectivity (De Zwart et al., 1998; Fredholm et al., 2001). Recently a new series of non-ribose agonists for adenosine receptors was described (Chang et al., 2005) including several highly potent agonists for the adenosine A_{2B} receptor (Beukers et al., 2004a). LUF5835 e.g. was a full agonist with an EC_{50} value of 10 nM. Still none of these agonists was truly selective for the A_{2B} receptor.

In contrast, several selective antagonists have been identified for the adenosine A_{2B} receptor. For example, the xanthine amide derivatives MRS1668, MRS1706 and MRS1754 are relatively new selective antagonists for the adenosine A_{2B} receptor with K_i values in the nanomolar range (Kim et al., 2000). Except for these new antagonists, prototypic adenosine receptor antagonists such as ZM241385, DPCPX, XAC and CGS15943 have a good to reasonable affinity for the adenosine A_{2B} receptor but are not selective versus other subtypes of adenosine receptors (Klotz et al., 1998; Ongini et al., 1999; Alexander et al., 1996).

The phenomenon of constitutive receptor activity i.e. receptor signaling in the absence of agonists allows the discrimination between neutral antagonists and inverse agonists. Although the therapeutic implications remain as yet unclear, many clinically used drugs turn out to be inverse agonists rather than neutral antagonists (Costa and Cotecchia, 2005; Bond and IJzerman, 2006). Whether the currently known antagonists of the adenosine A_{2B} receptor possess inverse agonistic properties is unknown as the wild-type (wt) adenosine A_{2B} receptor lacks constitutive activity (Beukers et al., 2004b).

In our previous work, we identified constitutively active mutant (CAM) adenosine A_{2B} receptors from a random mutation bank using a robust yeast selection assay (Beukers et al. 2004b; Beukers and IJzerman, 2005). These yeast cells were genetically engineered to not only communicate with human GPCRs, but also identify (constitutively) active receptors (Pausch, 1997; Dowell and Brown, 2002). To this end the signal transduction pathway of yeast was coupled to the production of the essential amino acid, histidine. Yeast cells expressing gain-of-function or constitutively active adenosine A_{2B} receptors were identified through their ability to grow in histidine-deficient medium.

Since CAM receptors provide a useful tool to discriminate inverse agonists from neutral antagonists, CAM adenosine A_{2B} receptors identified in our previous study were used in our current study to characterize three A_{2B} receptor antagonists: ZM241385, DPCPX and MRS1706. All three compounds proved to be inverse agonists for the adenosine A_{2B} receptor. The present study is the first to describe inverse agonists for the human adenosine A_{2B} receptor. Moreover, the use of CAM receptors with varying levels of constitutive activity enabled us to determine the different intrinsic efficacies of these compounds. Finally, mathematical simulation of our experimental data confirmed the intricate connection between the ligands' intrinsic efficacy and the receptor's constitutive activity.

aterials and methods

aterials

NECA (5'-*N*-ethylcarboxamidoadenosine) was purchased from Sigma Aldrich, Steinhem, Germany. ZM241385 (4-(2-[7-amino-2-(2-furyl[1,2,4]-triazolo[2,3-a[1,3,5]triazin-5-yl-aminoethyl)phenol)) was a gift from Dr. S.M. Poucher, AstraZeneca Pharmaceuticals, Macclesfield, UK. DPCPX (8-Cyclopentyl-1, 3-dipropylxanthine) was purchased from RBI (Natick, MA, USA). MRS1706 (*N*-(4-acetylphenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-

dipropyl-1*H*-purin-8-yl)phenoxy] acetamide) was obtained from Tocris Cookson Inc, Bristol, UK. 3-AT (3-amino-[1,2,4]-triazole) was purchased from Sigma Aldrich, St Louis, USA.

A genetically modified yeast *Saccharomyces cerevisiae* strain with the following genotype: *MAT*a*his3 leu2 trp1 ura3 can1 gpa1*Δ::Gα_{i3} *far1*Δ::ura3 sst2Δ::ura3 Fus1::FUS1-HIS3 LEU2::FUS1-lacZ ste2Δ::G418^R was a gift from Dr. S. J. Dowell, GSK (Stevenage, UK). Constitutively active adenosine A_{2B} receptor mutants (CAM) were obtained through random mutagenesis as previously described (Beukers et al., 2004b). These mutants were identified with a screening assay based on yeast growth.

ethods

east growth assay

Nine constitutively active adenosine A_{2B} receptor mutants were selected from a random mutation bank based on their ability to grow on solid or in liquid medium in the absence of agonists. Single colonies of these nine CAM receptors were used to guarantee a consistent phenotype.

To perform growth assays on solid agar medium, yeast cells from an overnight culture were diluted to around 400,000 cells/ml (OD_{600} 0.02) and droplets of 1.5 µl were spotted on growth assay plates containing minimal agar medium, 20 mg/l adenine, 20 mg/l tryptophan, with or without either ZM241385 (1 nM to 0.1 mM), DPCPX (1nM to 0.1 mM) or MRS1706 (0.1 nM to 10 µM). Receptor-independent growth was suppressed through the addition of 7 mM 3-AT. After incubation at 30 C for 48 hours, the plates were scanned and receptor-mediated yeast growth was quantified with Quantity One imaging software from Bio-Rad (Hercules, CA). The growth rate of yeast was calculated as the density of each spot with a correction for local background on the plate.

For growth assays in liquid medium, an overnight yeast culture was adjusted to OD_{600} 0.2 and 50 µl was seeded into each well of a 96-wells plate. Every well contained 200 µl minimal medium, 20 mg/l adenine, 20 mg/l tryptophan, 7 mM 3AT with or without the indicated concentrations of ZM241385, DPCPX or MRS1706. The cells were kept at 30 C for 35 hours and yeast growth in 96-wells plates was automatically recorded using a Genios plate reader (Tecan Inc.). OD_{600} of every well was used to determine yeast growth. Variations in the background OD values were insignificant hence no background correction was carried out in the liquid medium growth assay experiments.

Each experiment was repeated 3 to 5 times. The IC_{50} values and E_{max} values were calculated with Prism version 4.0 (GraphPad Software Inc., San Diego, CA).

Schild analysis

NECA concentration-growth curves of the wild-type adenosine A_{2B} receptor were obtained in the absence and presence of ZM241385, DPCPX or MRS1706. Dose ratios (DR) were calculated from the molar NECA concentrations producing a half-maximal response (EC_{50}) in the presence of one of these three compounds divided by the EC_{50} obtained in the absence of

these compounds. The DRs were subjected to Schild analysis to determine whether the compounds acted as competitive antagonists (Arunlakshana and Schild, 1959):

Log(DR-1) nLog[B] + logK_B

In this equation, [B] refers to the molar concentration of ZM241385, DPCPX or MRS1706 and K_B is the equilibrium dissociation constant of the complex of the receptor with one of the compounds. A plot of log (DR-1) values (y-axis) versus logarithm molar concentrations of these compounds (x-axis) yielded a straight line of which the intercept reflects the pK_B or pA_2 value of the compound and the slope (n) reveals whether the compound is a competitive antagonist (n 1) or not (n≠1).

Radioligand inding assay

Yeasts ($1*10^9$ cells) were harvested after overnight culture and resuspended in ligand binding buffer (50 mM Tris–HCl, 10 mM $MgCl_2$, 1mM EDTA and 0.01% CHAPS, PH8.26). Fifty microliter yeast cells (3 mg protein/ml) were incubated with 1.2 nM [^{3}H] MRS1754 and 1mM NECA to determine non-specific binding in a total volume of 200 µl. Yeasts were incubated at 25 C for 2 hours and harvested by millipore or harvester. After rinsing the GFC filters 3 times with ice cold binding buffer, radioactivity was measured as counts per minute using a Perkin–Elmer 1450 Microbeta liquid scintillation and luminescence counter. All samples were run at least three times in duplicate.

arameter fitting using rism

The two-state receptor model was originally applied to describe the function of ion-channels and adapted to explain the activation of receptors (Leff, 1995). In this study, it is used to interpret and simulate our experimental data. The two-state receptor model is described by three parameters: L, the isomerization constant which is the ratio of the receptor in the active R_a state versus the inactive R_i state; α, the intrinsic efficacy which refers to the affinity of a ligand for the active state of the receptor (R_a) over the inactive state of the receptor (R_i); K_A, the equilibrium dissociation constant of a ligand-receptor complex.

According to the two-state receptor model, the proportion of receptors in the active state can be calculated as:

$$\rho = \frac{\alpha L[A]/K_A + L}{[A]/K_A(1+\alpha L) + L + 1}$$ equation 1 (Kenakin, 2003)

In the absence of ligand ([A] 0) the proportion of receptors in the active state, the constitutively active receptors, can be stated as:

$$\rho_0 = L/(L+1)$$ equation 2

From these equations the L value, the ratio of activated receptors versus inactivated receptors, can be calculated:

$L = \rho_0/(1-\rho_0)$ equation 3

The value ρ_0 (the proportion of receptors in the active state in the absence of ligand) of each CAM receptor was determined by quantification of spontaneous yeast growth in histidine-deficient medium in the presence of 7 mM 3AT. Yeast growth is positively correlated to the proportion of receptors in the active state, thus yeast growth can be used to calculate the ρ values of the receptors. To determine the ρ values of the different CAM receptors we first defined the two extreme values detected in this study. The lower limit of ρ_0 was set at 0.001 (reflecting 0.1% of receptors in the active state) for the growth obtained upon expression of the wt receptor. The upper limit of ρ_0 was set at 0.999 (which means 99.9% of receptors are active) for the growth obtained upon expression of either one of the two mutant receptors, A18T/A23V/C83Y/A106V/R112S or Q214L/I230N/V240M/V250M/N254Y/T257S/ K269stop. These mutants exhibited the highest level of constitutive activity among all the mutants and could not be further activated by addition of agonists such as NECA (data not shown), which suggested that they have reached maximal levels of receptor activation. Thus, the L values of the wt and these two mutants were fixed at 0.001 and 999, respectively (see equation 3).

The ρ values of mutants in the presence and absence of concentrations of ZM241385, DPCPX or MRS1706 were scaled according to two defined extreme values: 0.001 and 0.999. Then Graphpad Prism was used to perform curve fitting according to equation 1 and to determine the optimal values for L, α and K_A. In the two-state receptor model, the α value is a characteristic of the compound and we assume that a compound has the same K_A value for all mutant A_{2B} receptors. Therefore, the L values of the different mutants were determined with the constraint that the α value and K_A value for each compound were shared among the different mutants.

Since the two highly CAM receptors A18T/A23V/C83Y/A106V/R112S and Q214L/I230N/ V240M/V250M/N254Y/T257S/K269stop could not be inhibited by any of the compounds tested, the growth of these two mutants in the presence and absence of inverse agonist could not be used for curve fitting in GraphPad Prism.

Simulation of growth curves using at a

The pharmacological two-state receptor model was implemented in the software package MatLab version 7.0 (The Mathwork, Inc, Natick, USA) and a graphic interface was composed to facilitate parameter input and to simulate curves. Concentration-proportion (ρ) curves were simulated and visualized with a fixed α and K_A value and variable L values to mimic the experimental curves.

Results

dentification of constituti ely acti e mutants

To select constitutively active mutant A_{2B} receptors, we employed a yeast assay based on growth which was previously described (Beukers et al., 2004b). In brief, human adenosine A_{2B} receptors couple to the endogenous signaling pathway of yeast (Brown et al., 2000) and activate the synthesis of histidine, which allows the yeast cells to grow in histidine-deficient medium. Hence, the growth of these yeast cells and the presence of active adenosine A_{2B} receptors are positively correlated and increased receptor activity results in increased yeast growth.

From our random mutation bank nine previously published A_{2B} receptor mutants with different levels of constitutive activity were selected (Beukers et al., 2004b) and used as screening tools to discover inverse agonists. Among them, the highest levels of constitutive activity were obtained with two multiple mutant receptors, A18T/A23V/C83Y/A106V/R112S and the truncated mutant Q214L/I230N/V240M/V250M/N254Y/T257S/K269stop, missing the C-terminal and transmembrane helix 7. Mutants N36S/T42A, N36S/T42A/T66A and T42A/V54A showed intermediate levels of agonist-independent growth. Finally, three point mutants T42A, F84L, F84S and the double mutant F84L/S95G exhibited relatively low levels

Table 1. Constitutive activity of wild-type and mutant human adenosine A_{2B} receptors.

Yeast cells expressing the wild-type or one of the 9 mutant adenosine A_{2B} receptors were screened on selection plates containing minimal agar medium plus tryptophan, adenine and 7 mM 3AT, the latter to suppress receptor-independent growth. - indicates that yeast cells did not grow on the selection plate due to the lack of constitutive receptor activity; + indicates that yeast cells did grow on the selection plates but only a weak constitutive activity was detectable; ++ indicates that the mutants exhibited constitutive activity 2 to 4 fold higher than + ; +++ indicates that the constitutive activity of the mutants was at least 5 fold greater than + .

WT/Mutants	Constitutive activity
Wild-type	-
T42A	+
F84L	+
F84S	+
F84L/S95G	+
N36S/T42A	++
N36S/T42A/T66A	++
T42A/V54A	++
A18T/A23V/C83Y/A106V/R112S	+++
Q214L/I230N/V240M/V250M/N254Y/T257S/K269stop	+++

of constitutive activity (Table 1).

To verify whether the constitutive activity of the mutants was a result of the mutation or caused by increased receptor expression levels, radioligand binding studies were carried out. As can be seen in figure 1, all mutant receptors did recognize the radioligand [^{3}H]MRS1754 and showed specific binding suggesting that the mutations did not affect ligand binding properties. In addition, the highest level of specific binding was obtained with the wild-type receptor suggesting that the constitutive activity was not a result from increased receptor expression levels.

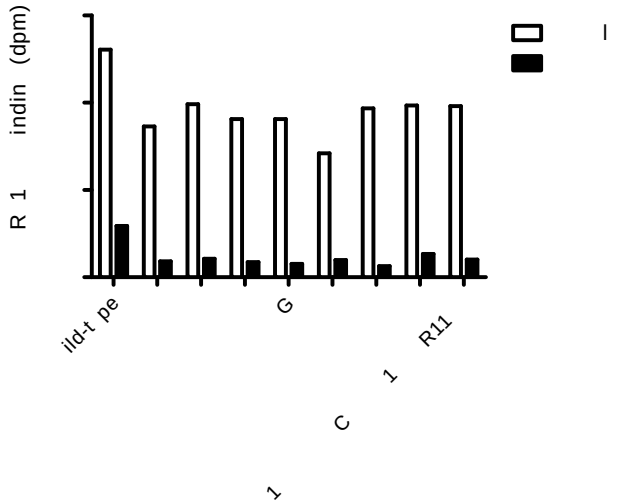


Figure. 1 Total binding and specific binding of wt and mutant A_{2B} receptors.

Antagonism of 241385 PCP and R 1706 at the wild-type adenosine A_{2B} receptor

The agonistic effect of NECA on the wild-type adenosine A_{2B} receptor was antagonized by

Table 2. Comparison of the pA_2 values of ZM241385, DPCPX and MRS1706 as determined through antagonism of NECA-induced activation of adenosine A_{2B} receptors in this study versus literature data obtained in different tissues and cells.

Compound	Tissue or cells	pA_2 from literature	pA_2 this study
ZM241385	Rat mesenteric artery[a]	7.20 0.12	7.32 0.29
	CHO cells[b]	7.32 (7.17-7.48)	
DPCPX	HEK293[c]	7.01	
	CHO.A2B4 cell[d]	7.16	6.41 0.16
	Guinea-pig cerebral cortex[e]	6.91	
	Guinea-pig tracheal epithelial cells[f]	6.51 0.29	
MRS1706	n. d.		7.38 0.18

[a]. Prentice et al., 1997, [b]. Ongini et al., 1999, [c]. Cooper et al., 1997, [d]. Alexander et al., 1996, [e]. Poucher et al., 1995, [f]. Pelletier et al., 2000. n. d. not determined

ZM241385, DPCPX, and MRS1706 in a concentration-dependent manner (Figure 2A, C, and E), which resulted in a rightward shift of the concentration-response curve without a change of E_{max}. Schild analysis of this antagonism (n 3) yielded pA_2 values of 7.32 0.29 for ZM241385, 6.41 0.16 for DPCPX and 7.38 0.18 for MRS1706. The slope of the Schild plot was 0.99 0.03 for ZM241385, 1.04 0.14 for DPCPX and 1.26 0.07 for MRS1706 indicating the competitive interaction of these compounds with the receptor (Figure. 2B, D, and F). Table 2 displays a comparison of the pA_2 values of ZM241385, DPCPX and MRS1706 obtained in this study with literature values.

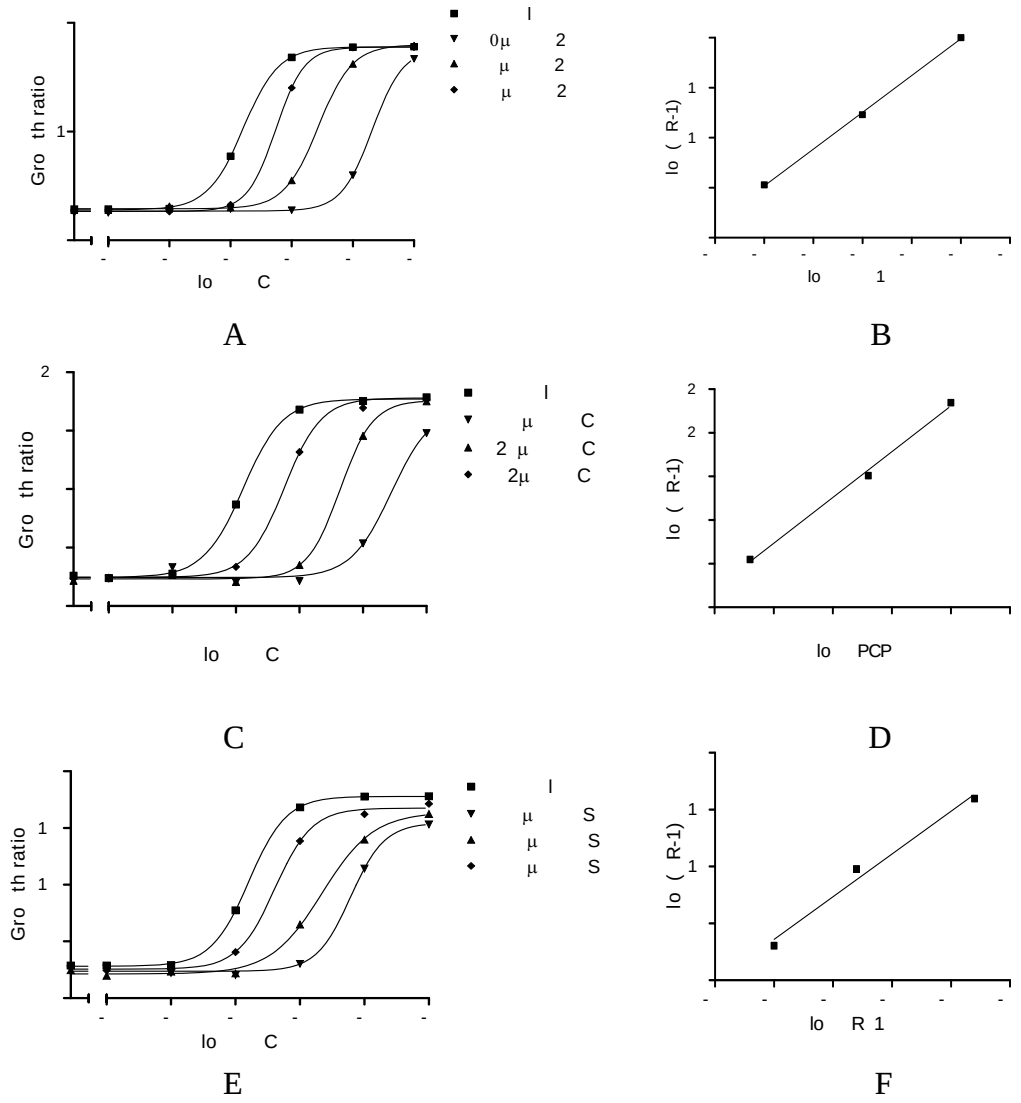


Figure. 2 Concentration-response curves of NECA in the absence or presence of 10 μM, 1 μM and 0.1 μM ZM241385 (A), 100 μM, 20 μM and 2 μM DPCPX (C), and 5 μM, 0.5μM and 0.1 μM MRS1706 (E). Schild plot analyses for the determination of pA_2 values of ZM241385, DPCPX, and MRS1706 at the adenosine A_{2B} receptor are shown in B), D) and F).

n erse agonistic properties of 241385 PCP and R 1706

Three types of response of the constitutively active mutants were observed when yeast cells expressing CAM A_{2B} receptors were cultured in the presence of ZM241385, DPCPX or MRS1706.

Both the mutants with low and intermediate levels of constitutive activity were inactivated by ZM241385, DPCPX, and MRS1706. Hence these compounds acted as inverse agonists on the

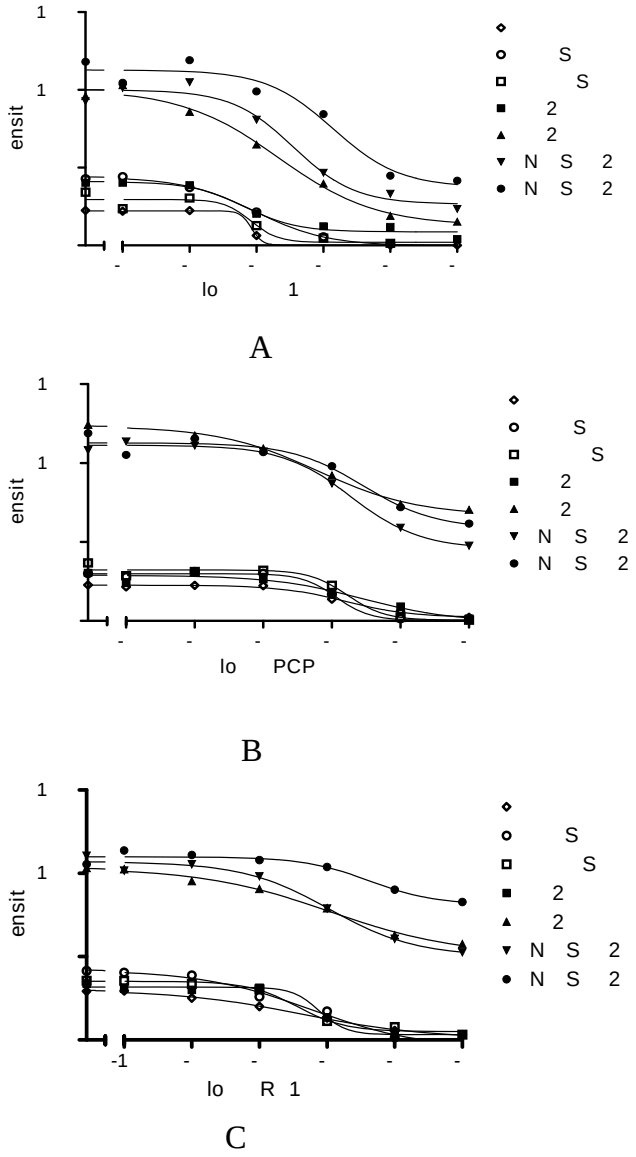


Figure. 3 Concentration-response curves of ZM241385, DPCPX, and MRS1706-induced inhibition of yeast growth. Yeast cells expressing 9 CAM adenosine A_{2B} receptors with different levels of constitutive activity were tested in histidine-deficient solid medium containing 7 mM 3AT and concentrations of ZM241385 (A), DPCPX (B) or MRS1706 (C) as indicated. Growth of the yeast cells was scanned and quantified with Quantity One imaging software. One representative experiment performed in duplicate is shown of at least three independent experiments.

constitutively active human adenosine A_{2B} mutants, albeit with varying intrinsic activities and potencies as described in detail below (Table 3, Figure 3).

Table 3. Comparison of the constitutive activity (CA), potency (IC_{50} value) and intrinsic activity (% inhibition) of ZM241385, DPCPX and MRS1706 towards CAM adenosine A_{2B} receptors with low and termediate levels of constitutive activity.

Mutants	CA	ZM241385		DPCPX		MRS	
		IC_{50} (nM)	I_{max} (%)	IC_{50} (nM)	I_{max} (%)	IC_{50} (nM)	I_{max} (%)
F84L	+	135 88	95 7	1440 1394	92 3	43 21	91 12
F84S	+	107 37	99 2	1907 675	97 3	54 12	94 6
F84L/S95G	+	102 60	97 4	1430 314	99 2	40 32	94 8
T42A	+	71 34	96 5	1549 981	96 2	98 62	93 5
T42A/V54A	++	210 86	79 7	798 406	40 12	166 138	54 5
N36S/T42A	++	186 147	74 7	1550 1222	58 3	133 66	57 11
N36S/T42A/T66A	++	522 239	63 4	6247 2460	39 11	[a]	28 16

Results are expressed as mean ± S.E.M. from at least 3 independent experiments. [a] IC_{50} value could not be determined due to small experimental window.

The basal growth of yeast cells expressing T42A, F84L, F84S or F84L/S95G mutant receptors was dose-dependently reduced by ZM241385, DPCPX and MRS1706 from 91% to 99%. In other words, the basal growth of yeast cells expressing these 4 low CAM receptors were almost completely inhibited in the presence of 10 μM of any of these inverse agonists. The mutants with intermediate levels of constitutive activity, N36S/T42A, N36S/T42A/T66A and T42A/V54A were inhibited by all three inverse agonists albeit with lower intrinsic activities, ranging from 28% to 79%. None of the tested inverse agonists could, however, completely inhibit the growth of yeast cells expressing any of these 3 medium level CAM receptors.

The multiple mutant A18T/A23V/C83Y/A106V/R112S and the truncated mutant Q214L/I230N/V240M/V250M/N254Y/T257S/K269stop did not respond to ZM241385, DPCPX or MRS1706 at any of the concentrations tested. A representative curve of ZM241385 is shown in Figure 4. Apparently, these 2 CAM A_{2B} receptors are endowed with robust activity and are locked in an active state.

Point mutants F84L and F84S behaved similar to each other with respect to both the potency and intrinsic efficacy of the inverse agonists, indicating that a mutation from phenylalanine to leucine or serine has the same effect. These two single mutants have very low constitutive activity and were completely inhibited by all three inverse agonists. An additional S95G mutation seemed to have no effect on either the constitutive activity or the inverse agonistic effect.

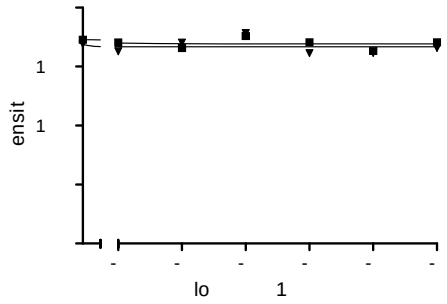


Figure. 4 Effect of ZM241385 on the constitutive activity of the highly constitutively active A18T/A23V/C83Y/A106V/R112S (■) and Q214L/I230N/V240M/V250M/N254Y/T257S/K269stop (▼) adenosine A_{2B} receptors. No significant inhibition of yeast growth was observed after treatment with ZM241385.

Like the point mutants F84L and F84S, the T42A mutant exhibited relatively low constitutive activity that was completely inhibited by all three inverse agonists. Comparison of the T42A point mutant with the T42A/V54A and the N36S/T42A double mutants showed that the IC_{50} values of the three inverse agonists were at most 3-fold altered when an additional V54A or N36S mutation was present. However, whereas the constitutive activity of the T42A mutant could be completely inhibited by all three inverse agonists, an additional V54A or N36S mutation resulted in a higher level of constitutive activity and could be only partially inhibited. Whereas the N36S mutation did not cause significant changes with respect to the potency of the three compounds, an additional T66A mutation along with the T42A/N36S double mutation increased the IC_{50} values of ZM241385 and DPCPX from 186 nM to 522 nM and from 1550 nM to 6247 nM, respectively. Due to the small window, the IC_{50} value of MRS1706 for the T42A/N36S/T66A triple mutant could not be determined. In addition, the level of constitutive activity of this N36S/T42A/T66A triple mutant is increased compared to the N36S/T42A double mutant. The most interesting observation concerning the CAM receptors containing a T42A mutation was that the additional mutations increased not only the constitutive activity of the receptor, but also made the receptor less sensitive towards the inverse agonists.

For ZM241385 and MRS1706, not only the intrinsic activity profoundly decreased on the mutants with increasing levels of constitutive activity, but their potency also slightly decreased. For instance, the IC_{50} values of ZM241385 for the medium level CAM N36S/T42A and T42A/V54A were approximately 2-fold higher than the IC_{50} values for this compound on the low level CAM receptors. In addition, the IC_{50} value of ZM241385 increased another 2.5- to 3-fold for N36S/T42A/T66A which had slightly higher constitutive activity than the other two medium level CAM receptors. Similarly, the IC_{50} values of MRS1706 for medium level CAM receptors were also slightly higher than for low level CAM receptors, varying from 1.5-fold to 4-fold. However, the potency of DPCPX for mutants with

increased level of constitutive activity did not increase except for N36S/T42A/T66A triple mutant.

The rank order of potencies of the inverse agonists showed that with the exception of the T42A/V54A double mutant, the IC_{50} values of DPCPX were 8- to 22-fold higher than the values for ZM241385. The IC_{50} values of MRS1706 in turn were 11- to 36-fold lower than the values for DPCPX which is in accordance with the weaker antagonism of DPCPX at the wt adenosine A_{2B} receptor (see Tables 2 and 3).

Estimation of parameters of the two-state receptor model

More than ten years ago the two-state receptor model was introduced to successfully explain some observations of GPCRs in cell lines and in recombinant receptor-expression systems (Leff, 1995); for example a partial agonist in one experimental system could behave as a full agonist in another. In this study, we applied this model to characterize both the constitutive activity of CAM adenosine A_{2B} receptors and the intrinsic efficacy of the tested compounds.

Three important parameters: L (the ratio of active receptors R_a versus inactive receptors R_i), α (intrinsic efficacy, which reflects the ratio of ligand affinity for R_a over R_i) and K_A (equilibrium dissociation constant of the ligand-receptor complex (AR_i)) were obtained from the scaled data of 3 to 5 independent experiments with GraphPad Prism. When $\alpha>1$, the ligand will enrich the R_a state and is classified as an agonist; conversely, if α 1, the ligand will enrich the R_i state and is classified as an inverse agonist. Thus we applied the constraint of shared value for all data set on these two parameters in curve fitting. Fitted α values were 0.14 0.03, 0.35 0.03 and 0.31 0.02 for ZM241385, DPCPX and MRS1706, respectively. Among these 3 inverse agonists, ZM241385 had the lowest intrinsic efficacy value whereas MRS1706 and DPCPX had comparable intrinsic efficacies. This rank order of fitted α values was consistent with the maximal inhibition rate (I_{max}) of the inverse agonists on mutants with medium levels of constitutive activity (Table 3).

Next to the intrinsic efficacies also the K_A values were fitted and were 89 17, 449 146 and 30 8 for ZM241385, DPCPX and MRS1706, respectively. According to the two-state receptor model, the dissociation constant K_A is proportional to the EC_{50} (EC_{50} $K_A(1+L)/[(1/\alpha)+L]$) (Kenakin, 2003). The general rank order of fitted K_A values was DPCPX > ZM241385 > MRS1706, which was in agreement with the IC_{50} data (see Table 3). We felt confident to assume similar K_A values for both wild type and mutant receptors, since IC_{50} values for a given compound did not vary to a great extent (Table 3).

The L values, representing the ratio of active versus inactive receptors, of low level and intermediate level CAM adenosine A_{2B} receptors were determined with respect to the A18T/A23V/C83Y/A106V/R112S mutant and the truncated receptor as described in the Materials and Methods section. The fitted L values differentiated into 2 groups (Table 4):

Table 4. Fitted parameters L for mutants with low level or medium level constitutive activity.

mutants	ZM241385	DPCPX	MRS1706
F84L	0.11 0.02	0.12 0.02	0.14 0.02
F84S	0.22 0.03	0.16 0.02	0.22 0.02
F84L/S95G	0.22 0.02	0.17 0.02	0.20 0.02
T42A	0.20 0.16	0.16 0.02	0.18 0.02
T42A/V54A	1.38 0.10	1.6 0.10	1.38 0.08
N36S/T42A	1.49 0.10	1.47 0.10	1.52 0.08
N36S/T42A/T66A	1.9 0.17	1.69 0.10	1.90 0.11

Low level CAM receptors have L values around or slightly smaller than 0.2; intermediate level CAM receptors have L values ranging from 1.38 for the T42A/V54A mutant to 1.90 for the N36S/T42A/T66A mutant. These levels were 7- to 15-fold higher than the average L value of the low level CAM receptors.

In order to discuss our experimental data further, we implemented the two-state receptor model in the software program MatLab and composed a graphic interface to facilitate parameter input and curve simulation. We varied L values but fixed α and K_A at 0.2 and 5* 10^{-8} M, respectively to see whether we could mimic our experimental data. When L values of 0.2, 2 and 200 were used, the overall shape of the simulated curves mimicked the curves that we obtained with receptors with varying levels of constitutive activity (Figure. 5). The constitutive activity of CAM receptors with low L values (L 0.2) was completely inhibited by the inverse agonist; CAM receptors with medium L values (L 2) were partially inhibited (around 50%); and CAM receptors with high L values (L 200) could not be inhibited by inverse agonists with an α value of 0.2. Careful analysis of the simulated curves at L 0.2 reveals that a slight amount of residual activity is expected to remain upon application of saturating levels of inverse agonists with an intrinsic efficacy of 0.2. Apparently, this residual growth of the yeast cells is beyond the detection limit.

Besides these three types of simulated curves that we have also observed in our experiments, there was another type of curve that was not represented among our series of CAM receptors. When L is 20, the receptor could be inhibited for 15% by an inverse agonist with an α value of 0.2. We did not observe this level of inhibition in our experiments, because none of our CAM adenosine A_{2B} receptors displayed an L value of around 20.

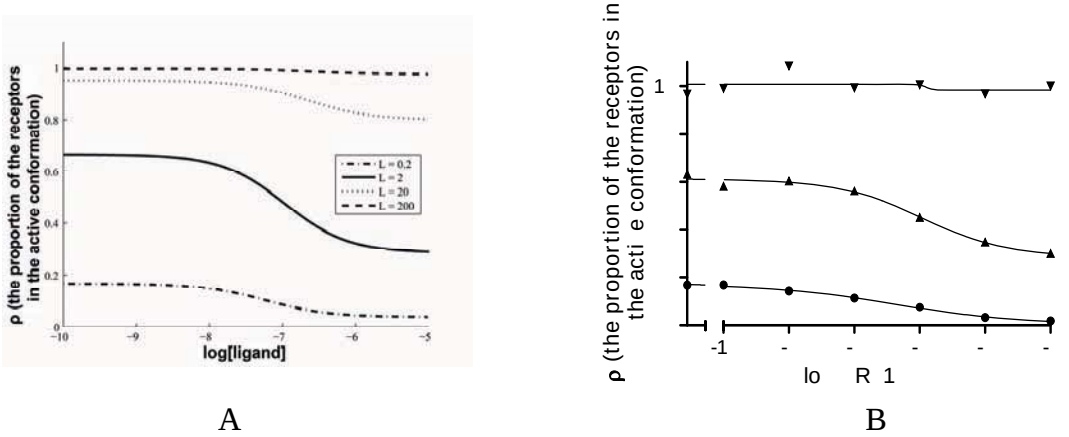


Figure. 5 Simulated curves versus experimentally determined curves. A) Simulated dose-proportion (ρ) curves. The parameters were: L 0.2 (mixed line), 2 (continuous line), 20 (dotted line) and 200 (dashed line); α 0.2; K_A 50 nM. B) Experimentally determined dose-proportion (ρ) curves from growth assays of mutants F84L (●), N36S/T42A (▲) and Q214L/I230N/V240M/V250M/N254Y/T257S/K269stop (▼) on agar plates containing a range of concentrations of the inverse agonist MRS1706. On the X-axis the logarithm of the concentration of the ligand is shown. The Y-axis in A) describes the portion of receptors in the active state while the Y-axis in B) reflects the portion of receptors in the active state as calculated by the ratio of the growth of yeast cells expressing various CAM receptors versus the highest amount of growth obtained in yeast cells expressing a locked receptor.

iscussion

Evidence of constitutively active GPCRs has accumulated over the past 10 years, which enabled the distinction between neutral antagonists and inverse agonists for several GPCRs (Costa and Cotecchia, 2005). However, there is no report on inverse agonism for the wt human adenosine A_{2B} receptor due to its lack of constitutive activity. One might speculate that a high basal activity would disturb the physiological function of the adenosine A_{2B} receptor due to its ubiquitous expression (Volpini 2003). The low constitutive activity of the wt adenosine A_{2B} receptor, however, does not allow discrimination between antagonists and inverse agonists for this receptor subtype.

In our previous work a random mutation bank was constructed and a collection of adenosine A_{2B} receptor mutants with varying levels of constitutive activity was identified with a yeast growth assay (Beukers et al., 2004b). Yeast cells enabled us to identify CAM receptors among the randomly mutated receptors and these CAM receptors make it possible to study inverse agonism on the adenosine A_{2B} receptor. In this study, 9 mutant receptors with different levels of constitutive activity were used to examine inverse agonistic properties of 3 structurally different compounds, ZM241385, DPCPX and MRS1706. All three compounds have been described before in the literature as antagonists for the wild-type adenosine A_{2B} receptor (Alexander et al., 1996; Cooper et al., 1997; Pelletier et al., 2000; Poucher et al., 1995; Prentice et al., 1997; Ongini et al., 1999).

Before characterizing these three compounds on CAM A_{2B} receptors, we tested them on the wild-type human adenosine A_{2B} receptor expressed in yeast to check whether our yeast assay is comparable to assays with mammalian cells. The three compounds were tested as antagonists by their ability to shift NECA-induced dose-growth curves and the data were compared with literature values. In our yeast assay, ZM241385 was able to antagonize NECA-induced yeast growth with a pA_2 value of 7.32 0.29 and DPCPX a pA_2 value of 6.41 0.16. As demonstrated in Table 2, the former was similar to the pA_2 value of 7.20 which was observed for antagonist ZM241385 to antagonize the A_{2B} receptor-mediated relaxant effect of NECA in the rat mesenteric artery (Prentice et al., 1997). The latter was close to the pA_2 value of 6.51 reported in guinea-pig tracheal epithelial cells for the ability of DPCPX to antagonize NECA-evoked cyclic AMP generation (Pelletier et al., 2000). No pA_2 value has been reported for MRS1706, but the pK_i value found in radioligand binding studies (8.86) confirms our findings that MRS1706 is a potent antagonist (Kim et al., 2000). As shown in Table 2, comparison of these literature data to our own results indicated that the potencies obtained in the yeast system were in good agreement with mammalian data. In both cell systems the rank of order of potency of MRS1706, ZM241385 and DPCPX on the wild-type adenosine receptor is MRS1706 $\geq$ ZM241385 > DPCPX.

Subsequent experiments on CAM adenosine A_{2B} receptors expressed in yeast provided firm evidence that these three compounds should be classified as inverse agonists rather than antagonists because they inhibited the growth of CAM receptors. Interestingly, all three structurally diverse inverse agonists tested in this study behaved in a similar manner, that is they all showed full inverse agonism on low level CAM receptors, partial inverse agonism on medium level CAM mutants and no significant inverse agonism on high level CAM mutants although they bound these CAM receptors as well as other CAM receptors in radioligand binding assays (data not shown).

To interpret the different intrinsic activities of the inverse agonists on the CAM receptors at the molecular level, the two-state receptor model was used. In this simplified model, two states are identified: an inactive state (usually designated as the R state) and an active state (usually designated as the R* state). The equilibrium between receptors in the R versus R* state may be altered by ligands (Lefkowitz et al., 1993; Leff, 1995; Kenakin, 1996). Agonists stabilize R* while the inverse agonists stabilize the R state, and neutral antagonists have no preference for either state. This simplified pharmacological model does not take into consideration the multiple conformations of receptors in each state, receptor reserves or receptor to G protein-coupling, but it sufficed to explain our experimental observations.

Simulation of our experimental data revealed that the ability of inverse agonists to inhibit constitutively active receptors depends on the intrinsic efficacy (α) of the compounds as well as on the level of constitutive activity of the CAM receptor. Thus, low (L 0.2) and medium level CAM receptors (L 2) could be fully and partially inhibited by inverse agonists with α 0.2, respectively. Stronger inverse agonists, with smaller α values, should in theory be able

to fully inhibit not only these medium level CAM receptors but also the two locked CAM receptors.

Two precedents for such locked-on receptors have been described in literature. For the adenosine A_1 receptor, we identified a locked mutant receptor containing a G14T mutation. Indeed this mutation led not only to constitutive activity but also to a locked phenotype in the sense that the basal activity of this receptor could not be modulated by either agonists or inverse agonists in both a GTPγS and a cAMP assay (de Ligt et al., 2005). In another report, accumulation of [^{3}H]IP in cells expressing Y368N mutant $5HT_{2C}$ receptors revealed that this mutant was also locked because none of the tested inverse agonists could inhibit the signaling of this receptor whereas these compounds were able to inhibit the constitutive activity of the wild-type receptor and of another mutant receptor (Prioleau et al., 2002).

For inverse agonists, the smaller the α values are, the stronger inverse agonism they will exhibit. The α value is a property that is intrinsic to a given compound and therefore independent of other factors in a test system. In contrast, the intrinsic activity may vary for example when a mutation alters the interaction between receptor and G protein. Such a direct effect of a mutation on the intrinsic activity was described by Ganguli et al. on CAM secretin receptors (Ganguli et al., 1998). Three CAM secretin receptors with comparable levels of constitutive activity were created. Whereas the natural hormone secretin was able to activate the two single point mutants H156R and T322P, secretin turned out to be an inverse agonist on the double mutant receptor containing both mutations. In this case, the double mutant affected the intrinsic activity due to a reduction of the basal coupling of the receptor with G_s proteins.

In our study, on the other hand, the various CAM receptors display 3 different levels of constitutive activity (reflected in parameter L). The variation in intrinsic activity could be explained by these different L values as demonstrated by the simulations with variable L values whereas the intrinsic efficacy (α) and KA values of the ligands were fixed. Based on the experimental data, the rank order of intrinsic efficacy of ZM241385, DPCPX and MRS1706 is ZM241385>MRS1706≈DPCPX. In other words, despite its higher potency, MRS1706 is a weaker inverse agonist than ZM241385.

In conclusion, we were able to characterize in a yeast growth assay ZM241385, DPCPX and MRS1706 as inverse agonists on the human adenosine A_{2B} receptor. The investigations allowed us to quantify the effects of inverse agonists on receptors with different levels of constitutive activity, which to our knowledge is the first study of such nature. We learned that mutated adenosine A_{2B} receptors with different levels of constitutive activity responded differently to three inverse agonists. Two high level CAM receptors were locked in an active state and were insensitive to the inverse agonists. The three intermediate level CAM receptors were partially inhibited, whereas the four low level CAM receptors were almost completely inhibited. These differences can be explained with different isomerization constant (L) values according to the two-state receptor model.

References

Alexander SP, Cooper J, Shine J and Hill SJ (1996) Characterization of the human brain putative A_{2B} adenosine receptor expressed in Chinese hamster ovary (CHO.A_{2B4}) cells. *Br J Pharmacol* 119:1286-1290.

Arunlakshana O and Schild HO (1959) Some quantitative uses of drug antagonists. *Br J Pharmacol Chemother* 14:48-58.

Beukers MW, Chang LC, von Frijtag Drabbe Künzel JK, Mulder-Krieger T, Spanjersberg RF, Brussee J and IJzerman AP (2004a) New, non-adenosine, high-potency agonists for the human adenosine A_{2B} receptor with an improved selectivity profile compared to the reference agonist N-ethylcarboxamidoadenosine. *J Med Chem* 47:3707-3709.

Beukers MW, van Oppenraaij J, van der Hoorn PP, Blad CC, den Dulk H, Brouwer J and IJzerman AP (2004b) Random mutagenesis of the human adenosine A_{2B} receptor followed by growth selection in yeast. Identification of constitutively active and gain of function mutations. *Mol Pharmacol* 65:702-710.

Beukers MW and IJzerman AP (2005) Techniques: how to boost GPCR mutagenesis studies using yeast. *Trends Pharmacol Sci* 26:533-539.

Bond RA and IJzerman AP (2006) Recent developments in constitutive receptor activity and inverse agonism, and their potential for GPCR drug discovery. *Trends Pharmacol Sci* 27:92-96.

Brown AJ, Dyos SL, Whiteway MS, White JH, Watson MA, Marzioch M, Clare JJ, Cousens DJ, Paddon C, Plumpton C, Romanos MA and Dowell SJ (2000) Functional coupling of mammalian receptors to the yeast mating pathway using novel yeast/mammalian G protein alpha-subunit chimeras. *Yeast* 16:11-22.

Chang LC, von Frijtag Drabbe Künzel JK, Mulder-Krieger T, Spanjersberg RF, Roerink SF, van den Hout G, Beukers MW, Brussee J and IJzerman AP (2005) A series of ligands displaying a remarkable agonistic-antagonistic profile at the adenosine A_1 receptor. *J Med Chem* 48:2045-2053.

Cooper J, Hill SJ and Alexander SP (1997) An endogenous A_{2B} adenosine receptor coupled to cyclic AMP generation in human embryonic kidney (HEK 293) cells. *Br J Pharmacol* 122:546-550.

Costa T and Cotecchia S (2005) Historical review: Negative efficacy and the constitutive activity of G-protein-coupled receptors. *Trends Pharmacol Sci* 26:618-624.

de Ligt RA, Rivkees SA, Lorenzen A, Leurs R and IJzerman AP (2005) A locked-on, constitutively active mutant of the adenosine A1 receptor. *Eur J Pharmacol* 510:1-8.

de Zwart M, Link R, von Frijtag Drabbe Künzel JK, Cristalli G, Jacobson KA, Townsend-Nicholson A and IJzerman AP (1998) A functional screening of adenosine analogues at the adenosine A_{2B} receptor: a search for potent agonists. *Nucleosides Nucleotides* 17:969-985.

Dowell SJ and Brown AJ (2002) Yeast assays for G-protein-coupled receptors. *Receptors Channels* 8:343-352.

Drews J (2000) Drug discovery: a historical perspective. *Science* 287:1960-1964.

Feoktistov I, Wells JN and Biaggioni (1998) Adenosine A_{2B} receptors as therapeutic targets. *Drug Dev Res* 45: 198-206.

Fredholm BB, IJzerman AP, Jacobson KA, Klotz KN and Linden J (2001) International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol Rev* 53:527-552.

Ganguli SC, Park CG, Holtmann MH, Hadac EM, Kenakin TP and Miller LJ (1998) Protean effects of a natural peptide agonist of the G protein-coupled secretin receptor demonstrated by receptor mutagenesis. *J Pharmacol Exp Ther* 286:593-598.

Holgate ST (2005) The Quintiles Prize Lecture 2004. The identification of the adenosine A_{2B} receptor as a novel therapeutic target in asthma. *Br J Pharmacol* 145:1009-1015.

Hopkins AL and Groom CR (2002) The druggable genome. *Nat Rev Drug Discov* 1:727-730.

Kenakin T (1996) The classification of seven transmembrane receptors in recombinant expression systems. *Pharmacol Rev* 48:413–463

Kenakin T (2003) *A Pharmacology Primer*. Elsevier Academic Press, California and London.

Kim YC, Ji X, Melman N, Linden J and Jacobson KA (2000) Anilide derivatives of an 8-phenylxanthine carboxylic congener are highly potent and selective antagonists at human A_{2B} adenosine receptors. *J Med Chem* 43:1165-1172.

Klco JM, Wiegand CB, Narzinski K and Baranski TJ (2005) Essential role for the second extracellular loop in C5a receptor activation. *Nat Struct Mol Biol* 12:320-326.

Klotz KN, Hessling J, Hegler J, Owman C, Kull B, Fredholm BB, Lohse MJ (1998). Comparative pharmacology of human adenosine receptor subtypes - characterization of stably transfected receptors in CHO cells. *Naunyn-Schmiedeberg s Arch Pharmacol* 357:1-9

Leff P (1995) The two-state model of receptor activation. *Trends Pharmacol Sci* 16:89-97.

Lefkowitz RJ, Cotecchia S, Samama P, Costa T (1993) Constitutive activity of receptors coupled to guanine nucleotide regulatory proteins. *Trends Pharmacol Sci* 14:303–307.

Ongini E, Dionisotti S, Gessi S, Irenius E, Fredholm BB (1999) Comparison of CGS 15943, ZM 241385 and SCH 58261 as antagonists at human adenosine receptors. *Naunyn Schmiedebergs Arch Pharmacol* 359:7-10.

Pausch MH (1997) G-protein-coupled receptors in Saccharomyces cerevisiae: high-throughput screening assays for drug discovery. *Trends Biotechnol* 15:487-494.

Pelletier S, Dube J, Villeneuve A, Gobeil F, Jr., Bernier SG, Battistini B, Guillemette G and Sirois P (2000) Adenosine induces cyclic-AMP formation and inhibits endothelin-1 production/secretion in guinea-pig tracheal epithelial cells through $A_{(2B)}$ adenosine receptors. *Br J Pharmacol* 129:243-250.

Poucher SM, Keddie JR, Singh P, Stoggall SM, Caulkett PW, Jones G and Coll MG (1995) The in vitro pharmacology of ZM 241385, a potent, non-xanthine A_{2A} selective adenosine receptor antagonist. *Br J Pharmacol* 115:1096-1102.

Prentice DJ, Payne SL and Hourani SM (1997) Activation of two sites by adenosine receptor agonists to cause relaxation in rat isolated mesenteric artery. *Br J Pharmacol* 122:1509-1515

Prioleau C, Visiers I, Ebersole BJ, Weinstein H and Sealfon SC (2002) Conserved helix 7 tyrosine acts as a multistate conformational switch in the 5HT2C receptor. Identification of a novel locked-on phenotype and double revertant mutations. *J Biol Chem* 277:36577-36584

Ralevic V and Burnstock G (1998) Receptors for purines and pyrimidines. *Pharmacol Rev* 50:413-492.

Yaar R, Jones MR, Chen JF and Ravid K (2005) Animal models for the study of adenosine receptor function. *J Cell Physiol* 202:9-20.

Volpini R, Costanzi S, Vittori S, Cristalli G, Klotz KN (2003) Medicinal chemistry and pharmacology of A_{2B} adenosine receptors. *Curr Top Med Chem* 3:427-443

Chapter

easuring negati e intrinsic efficacy: the role of constituti e acti ity le els

ummar

Traditionally it is believed that the inhibitory effect of an inverse agonist increases with an increase in constitutive activity of its receptor. However this is not always the case. Previously we found that some inverse agonists do not show any inhibitory effect on two mutant adenosine A_{2B} receptors with very high constitutive activity. In the present study, three groups of mutant human adenosine A_{2B} receptors displaying different phenotypes were studied. N43F and I93N mutant receptors lacked detectable constitutive activity and were insensitive to agonists; mutant N43S behaved as a locked receptor with strong constitutive activity and no longer responded to any ligand; the mutant A51V receptor possessing intermediate constitutive activity was sensitive to both agonists and inverse agonists. The two-state receptor model was used to analyze and simulate the behavior of locked receptors and to determine optimal conditions for the screening of inverse agonists. The response of an inverse agonist was shown to depend on both the intrinsic efficacy of the inverse agonist and the constitutive activity of a receptor it interacts with. The optimal window for measurements is achieved when the value of the isomerization constant (L) between the active (R*) and inactive (R) states of the receptor equals the reciprocal square root of the intrinsic efficacy (α) of the inverse agonist. When L is smaller than this value, the window will increase with an increase of the constitutive activity of the receptor; when L is bigger, the window will decrease with an increase of the constitutive activity.

ntroduction

Constitutive activity, the spontaneous basal signalling activity of GPCRs in the absence of agonists (Leff, 1995; Lefkowitz et al., 1993; Milligan and Bond, 1997) has been identified for a considerable number of GPCRs, not only for mutant but also for wild-type receptors (de Ligt et al., 2000). The first two receptors for which constitutive activity was described are the δ opioid receptor (Koski et al., 1982) and β_2-adrenoceptor (Cerione et al., 1984). Currently, constitutive activity has been identified for more than 60 wild-type receptors such as the biogenic amine, nucleoside, lipid, amino acid, peptide and protein receptors (for a review, see Seifert and Wenzel-Seifert, 2002). Constitutively active mutant receptors are considered to mimic an active conformation of the receptor probably through the release of an intermolecular restraint that retains the wild-type receptor in an inactive conformation (Parnot et al., 2002).

Constitutive activity can be specifically suppressed by inverse agonists. Discrimination between antagonists and inverse agonists was first described in detail by Costa and Herz (1989). They identified two types of antagonists for the δ opioid receptor: one exhibited no

intrinsic activity while the other displayed negative intrinsic activity measured as the ability to inhibit the GTPase activity of G proteins. This negative intrinsic activity is usually referred to as inverse agonism. So far, numerous inverse agonistic phenomena have since been described in various recombinant cell systems, *in vivo* systems and in animal models.

Quite a few constitutively active mutant receptors are disease-related. Cases in point are retinitis pigmentosa and night blindness that are caused by constitutively active mutations of rhodopsin (Rao et al., 1994). Similarly, mutations in the V_2 vasopressin receptor result in diabetes insipidus (Morello et al., 2000) and mutant Ca^{2+} sensing receptors are responsible for certain forms of hypocalcaemia and hypercalciuria (Chattopadhyay et al., 1996). It was also reported that several mutations in the LH receptor cause male precocious puberty (Shenker et al., 1993). Inverse agonists have special therapeutic benefits for the treatment of constitutive activity-related diseases because these compounds may bring down the constitutive activity. In fact, many of the antagonists' that are clinically used are inverse agonists (for a review, see Bond and IJzerman, 2006; Milligan, 2003).

Traditionally, it is believed that an inhibitory effect of an inverse agonist will increase with an increase in constitutive activity of the receptor it interacts with as reviewed by Seifert and Wenzel-Seifert (2002). However, this is not always valid. For example the G14T mutant adenosine A_1 receptor has been reported to be a locked-on receptor. Neither agonists nor inverse agonists were capable of modulating its basal activity in either a GTPγS or a cAMP assay. Still the G14T mutant receptor was able to bind both the radiolabeled agonist [^{3}H]CCPA and the inverse agonist [^{3}H]DPCPX (de Ligt et al., 2005). In another study, substitution of a tyrosine in the highly conserved NP Y motif of the $5HT_{2C}$ receptor with any of the naturally occurring amino acids revealed a locked-on Y7.53N mutant. Although this mutant had a similar affinity as the wild-type receptor for all inverse agonists tested and even an increased affinity for all the agonists tested, the high basal signaling of this receptor was neither increased by agonists nor decreased by inverse agonists (Prioleau et al., 2002). Recently we reported on two highly constitutively active mutant receptors, the A18T/A23V/C83Y/A106V/R112S and the Q214L/I230N/V240M/V250M/N254Y/T257S/

K269stop adenosine A_{2B} receptor. Both mutant receptors could not be inactivated by the inverse agonists ZM241385, DPCPX or MRS1706 (Li et al., 2007). These examples suggest that such very constitutively active receptors may no longer respond to inverse agonists questioning the general theory that the inhibitory effect of an inverse agonist will increase with an increase in constitutive activity.

In the present study we aimed at a thorough understanding of the relationship between negative intrinsic efficacy and the level of constitutive activity for which the two-state receptor model provided the theoretical framework. It allowed us to explain how receptors can be locked in an active conformation and become irresponsive to agonists and inverse agonists. In addition, we were able to define conditions for optimal assay sensitivity using the same theoretical concept.

aterials and methods

east plasmid and A_{2B} receptor antibody

A genetically modified yeast *Saccharomyces cerevisiae* strain with the following genotype: *MAT*a*his3 leu2 trp1 ura3 can1 gpa1*Δ::Gα_{i3} *far1*Δ::ura3 sst2Δ::ura3 Fus1::FUS1-HIS3 LEU2::FUS1-lacZ ste2Δ::G418^R and expressing vector pDT-PGK was a gift from Dr. S. J. Dowell. Constitutively active adenosine A_{2B} receptor mutants were either identified upon screening of a randomly mutated receptor library (Beukers et al., 2004) or constructed through site-directed mutagenesis. The A_{2B} receptor antibody was kindly provided by Dr. I. Feoktistov.

ite-directed mutation

Site-directed mutation was introduced by PCR with mutation-inducing primers: 5'-ACGCCCACCTTCTACTTCCTG3' for the N43F mutant and 5'-AAGGCTGAAGTTGGAGCTCTG3' for the I93N mutant. Mutant genes were inserted into pDT-PGK vector at EcoRI/HindIII sites and were confirmed by DNA sequencing.

east membrane preparation and immunoblot

Transformed yeast cells were harvested in mid-exponential phase. Yeast cells were collected and washed with cold water and resuspend in ice cold lysis buffer (50 mM Tris-HCl PH7.5, 1 mM EDTA and 0.1 mM PMSF). Yeast cells were broken by vigorous vortexing with glass beads. Unbroken cells were precipitated and removed after 5,000 g centrifugation for 10 min at 4 C. Subsequently, the supernatant was centrifuged at 40,000 g for 40 min at 4 C. The pellets were resuspended with cold collection buffer (50 mM Tris-HCl PH7.5, 1mM EDTA) and used to measure protein concentrations.

Yeast membranes containing 0.5 mg protein/ml were denatured (3 - 5 min, 100 C), separated on SDS-PAGE and blotted on Hybond-ECL membrane (GE Healthcare). Hybond-ECL membrane was blocked with PBS containing 5% milk powder for 30 min, and then incubated with 1: 1,125 diluted A_{2B} receptor antibody for 1 hr. After thorough removal of unbound antibody by rinsing the membrane with PBST (0.05% Tween-20, PBS pH 7.6), the membrane was incubated with 1: 2,000 diluted HRP-conjugated anti-rabbit IgG (Jackson Immuno Reseach) for 1 hr. Hybond-ECL membrane was washed again and enhanced chemiluminescence was used to visualize the A_{2B} receptor.

east screening assay

Yeast screening assays were performed as described before (Beukers et al., 2004). In brief, yeast cells from an overnight culture were diluted to around 400,000 cells/ml (OD_{600} 0.02). In solid medium assays, droplets of 1.5 µl were spotted on histidine-deficient agar medium containing various concentrations of test compounds. In liquid medium assays, 50 µl was seeded into each well of a 96-wells plate. Receptor-independent yeast growth was suppressed through the addition of 7 mM 3-AT. After incubation at 30 C for 48 hours for solid medium

assays and 35 hours for liquid medium assays, the growth of yeast was recorded and quantified with Quantity One imaging software from Bio-Rad (Hercules, CA). Each experiment was repeated 3 times. Prism software (GraphPad Software Inc., San Diego, CA) was used for curve fitting and to calculate the potency and efficacy of the compounds.

Pharmacological modeling (two-state receptor model

The two-state receptor model is described by three parameters: L, the isomerization constant which is the ratio of the receptor in the active R_a state versus the inactive R_i state; α, the intrinsic efficacy which refers to the affinity of a ligand for the active state of the receptor (R_a) over the inactive state of the receptor (R_i); K_A, the equilibrium dissociation constant of a ligand-receptor complex. According to this model, the proportion of receptors in the active state is:

$$\rho = \frac{\alpha L[A]/\ _A + L}{[A]/\ _A(1+\alpha L) + L + 1}$$ equation 1 (Kenakin, 2003)

In the absence of ligand ([A] 0), the proportion of receptors in the active state is:

$$\rho_0 = L/(L+1)$$ equation 2

When the saturation concentration of the ligand is reached ([A] ∞), the proportion of receptors in the active state becomes:

ρ αL/(1+αL) equation 3

Thus, the theoretical activity window between unliganded and ligand-occupied receptor is:

ρ_0-ρ L/(L+1)- αL/(1+αL) equation 4

To obtain a graphical display of the theoretical window (ρ_0-ρ), equation 4 was implemented in the MatLab software package version 8.0 (The Mathwork, Inc, Natick, USA). A bell-shaped curve was obtained by plotting the theoretical window (ρ_0-ρ) against logarithmic L values (L was varied from 0.01 to 100 with an interval of 0.01) with fixed α values, on which the experimental data were superimposed. Similarly, the effect of α on the bell-shaped curve was investigated and displayed as a 3-D mesh with the MatLab software.

Results

election of mutant A_{2B} receptors

To test the interaction of inverse agonists with human adenosine A_{2B} receptors, we used our previously described yeast assay (Li et al., 2007). The relationship between the level of constitutive activity of a receptor and the sensitivity to measure the relative intrinsic efficacy of a ligand was determined with mutant receptors displaying different levels of constitutive activity. We selected mutant receptors with mutations at amino acids positions N43 (2.40), A51 (2.48) and I93 (3.40), i.e. located distant to the ligand binding site to avoid disturbance of

the interaction between ligands and the receptor (see snake plot' in the supplementary data for the location of these positions). We feel confident that these amino acids do not affect ligand binding, as is evident from mutagenesis studies on adenosine receptors (Kim et al., 2003) and from results of two-entropies analysis (Ye et al., 2006). Detailed information can be found in the supplementary material.

All in all we examined four mutants, two point mutants N43F and I93N created through site-directed mutagenesis, and another two point mutants A51V and N43S selected from a random mutation bank (Beukers et al., 2004).

These four mutant receptors represent three different phenotypes: 1) receptors without detectable constitutive activity that are insensitive to agonists (N43F and I93N), 2) a strongly constitutively active receptor that no longer respond to ligands (N43S) and 3) a receptor with some constitutive activity, sensitive to both agonists and inverse agonists (A51V). Our findings with these three categories will be described below.

Receptors without detectable constituti e acti ity and insensiti e to agonists

Neither the wild-type nor the I93N or N43F mutant receptors were able to induce yeast growth in the absence of an agonist. Upon addition of NECA, a prototypic A_{2B} receptor agonist, the wild-type receptor induced maximum yeast growth with an EC_{50} value for NECA of 110 26 nM. However, in the presence of NECA, little growth was observed for yeast cells expressing the N43F mutant receptor while no growth was seen with yeast cells expressing the I93N mutant receptor (Figure 1A). To examine whether the N43F and I93N mutant receptors were expressed in the yeast cells, yeast membranes were prepared and analyzed with a western blot (Figure 1B).

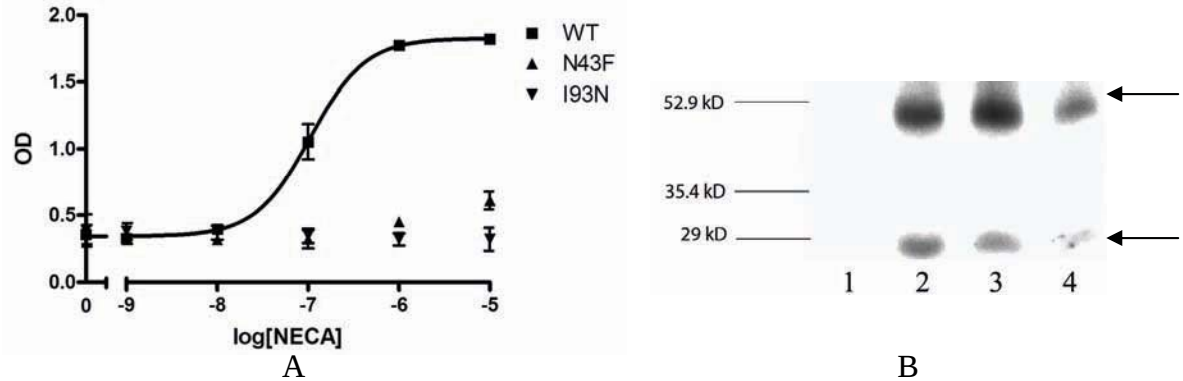


Figure. 1 A) Growth of yeast cells expressing the wild-type adenosine A_{2B} receptor or the N43F or I93N mutant receptor. Growth was measured in the absence or presence of increasing concentrations of NECA. The experiment was repeated 3 times, of which the means SEM are shown. B) Western blot analysis of control, empty vector (lane 1), or wild-type (lane 2), N43F (lane 3) and I93N (lane 4) A_{2B} receptors expressed in the yeast membrane. Two bands with an apparent molecular mass around 29 kD and 52 kD (indicated by arrows) were detected as A_{2B} receptor specific bands.

On the membranes expressing the wild-type receptor, the anti-A_{2B} receptor antibody labeled a band with an apparent molecular mass around 29 kD which is close to the molecular weight of the A_{2B} receptor. In addition, bands with apparent molecular masses bigger than 50 kD were labeled. These higher-mass bands may be the result of receptor oligomerization and/or posttranslational modifications. This western blot result is similar to that obtained on mammalian cells (Feoktistov et al., 2003). The blot showed that the N43F mutant receptor was expressed at similar levels as the wild-type receptor while the I93N mutant receptor showed around 50% decreased expression compared to the wild-type receptor.

Since the expression level of the two mutant receptors could not account for the insensitivity to the agonist, we analyzed this behavior with a pharmacological model. Matlab was used to mimic the activation of receptors with little constitutive activity (L values ranging from 0.01 to 0.0001) by an agonist with an α value of 100 and K_A Figure 2, a smaller L value corresponds to a less constitutively active receptor that is also less responsive to the agonist. When its L value is very small (L 0.0001), a receptor can hardly be activated by the same agonist. In functional assays, such a receptor will be unresponsive to an agonist and characterized as being locked in an inactive conformation.

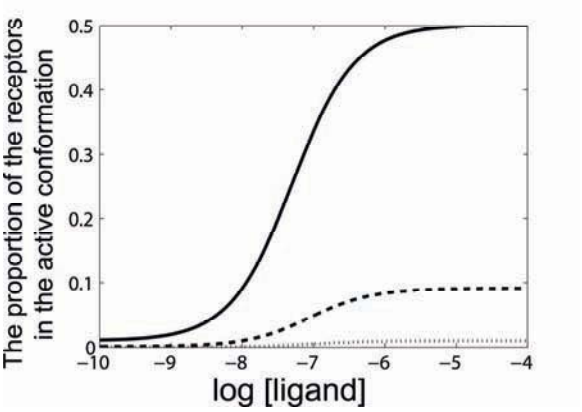


Figure. 2 Simulation of receptor activation with different ratios of active versus inactive receptors as represented by different L values. Receptor activation by an agonist with an intrinsic efficacy (α value) of 100 and an affinity (K_A) of 100 nM is shown. Continuous line L 0.01; dashed line L 0.001; dotted line L 0.0001.

trongly constituti ely acti e receptors insensiti e to both agonists and in erse agonists

The constitutively active mutant N43S receptor was retrieved from a random mutation bank (Beukers et al., 2004). It was characterized by a high level of constitutive activity and could not be further activated by 10 μM of the agonist NECA (Figure 3). It also did not respond to the inverse agonist ZM241385 (10 μM) which exhibited the greatest relative intrinsic efficacy among the three tested inverse agonists DPCPX, MRS1706 and ZM241385 as shown in our previous study (Li et al., 2007).

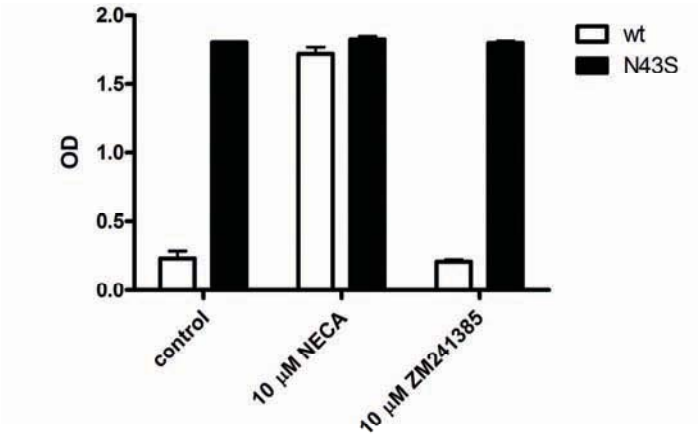


Figure. 3 Growth of yeast cells expressing the wild-type adenosine A_{2B} receptor or the N43S mutant receptor. Growth was measured in the absence (control) or presence of 10 μM NECA or 10 μM ZM241385. Each experiment was repeated 3 times, of which the means SEM are shown. *** p 0.001, student's unpaired *t* test.

Matlab was used to mimic the activation and inhibition of receptors with a high level of constitutive activity (L 1000). The α values were set from 100 for an agonist to 0.1 and 0.01 for inverse agonists. As shown in figure 4, it is hard to detect agonism and inverse agonism of a ligand on receptors with such a big L value. In other words, this receptor is locked in an active state and can not be further activated or inhibited.

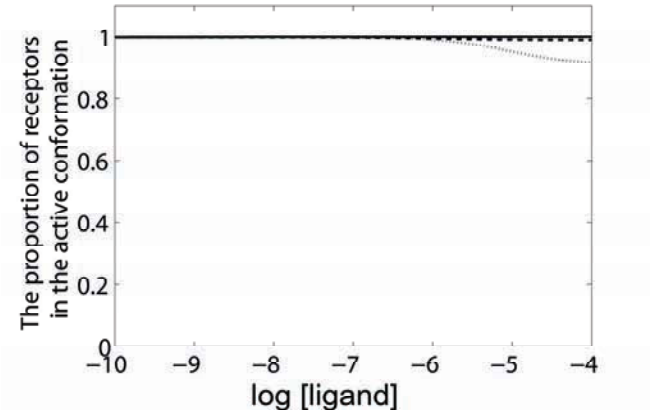


Figure. 4 Simulation of a concentration-response curve for a receptor with a ratio of active versus inactive receptors (L value) of 1000 and an inverse agonist with intrinsic efficacies (α values) varying from 100 (straight line), 0.1 (dashed line) or 0.01 (dotted line).

odestly constituti ely acti e receptors sensiti e to both agonists and in erse agonists

From our random screening assay we identified a mutant A51V receptor which exhibited an intermediate level of constitutive activity. It appeared sensitive to both agonists and inverse agonists. Thus in a single experiment the efficacy of the agonist NECA as well as the efficacy of the inverse agonist ZM241385 could be determined (Figure 5). The EC_{50} value was 96 25 nM for NECA and 203 37 nM for ZM241385.

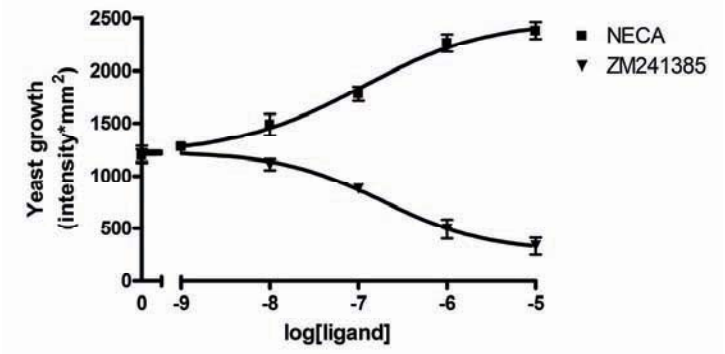


Figure. 5 Activation and inhibition of the growth of yeast cells expressing the A51V mutant A_{2B} receptor by the agonist NECA or the inverse agonist ZM241385, respectively. The experiment was repeated 3 times, of which the means SEM are shown.

Prediction of the experimental window with pharmacological modeling of the two-state receptor model

The two-state receptor model was used to determine the optimal experimental window to measure the intrinsic efficacy of compounds. Theoretically, when all the receptors are in an inactive state, the proportion of activated receptors (ρ) is 0 and no test system would be able to detect any signal. Conversely, when all the receptors are in an active state, ρ_∞ equals 1 and the test system will detect a maximal signal. This simplified model is based on two assumptions. First, only the R and R* states of the receptor and the interaction between the receptor and ligand are considered while receptor/G protein interactions are not. Second, the reported signal is assumed to be positively correlated with either agonist-induced or constitutive receptor activation in a given system. Based on these assumptions, the readout signal will reflect the ability of ligands to activate or inactivate the receptor. In addition, the theoretical window of a ligand in a functional assay can be calculated by the difference between the proportion of activated receptors in the absence and presence of the ligand. For agonists the proportion of active receptors in the absence of ligand (ρ_0) is subtracted from the proportion of active receptors in the presence of ligand (ρ_∞). Conversely, to determine the experimental window for inverse agonists, the proportion of receptors in the presence of excess amount ligand (ρ_∞) is subtracted from the proportion of active receptors in the absence of ligand (ρ_0). The absolute value of the response (ρ_0-ρ) is thus between 0 and 1. The experimental window, (ρ-ρ_0) for agonists and (ρ_0-ρ) for inverse agonists, is expressed as a percentage of the theoretically maximal response.

The absolute value of equation 4 in the two-state receptor model was implemented in the software package MatLab. Figure 6 displays the theoretical window (ρ_0-ρ), that is obtained for a hypothetical inverse agonist (α value equals 0.2) on a series of hypothetical receptors (L values vary from 0.01 to 100). The largest experimental window is obtained on a hypothetical receptor with an L value of 2.3. On such a receptor the maximal hypothetical window is 38% of the maximal response that the system can produce.

The ability to determine the intrinsic efficacy of ligands is also affected by the reproducibility and sensitivity of the assay. For example, when the standard deviation equals 10% of the maximum response, no significant response can be detected for an inverse agonist with an α value of 0.2 on receptors with L values below 0.15 ($10^{-0.83}$) or above 34 ($10^{1.53}$) (see Figure 6).

At receptors with this level of constitutive activity the effect of the ligand will not be significantly different from the standard deviation of the signal caused by the constitutive activity. In a more reproducible assay with a standard deviation of 5% of the maximum response, the range of receptors for which a response of this inverse agonist can be detected will increase to receptors with L values between 0.07 ($10^{-1.17}$) and 74 ($10^{1.87}$) (Figure 6).

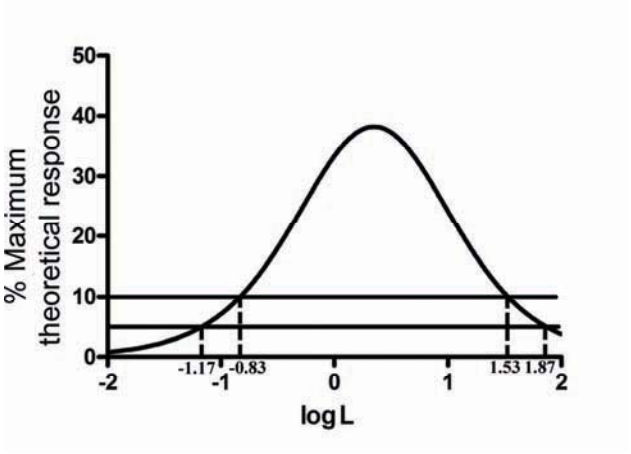


Figure. 6 The bell-shaped curve represents the plot of the theoretical window (ρ_0-ρ) versus the logarithm of L (the ratio of active versus inactive receptors) using series of L values (from 0.01 to 100 with an interval of 0.01) and a single α value equal to 0.2. The horizontal lines represent the detection limit with standard deviations of 5% and 10% of the maximal response for the lower and upper line, respectively.

The level of constitutive activity is lin ed to the ma imum response in a non-linear manner

Our results suggest that the level of constitutive activity of a receptor affects the ability to determine the intrinsic activity of a ligand. To test this hypothesis, the theoretical curve and available experimental data are shown in one figure (Figure 7). The experimental data stem from a previous study where we reported on 7 mutant human adenosine A_{2B} receptors. F84L, F84S, F84L/S95G, T42A are mutants with low levels of constitutive activity and T42A/V54A, N36S/T42A, N36S/T42A/T66A are mutants with intermediate levels of constitutive activity (Li et al., 2007). The inverse agonist ZM241385 had the highest intrinsic efficacy of the three inverse agonist tested with an α value of 0.14. In this study, we normalized the experimental

window of ZM241385 on these mutants with respect to the maximal response in the yeast assay, and obtained the following percentages of maximum responses: 10%, 16.5%, 17.5%, 14.6%, 47.4%, 45% and 39.6% for F84L, F84S, F84L/S95G, T42A, T42A/V54A, N36S/T42A and N36S/T42A/T66A, respectively. The L values of these mutants were 0.12, 0.20, 0.20, 0.18, 1.45, 1.49 and 1.83, respectively. These experimental data were superimposed on the bell-shaped curve representing the theoretical maximum window (caused by an inverse agonist with an α value equal to 0.14) obtained with L values ranging from 0.01 to 100 (Figure 7). The plot shows that the experimentally determined data were located on or very close to the left-hand side of the theoretical curve. In our studies, receptors with high levels of constitutive activity (higher constitutive activity than N36S/T42A/T66A mutant, but less constitutively active than locked-on mutant) have not been identified so far. As a consequence we have no experimental data available to explore the right-hand side of the curve in figure 7. The L value of the highly constitutively active mutants which were non-responsive to ligands could not be determined experimentally because of the very high L values. This is due to the fact that a very small experimental standard deviation leads to huge standard deviations in L values when almost all the receptors are constitutively active and maximum activation is achieved. Thus, we could not calculate the L value of the N43S mutant receptor and superimpose its data on the bell-shaped curve. Although the L value of the N43S mutant receptor can not be quantified, the mutant receptor does show a decreased response at such high L values.

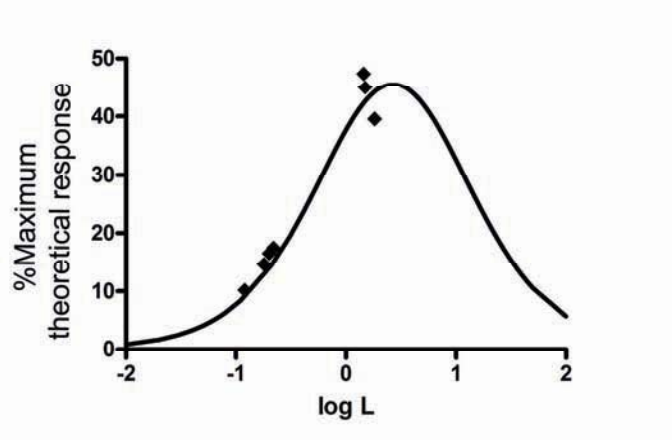
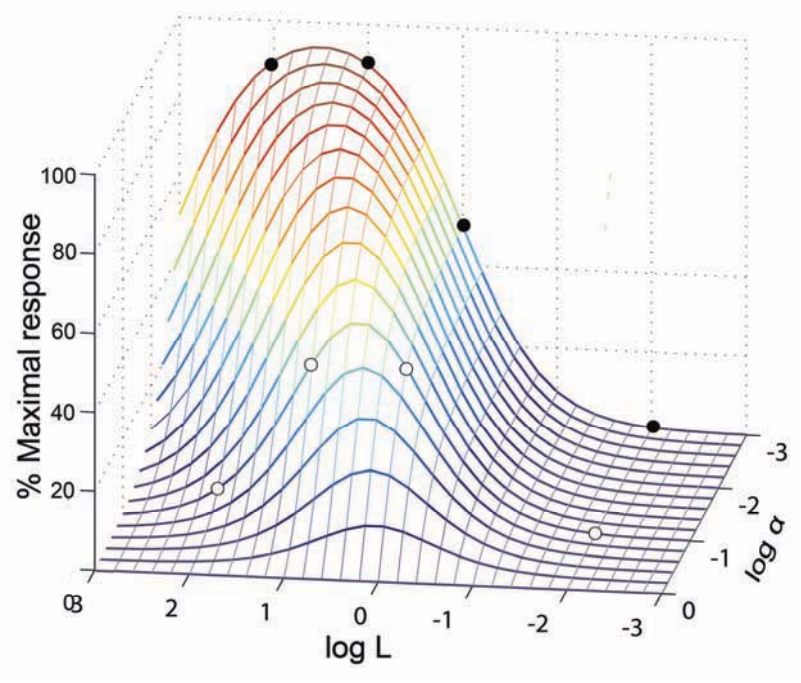


Figure.7 The bell-shaped curve represents the plot of the experimental window (ρ_0-ρ) versus the logarithm of L (the ratio of active versus inactive receptors) using series of L values (from 0.01 to 100 with an interval of 0.01) and a single α value equal to 0.14. The data points represent the previously reported experimental data of ZM241385 tested on 7 mutant adenosine A_{2B} receptors (Li et al., 2007). From left to right, the mutants were F84L, T42A, F84S, F84L/S95G, T42A/V54A, N36S/T42A and N36S/T42A/T66A. The L values of these mutants were 0.12, 0.18, 0.20, 0.20, 1.45, 1.49 and 1.83, respectively. The calculated experimental windows were 10%, 14.6%, 16.5%, 17.5%, 47.4%, 45% and 39.6%, respectively.

oth the intrinsic efficacy α and the isomeri ation constant determine the e perimental window

Both the intrinsic efficacy α and the isomerization constant L determine the experimental window (equation 4). The relationship between the logarithm of L (x-axis), the logarithm of α



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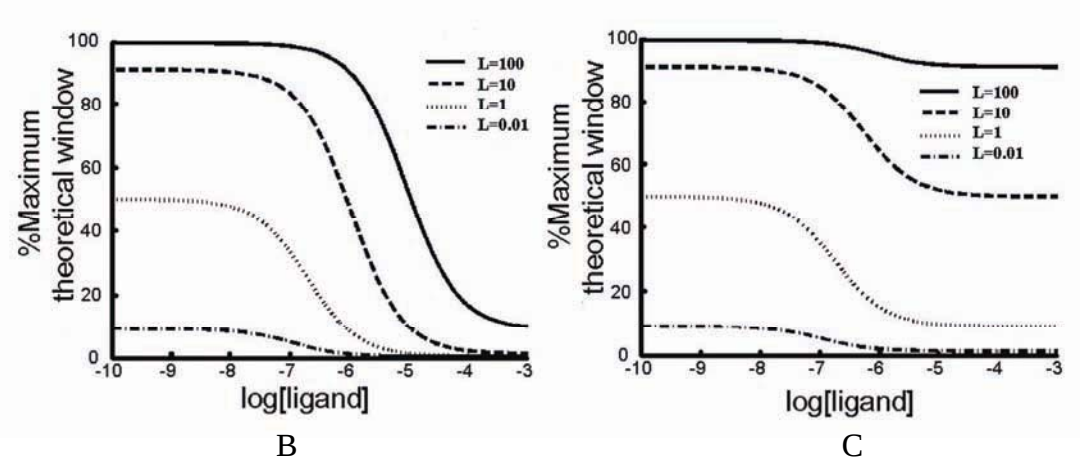


B C

Figure. 8 A) Three-dimensional mesh of the percentage of maximum theoretical window for hypothetic inverse agonists with continuous intrinsic efficacy (α) values between 0.001 to 1 on hypothetic receptors with continuous ratios of active versus inactive receptors (L values) between 0.001 to 1000. In figure 8B, concentration-response curves are shown for receptors with L values of 0.01, 1, 10 and 100 for a strong inverse agonist with an α value of 0.001 (closed circles in figure 8A). Similarly, in figure 8C, concentration-response curves are shown for a weak inverse agonist with an α value of 0.1 (open circles in figure 8A).

(z-axis) and the percentage of the maximum theoretical window (y-axis) can be visualized in a 3-D mesh (see Figure 8A). A series of bell-shaped curves represent the percentage of the maximum theoretical window that can be achieved when log α and log L are varied. This mesh shows that for a ligand with an α value between 0.001 and 1 an optimal theoretical window can be achieved for receptors with a log L value close to 1.

Within the framework of the two-state receptor model, the theoretical window (ρ_0-ρ_∞) is calculated as L/(L+1)- αL/(1+αL) . An optimal theoretical window will be obtained when the relationship between the level of constitutive activity (L) of a receptor and the α value of the ligand obeys the following equation

$$L = \frac{1}{\sqrt{\alpha}} \qquad \text{equation 5}$$

The 3-D mesh is composed of a series of bell-shaped curve according to the different intrinsic efficacies of a ligand. The maximum is achieved when L equals the reciprocal square root of α. When L is bigger than the reciprocal square root of α, the window will decrease with an increase of L. Conversely, when L is smaller than the reciprocal square root of α, the window will increase with an increase of L.

We chose two positions in the mesh to simulate dose-response curves and analyzed the theoretical windows. Although the theoretical window is independent of K_A, K_A is necessary to simulate concentration-effect curves because it determines the relative EC_{50} value of a concentration-effect curve. In the simulation, we used a fixed K_A value of 100 nM. In figure 8B and 8C we simulated the situation where α is fixed and L is variable. If the ligand is a very strong inverse agonist ($\alpha = 0.001$), a bigger window is observed on receptors with higher levels of constitutive activity provided that the ligand is soluble enough to allow testing at mM concentrations (Figure 8B). If the ligand is a very weak inverse agonist ($\alpha = 0.1$), the window will first increase and then decrease with increasing levels of constitutive activity of the receptor (Figure 8C). Fixing α while varying L resulted in concentration-effect curves similar to the ones shown in figure 2 and figure 4.

iscussion

In our previous study, we found two remarkable mutant human adenosine A_{2B} receptors which are highly constitutively active and did not respond to the tested inverse agonists (Li et al., 2007). This finding suggested that the inhibitory effect of an inverse agonist could decrease with an increase in constitutive activity of the receptors and contrasts with the positive relationship between the inhibitory effect and constitutive activity that is often suggested.

In the current study, we aimed at providing a theoretical framework to unravel the relationship between the inhibitory effect of an inverse agonist and the constitutive activity of a receptor. We considered our experimental findings within the boundaries of the two-state receptor model. In this context, all the conformations a receptor might adopt and all the subsequent effector systems activated by such conformations (Kenakin, 2005) can be

classified into two states: 1) an active state, resulting in a measurable readout signal and 2) an inactive state, which cannot induce a readout signal. In our yeast assay, a single downstream signaling event was measured, i.e. the growth of yeast cells, which is positively correlated to the activation of the receptors. In this situation the two-state receptor model can be usefully applied as we have shown in our previous work (Li et al., 2007). Similar to our previous work, the reported intrinsic efficacies are relative intrinsic efficacies as they are system-dependent (Li et al., 2007). In addition, we assume that the downstream signaling effects in our yeast system are identical among the mutants ratifying the use of the simplified two-state receptor model. If downstream signaling events need to be accounted for, the two-state model should be extended with an intervening forcing function (supplementary data to Li et al., 2007).

The two-state model assumes that the receptor states exist in equilibrium between R* (active) and R (inactive), in which R* represents the receptor populations that lead to yeast growth. The isomerization constant (L) determines the ratio between the two receptor populations. The L value is a measure for the percentage of receptors that are present in the active conformation in the absence of ligand and therefore L reflects the level of constitutive activity of a receptor. Bigger L values correspond to more active receptors in a ligand-free system.

L also determines whether the receptor can be easily activated or not. A receptor with a preference for the inactive state will require an agonist with a large intrinsic efficacy to activate it. This situation is depicted in figure 2, in which an agonist can behave as a partial agonist on a receptor with a small L value (L 0.001). When the L value is even smaller (L 0.0001), the effect of the agonist will be beyond the detection limit.

Several observations in the literature may be explained by such small L values. For instance, the V60L mutant human melanocortin receptor showed impaired ability to respond to α-MSH although expression levels and ligand binding were indistinguishable from that of the wild-type receptor (Schioth et al., 1999). Similarly, the I51A mutant cholecystokinin (CCK) receptor showed decreased efficacies upon CCK stimulation while binding features were very similar to those of the wild-type CCK1 receptor (Escrieut et al., 2002). A third example is the R68L mutation in the first intracellular loop of the CCK-B receptor. This mutant completely lost its ability to respond to both CCK and gastrin as determined in cAMP experiments, although it was expressed at a comparable level as the wild-type receptor without impaired binding affinity for gastrin (Wu et al., 1999). These mutant receptors, as well as the N43F and I93N human adenosine A_{2B} receptor in this study, have an impaired response to their agonist and this may be due to rather small L values.

Whereas receptors with small L values respond poorly to agonists, highly constitutively active receptors seem to be insensitive to inhibition by inverse agonists (Figure 4). To inhibit receptors with an L value of 1000, inverse agonists with a large intrinsic efficacy (α 0.01 in this example) are required to obtain at least some inhibitory effect. Inverse agonists with a smaller intrinsic efficacy (α 0.1) hardly show any effect. In other words, such a receptor appears to be locked in an active state unless highly efficacious inverse agonists are applied.

Some receptors indeed possess such high levels of constitutive activity rendering them unresponsive to any known inverse agonist. A couple of examples of such receptors have been mentioned and referred to in the introduction: the G14T mutant adenosine A_1 receptor, the Y7.53N mutant $5HT_{2C}$ receptor and two human adenosine A_{2B} mutants, the A18T/A23V/C83Y/A106V/R112S and the Q214L/I230N/V240M/V250M/N254Y/T257S/K269stop mutant. In the current study, the human adenosine A_{2B} N43S mutant receptor was identified as a receptor that is too constitutively active to be silenced by known inverse agonists.

As a consequence of the above, one should be cautious using receptors with a high constitutive activity to screen for inverse agonists, because only high-efficacy inverse agonists can be identified while weaker inverse agonists will be overlooked. These weaker ligands might however be good lead compounds for drug development and may also provide useful information for structure-activity relationships.

To detect inverse agonists with low efficacy or to rank compounds based on their intrinsic efficacy, receptors with intermediate levels of constitutive activity provide a better test system than receptors with either a rather high constitutive activity or negligible constitutive activity. This is true for wild-type receptors displaying constitutive activity as well as for mutant receptors provided that the mutation does not affect ligand binding, G protein-coupling and receptor expression levels. As explained in the results section, the greatest sensitivity will be obtained with a receptor for which the L value is equal to the reciprocal square root of the α value. When L is smaller than this value, an increase of L will increase the window. On the contrary, when L is bigger than this value, the window will decrease with an increase of L.

As we mentioned above, the assumption that the inhibitory effect of an inverse agonist will increase with an increase in constitutive activity of the receptors is valid only for highly efficacious inverse agonists. Such inverse agonists have small α values, thus the reciprocal square root value of α is very big. In that case a maximum experimental window can only be achieved when L is very big or, in other words, when the receptor is very constitutively active. However, receptors with such high constitutive activity are not common; usually the L values are smaller than the values required to achieve the maximum windows. In that case, the window increases with increasing constitutive activity (Figure 8B).

On the other hand, for weaker inverse agonists with larger α values, the reciprocal square root value of α is relatively small and receptors may have L values exceeding the optimal value for achieving the maximum window. Thus, for weak inverse agonists, there are two possibilities: when L values of the receptors are smaller than the reciprocal square root value of α, the window is increased with an increase of L; when L values are bigger than that, the window is decreased with an increase of L (Figure 8C), such that the window disappears with the highest L values, effectively yielding a locked' receptor.

In conclusion, we explored in this study the relationship between the inhibitory effect of inverse agonists and the level of constitutive activity of receptors. High constitutive activity

may yield lock receptors that do not respond to inverse agonists, as a consequence of the delicate balance between receptor isomerization constant and ligand intrinsic efficacy. Receptors with intermediate levels of constitutive activity however allow detection of both agonists and inverse agonists as the three groups of mutant A_{2B} receptors with different phenotypes show. Such receptors are in fact good screening tools since they provide an optimal window to sense the intrinsic efficacy of ligands.

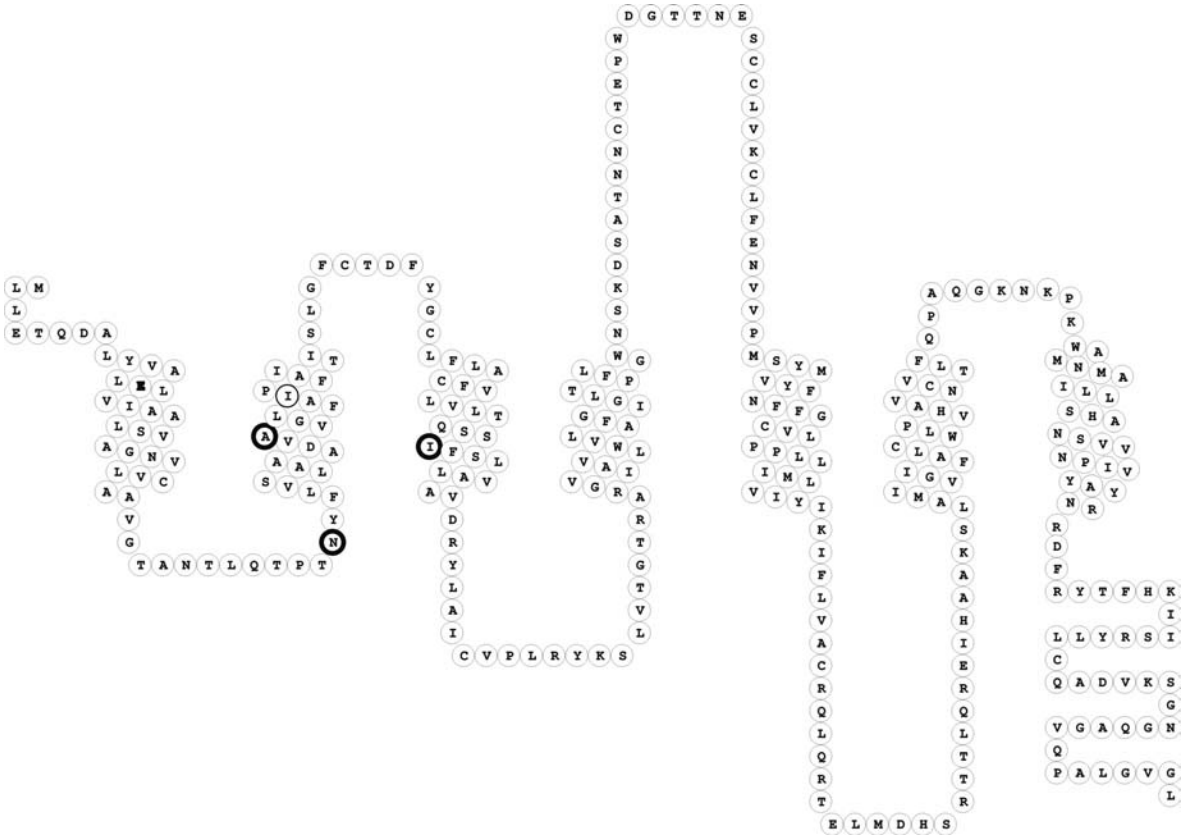
c no led ements

We would like to thank Dr. I. Feoktistov (Vanderbilt University, USA) for providing the A_{2B} receptor antibody, the experimental protocol how to use it and the helpful discussions.

upplementar data

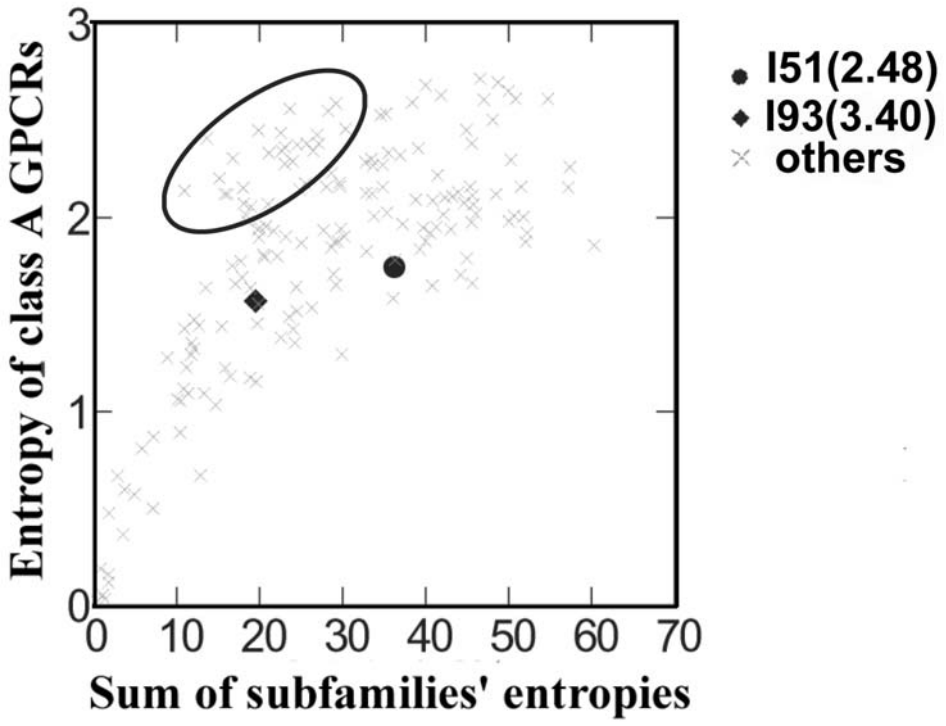
1. na e plot of human adenosine A_{2B} receptor

Snake plot of human adenosine A_{2B} receptor with studied positions N43 (2.40), A51 (2.48) and I93 (3.40) in bold circles. N43 (2.40) is located in the 1st intracellular loop, close to helix 2, A51 (2.48) and I93 (3.40) are in helix 2 and helix 3, respectively.



2. Two-entropies analysis

The mutated residues are located distant from the ligand binding site as shown in the two-entropy analysis. This is a newly developed bioinformatics method and was shown to successfully discriminate positions involved in the ligand binding sites from the other positions (Ye et al., 2006). In short, the principle of the two-entropy analysis to find amino acids involved in ligand binding is based on the assumption that such amino acids must be divergent among subfamilies recognizing different endogenous ligands and be similar or even identical within a subfamily recognizing the same endogenous ligand. Scores are given to individual positions, the smaller a value is, the more conserved the position is, and vice versa. According to this principle, the amino acids involved in ligand binding should appear in the upper left corner (as shown in the ellipse) of the two-entropy plot.



Two of the mutated positions in the A_{2B} receptor, A51 (2.48) and I93 (3.40), were found in the center of the plot which suggested that they are not likely to be involved in ligand binding. These two positions are highlighted in the two-entropy plot. The other position, N43 (2.40), is located at the interface of helix 2 and intracellular loop 1. The intra- and extracellular loops of the receptors were not evaluated in the two-entropy analysis, but in general the intracellular loops are not considered to be involved in direct ligand binding. The reader is referred to Ye et al., Proteins 63 (2006) 1018-1030 for more information.

References

Beukers MW, van Oppenraaij J, van der Hoorn PP, Blad CC, den Dulk H, Brouwer J and IJzerman AP (2004) Random mutagenesis of the human adenosine A_{2B} receptor followed by growth selection in yeast. Identification of constitutively active and gain of function mutations. *Mol Pharmacol* 65:702-710.

Bond RA and IJzerman AP (2006) Recent developments in constitutive receptor activity and inverse agonism, and their potential for GPCR drug discovery. *Trends Pharmacol Sci* 27:92-96.

Cerione RA, Sibley DR, Codina J, Benovic JL, Winslow J, Neer EJ, Birnbaumer L, Caron MG and Lefkowitz RJ (1984) Reconstitution of a hormone-sensitive adenylate cyclase system. The pure beta-adrenergic receptor and guanine nucleotide regulatory protein confer hormone responsiveness on the resolved catalytic unit. *J Biol Chem* 259:9979-9982.

Chattopadhyay N, Mithal A and Brown EM (1996) The calcium-sensing receptor: a window into the physiology and pathophysiology of mineral ion metabolism. *Endocr Rev* 17:289-307.

Costa T and Herz A (1989) Antagonists with negative intrinsic activity at delta opioid receptors coupled to GTP-binding proteins. *Proc Natl Acad Sci U S A* 86:7321-7325.

de Ligt RA, Kourounakis AP and IJzerman AP (2000) Inverse agonism at G protein-coupled receptors: (patho)physiological relevance and implications for drug discovery. *Br J Pharmacol* 130:1-12.

de Ligt RA, Rivkees SA, Lorenzen A, Leurs R and IJzerman AP (2005) A locked-on, constitutively active mutant of the adenosine A_1 receptor. *Eur J Pharmacol* 510:1-8.

Escrieut C, Gigoux V, Archer E, Verrier S, Maigret B, Behrendt R, Moroder L, Bignon E, Silvente-Poirot S, Pradayrol L and Fourmy D (2002) The biologically crucial C terminus of cholecystokinin and the non-peptide agonist SR-146,131 share a common binding site in the human CCK1 receptor. Evidence for a crucial role of Met-121 in the activation process. *J Biol Chem* 277:7546-7555.

Feoktistov I, Ryzhov S, Goldstein AE and Biaggioni I (2003) Mast cell-mediated stimulation of angiogenesis: cooperative interaction between A_{2B} and A_3 adenosine receptors. *Circ Res* 92:485-492.

Kenakin T (2003) *A Pharmacology Primer*. Elsevier Academic Press, California and London.

Kenakin T (2005) New concepts in drug discovery: collateral efficacy and permissive antagonism. *Nat Rev Drug Discov* 4:919-27.

Kim SK, Gao ZG, Van Rompaey P, Gross AS, Chen A, Van Calenbergh S and Jacobson KA (2003) Modeling the adenosine receptors: comparison of the binding domains of A_{2A} agonists and antagonists. *J Med Chem* 46:4847-4859.

Koski G, Streaty RA and Klee WA (1982) Modulation of sodium-sensitive GTPase by partial opiate agonists. An explanation for the dual requirement for Na^+ and GTP in inhibitory regulation of adenylate cyclase. *J Biol Chem* 257:14035-14040.

Leff P (1995) The two-state model of receptor activation. *Trends Pharmacol Sci* 16:89-97.

Lefkowitz RJ, Cotecchia S, Samama P and Costa T (1993) Constitutive activity of receptors coupled to guanine nucleotide regulatory proteins. *Trends Pharmacol Sci* 14:303-307.

Li Q, Ye K, Blad CC, den Dulk H, Brouwer J, IJzerman AP and Beukers MW (2007) ZM241385, DPCPX, MRS1706 are inverse agonists with different relative intrinsic efficacies on constitutively active mutants of the human adenosine A_{2B} receptor. *J Pharmacol Exp Ther* 320:637-645.

Milligan G (2003) Constitutive activity and inverse agonists of G protein-coupled receptors: a current perspective. *Mol Pharmacol* 64:1271-1276.

Milligan G and Bond RA (1997) Inverse agonism and the regulation of receptor number. *Trends Pharmacol Sci* 18:468-474.

Morello JP, Salahpour A, Laperriere A, Bernier V, Arthus MF, Lonergan M, Petaja-Repo U, Angers S, Morin D, Bichet DG and Bouvier M (2000) Pharmacological chaperones rescue cell-surface expression and function of misfolded V_2 vasopressin receptor mutants. *J Clin Invest* 105:887-895.

Parnot C, Miserey-Lenkei S, Bardin S, Corvol P and Clauser E (2002) Lessons from constitutively active mutants of G protein-coupled receptors. *Trends Endocrinol Metab* 13:336-343.

Prioleau C, Visiers I, Ebersole BJ, Weinstein H and Sealfon SC (2002) Conserved helix 7 tyrosine acts as a multistate conformational switch in the $5HT_{2C}$ receptor. Identification of a novel locked-on phenotype and double revertant mutations. *J Biol Chem* 277:36577-36584.

Rao VR, Cohen GB and Oprian DD (1994) Rhodopsin mutation G90D and a molecular mechanism for congenital night blindness. *Nature* 367:639-642.

Schioth HB, Muceniece R, Mutulis F, Bouifrouri AA, Mutule I and Wikberg JE (1999) Further pharmacological characterization of the selective melanocortin 4 receptor antagonist HS014: comparison with SHU9119. *Neuropeptides* 33:191-196.

Seifert R and Wenzel-Seifert K (2002) Constitutive activity of G-protein-coupled receptors: cause of disease and common property of wild-type receptors. *Naunyn Schmiedebergs Arch Pharmacol* 366:381-416.

Shenker A, Laue L, Kosugi S, Merendino JJ, Jr., Minegishi T and Cutler GB, Jr. (1993) A constitutively activating mutation of the luteinizing hormone receptor in familial male precocious puberty. *Nature* 365:652-654.

Wu SV, Yang M, Avedian D, Birnbaumer M and Walsh JH (1999) Single amino acid substitution of serine82 to asparagine in first intracellular loop of human cholecystokinin (CCK)-B receptor confers full cyclic AMP responses to CCK and gastrin. *Mol Pharmacol* 55:795-803.

Ye K, Lameijer EW, Beukers MW and IJzerman AP (2006) A two-entropies analysis to identify functional positions in the transmembrane region of class A G protein-coupled receptors. *Proteins* 63:1018-1030.

Chapter

hedding light on the acti ation of the human adenosine A_{2B} receptor with site-directed mutagenesis

ummar

The human adenosine A_{2B} receptor belongs to class A GPCRs and is involved in important physiological functions. In our previous study, we applied random mutagenesis followed by functional screening to identify gain-of-function and/or constitutively active mutant receptors. To our surprise, none of the identified mutant receptors contained mutations in the conserved NPxxY motif or, in an extended version, the NxxxNPxxY motif. In this project we applied site-directed mutagenesis to investigate the role of the NxxxNPxxY motif and the potential salt bridge between TM1 (E14) and TM7 (H280) in receptor activation. In total 12 mutant receptors were constructed and tested with 5 agonists: NECA, CPA, CGS21680, IBMECA and LUF5833. The results confirmed our previous random mutagenesis experiments because none of the site-directed mutants displayed either gain-of-function or constitutive activity. With the exception of the N294I (increased expression levels), N286A (decreased expression levels) and H280E (abolished expression) mutant receptors, all constructed mutants were expressed at levels comparable to the wild-type receptor. Except for N294I which behaved similar to the wild-type receptor, the agonist potency and/or efficacy for the other mutant receptors were reduced to varying degrees. Despite normal expression levels, mutants E14H, I61A, I61D, I61K and Y290F did not respond to any of the tested agonists. Apparently, both the potential salt bridge and the NxxxNPxxY motif play an important role in the process of receptor activation. Interestingly, analysis of two homology models of the adenosine A_{2B} receptor based on the crystal structures of the human β_2 adrenergic receptor and bovine rhodopsin, respectively, revealed subtle differences among these receptors. These differences may explain why the role of the NxxxNPxxY motif differs among Class A GPCRs.

ntroduction

The adenosine receptor is a member of the rhodopsin-like family of G protein-coupled receptors (GPCRs). In human, there are 4 subtypes of adenosine receptors: A_1, A_{2A}, A_{2B}, and A_3. Among them, the A_{2B} subtype is implied in atherosclerosis of smooth muscle cells, in mast cell activation in asthma, resulting in bronchoconstriction, in intestinal (dys)function, and in dilation of certain vascular beds (Feoktistov et al., 1998; Ralevic and Burnstock, 1998; Holgate, 2005; Yaar et al., 2005).

Despite extensive efforts, structural information on GPCRs is still scarce. To date, two crystal structures are available, that of the inactive state of bovine rhodopsin (Palczewski et al., 2000) and, very recently, of the human β_2 adrenergic receptor (Cherezov et al., 2007; Rasmussen et al., 2007; Rosenbaum et al., 2007). Until now the structure of bovine rhodopsin has been used as a template to build homology models for GPCRs (Bissantz et al., 2003; Fano et al., 2006; Hobrath and Wang, 2006; Nowak et al., 2006). The availability of the β_2-adrenergic receptor structure enabled us to build and compare two homology models of the human adenosine A_{2B} receptors.

Rhodopsin-like GPCRs (class A GPCRs) share a large number of conserved sequence patterns. For example, the most conserved residues in each transmembrane (TM) helix are:

N1.50, D2.50, R3.50, W4.50, P5.50, P6.50, and P7.50, following the numbering scheme introduced by Ballesteros and Weinstein (1995). These patterns are easily identified and used for multiple sequence alignment of rhodopsin-like GPCRs to allow sequence comparison and model building (Mirzadegan et al., 2003).

Highly conserved patterns imply an important structural and/or functional role. Several conserved patterns have been identified among class A GPCRs: a disulfide bridge formed by the two Cys residues located in extracellular loop 2 (IL2) and at the beginning of TM3 (Klco et al., 2005); salt bridges between TM3 and TM6 or TM1 and TM7 (Kim et al., 1997; Ballesteros et al., 2001); the DRY/ERY motif at the end of TM3 (Alewijnse et al., 2000); CWxP in TM 6 (Shi et al., 2002; Ruprecht et al., 2004); and an extended motif consisting of the NPxxY motif in TM 7 with the F in TM8 5 or 6 amino acids downstream of the NPxxY motif (Govaerts et al., 2001; Urizar et al., 2005; Mirzadegan, et al., 2003) This NPxxY motif is highly conserved and is part of the NxxxNPxxY motif.

In our previous study (Beukers et al., 2004a), we applied random mutagenesis followed by functional screening to identify gain-of-function and/or constitutive active receptors. To our surprise, none of the identified mutant receptors contained mutations either in the conserved NPxxY motif or in the NxxxNPxxY motif. The random mutagenesis method can be limited in that not all potential amino acid substitutions are obtained. This is because chances are small that a simultaneous substitution of more than one nucleotide occurs within a single codon. In the present study we therefore investigated the activation of the human adenosine A_{2B} receptor through site-directed rather than random mutagenesis, while still using our robust and straightforward yeast screening assay.

The site-directed mutations were made based on the following criteria 1) sequence comparison among adenosine receptors and receptors with known structures 2) general GPCR receptor activation domains and 3) comparison of receptor homology models built on two different templates.

aterial and methods

east plasmid and A_{2B} receptor antibody

A genetically modified yeast *Saccharomyces cerevisiae* strain with the following genotype: *MAT*a*his3 leu2 trp1 ura3 can1 gpa1*Δ::Gα_{i3} *far1*Δ::ura3 sst2Δ::ura3 Fus1::FUS1-HIS3 LEU2::FUS1-lacZ ste2Δ::G418^R and expressing vector pDT-PGK was a gift from Dr. S. J. Dowell. Adenosine A_{2B} receptor mutants were constructed through site-directed mutagenesis. The A_{2B} receptor antibody was kindly provided by Dr. I. Feoktistov.

ite-directed mutagenesis

Site-directed mutation was introduced by PCR with mutation-inducing primers: 5'- TGAC AACTGACTGGGCATGTGACA3' for the N282Q mutant, 5'- TGACAACTGATCTGG CATGTGA3' for N282R mutant, 5'-AGACAATGGGAGCGACAACTGAAT3' for N286A mutant, 5'-AGACAATGGGCTGGACAACTGAAT3' for N286Q mutant, 5'- AGACAA TGGGTCTGACAACTGAAT3' for N286R mutant, 5'- CCGGTAAGCAAAGACAATG GG3' for Y290F mutant, 5' CGGTAAGCATTGACAATGGGA3' for Y290N mutant and 5'-GAAGTCTCGGATCCGGTAAGC3' for N294I mutant; 5'-CAT*GGTCTCC*GCTC TTCGCCGCCCCCTTTGCCATC3' for I61A; 5'-CAT*GGTCTCC*GCTCTTCGCCGAC

CCCTTTGCCATC3’ for I61D; 5’-CAT*GGTCTCC*GCTCTTCGCCAAACCCTTTGCC ATC3’ I61K; 5’CAT*GAATTC*ATGCTGCTGGAGACACAGGACGCGCTGTACGTGGC GCTGCACCTGGTCATCG 3’ for E14H and 5'-CAT*GGTCTCC*TTCTTCTGTCAGAG GCCAATTCAGTTG3' for H280E.

Restriction sites in the primers are indicated in italics. Mutant genes were inserted into pDT-PGK vector at EcoRI/HindIII sites and were confirmed by DNA sequencing. The plasmids containing any of the above mentioned mutant genes were transferred into yeast *Saccharomyces cerevisiae* and selected on plates with minimal media without uracil, which enabled us to isolate the transformants containing the mutation.

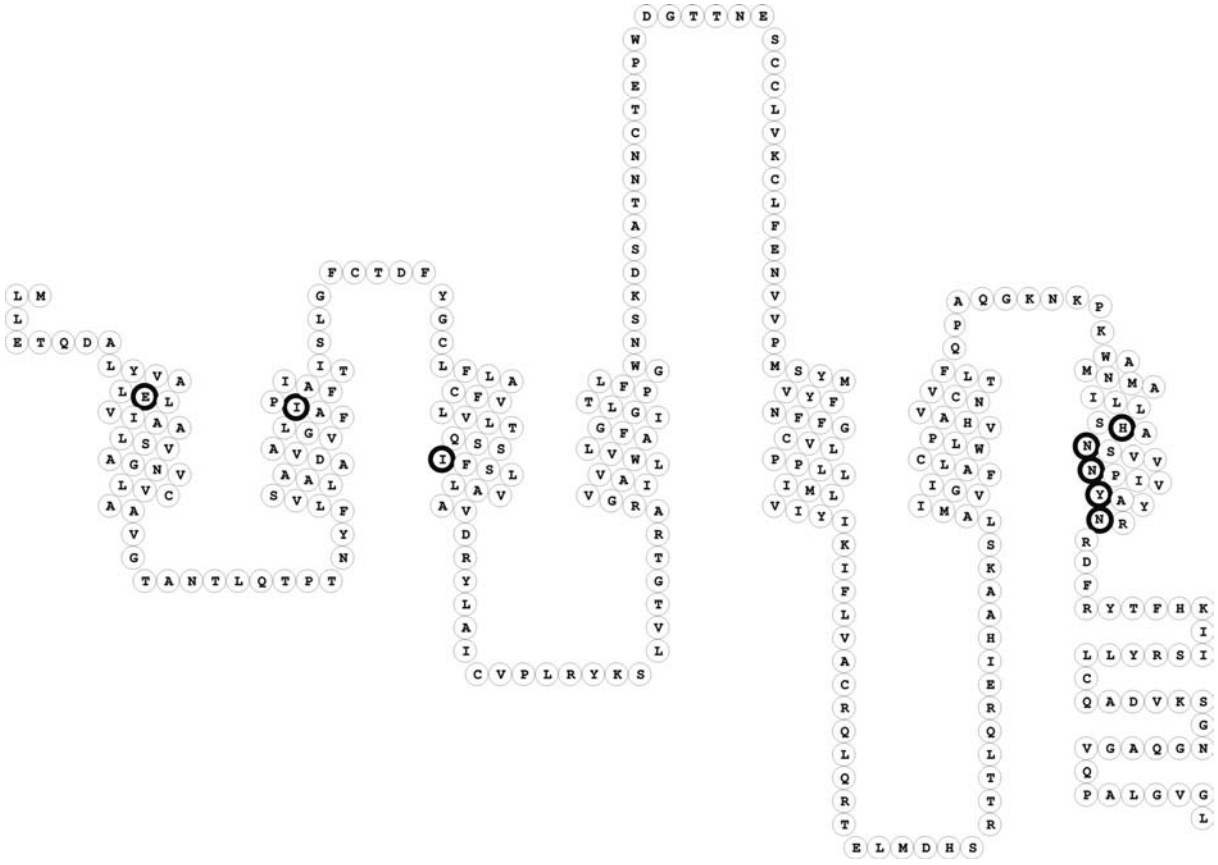


Figure 1. Snake plot of the human adenosine A_{2B} receptor with studied positions E14 (1.39), I61 (2.58), I93 (3.40), N282 (7.45), N286 (7.49), Y290 (7.53) and N294 (7.57) in bold circles. E14 (1.39), I61 (2.58) and I93 (3.40) are located in the first, second, and third helices respectively. N282 (7.45), N286 (7.49), Y290 (7.53) and N294 (7.57) are located in helix seven.

east cells immunoblotting

Transformed yeast cells were cultured overnight at 30 C and around $4*10^5$ yeast cells were harvested in mid-exponential phase. These cells were broken by vigorous vortexing with glass beads 2 times of 1 min after washing with water and 20% TCA (20% w/v trichloro acetic acid). Broken yeast cells were collected and denatured (3 - 5 min, 100 C), separated on SDS-PAGE and blotted on Hybond-ECL membrane (GE Healthcare). Hybond-ECL membrane was blocked with PBS containing 5% milk powder for 30 min, and then incubated

with 1: 1,125 diluted A_{2B} receptor antibody for 1 hr. After thorough removal of unbound antibody by rinsing the membrane with PBST (0.05% Tween-20, PBS PH 7.6), the membrane was incubated with 1: 2,000 diluted HRP-conjugated anti-rabbit IgG (Jackson Immuno Reseach) for 1 hr. Hybond-ECL membrane was washed again and the specific signal of A_{2B} receptors was probed according to the ECL Western blotting analysis system (GE Healthcare).

east screening assay

Yeast screening assays were performed as described before (Beukers et al., 2004a). In brief, yeast cells from an overnight culture were diluted to around 400,000 cells/ml (OD_{600} 0.02), 50 μl was seeded into each well of a 96-wells plate. Receptor-independent yeast growth was suppressed through the addition of 7 mM 3-AT. After incubation at 30 C for 35 hours the yeast growth revealed by optical density (OD) was recorded. Each experiment was repeated at least 3 times. Prism software (GraphPad Software Inc., San Diego, CA) was used to calculate the potency and efficacy of the compounds.

A_{2B} model

All the primary sequences were obtained from NCBI. The crystal structure of bovine rhodopsin was obtained from the Protein Data Bank (http://www.rcsb.org/pdb) (Berman HM, 2000) and the primary sequence was extracted from the crystal structure. The primary sequence of the human β_2 adrenergic receptor was extracted from the crystal structure. The primary sequences of the human adenosine A_1 (NP 001041695.1), A_{2A} (NP 000666.2), A_{2B} (NP 000667.1), A_3 (NP 065734.5) receptors were obtained from NCBI. Sequence alignment of human adenosine A_1, A_{2A}, A_{2B}, A_3, human β_2 adrenergic receptor and bovine rhodopsin was performed using CLUSTALW (Chenna et al., 2003). The following helical segments of rhodopsin-like GPCRs were properly aligned: Asn (1.50); Asp (2.50); Asp/Glu (3.49)-Arg (3.50)-Tyr (3.51); Trp (4.50); Tyr (5.50); Tyr (6.48)-Leu (6.49)-Pro (6.50); and Asn(7.49)-Pro (7.50)-(Xaa)2-Tyr(7.53). We applied the Ballosteros numbering scheme (Ballesteros and Weinstein, 1995), labeling the most conserved position in each helix with 50 and numbering the remaining residues according to their distance and direction with respect to the conserved residues.

The structural homology models were created using InsightII (San Diego, CA, USA).

oc ing

Docking simulations were performed with AutoDock (http://autodock.scripps.edu/) Grid maps of 20x20x20 angstrom representing the protein were calculated with AutoGrid. Such maps were centered on the oxygen atom of the β-hydroxy group of residue T89. Docking simulations were carried out using the Lamarckian genetic algorithm, with an initial population of 100 individuals, a maximum number of 10,000,000 energy evaluations and a maximum number of 50,000 generations (http://autodock.scripps.edu/faqs-help/tutorial/using-autodock-withautodocktools/UsingAutoDockWithADTv2e.pdf). Resulting orientations lying within 1.5 angstrom in the RMSD were clustered together. Finally the configuration with the most favorable free energy of binding was further optimized by 1500 energy minimization steps with InsightII.

Results

ultiple se uence alignment

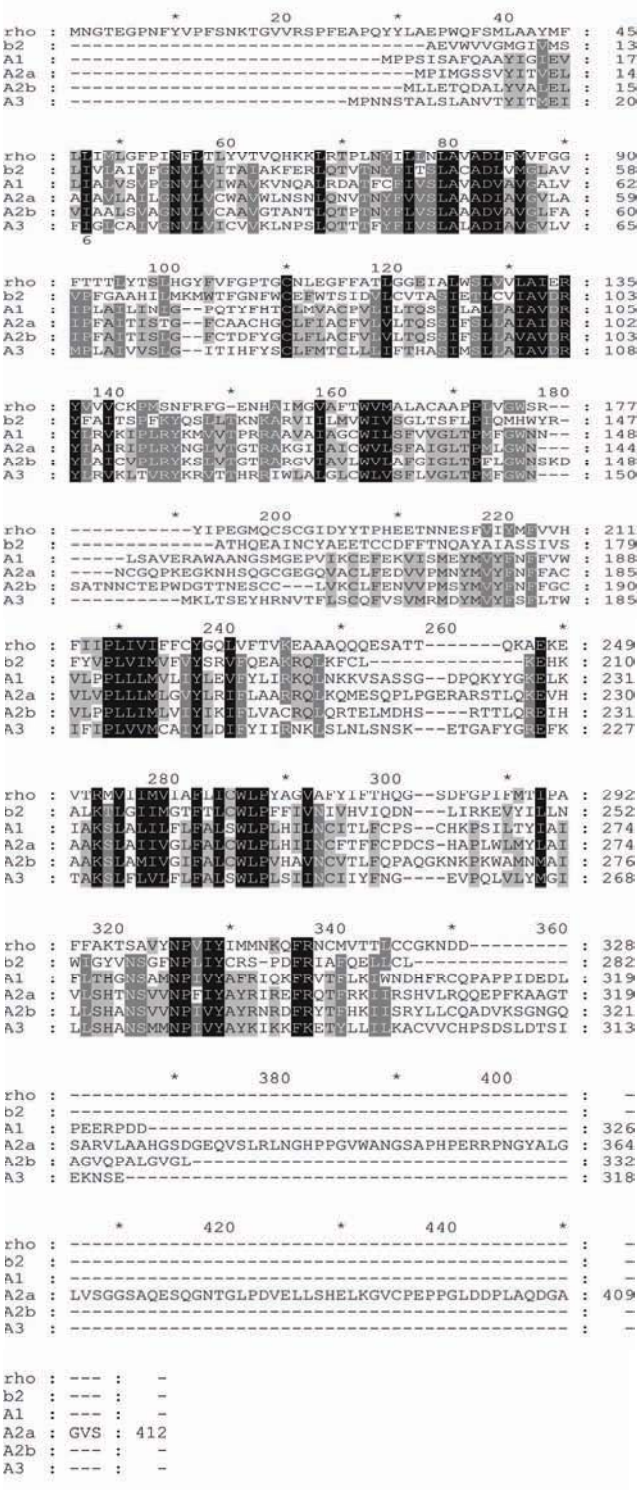


Figure 2. Multiple amino acid sequence alignment of the human adenosine A_1, A_{2A}, A_{2B}, A_3, human β_2 adrenergic receptors and bovine rhodopsin. Conserved residues are marked in black. Similar residues are marked in grey.

CLUSTALW was applied to create a sequence alignment of the human adenosine A_1, A_{2A}, A_{2B}, A_3, human β_2 adrenergic receptor and bovine rhodopsin. In the alignment, the most conserved residues were properly aligned as shown in figure 2.

Overall, the sequences of the human adenosine A_{2B} and bovine rhodopsin are 12% identical, whereas the A_{2B} and β_2 adrenergic receptor are 17% identical. The sequence identity between the A_{2B} receptor and other adenosine subfamily receptors is greater than 25%; among these the A_{2A} receptor has the highest identity 41% (Table 1).

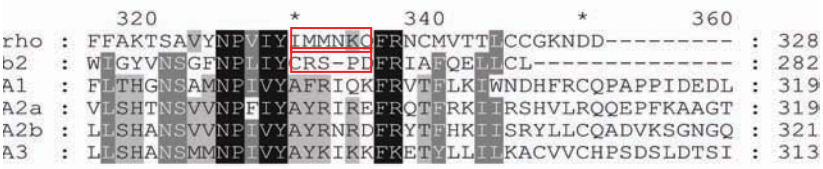
Table 1. Identity among bovine rhodopsin, β_2 adrenergic receptor and the 4 subtypes of human adenosine receptors.

Identity (%)	rhodopsin	β_2	A_1	A_{2A}	A_{2B}
β_2	11.0				
A_1	9.3	15.7			
A_{2A}	11.9	17.9	32.9		
A_{2B}	11.9	17.4	31.1	40.8	
A_3	9.5	15.5	32.2	26.5	25.6

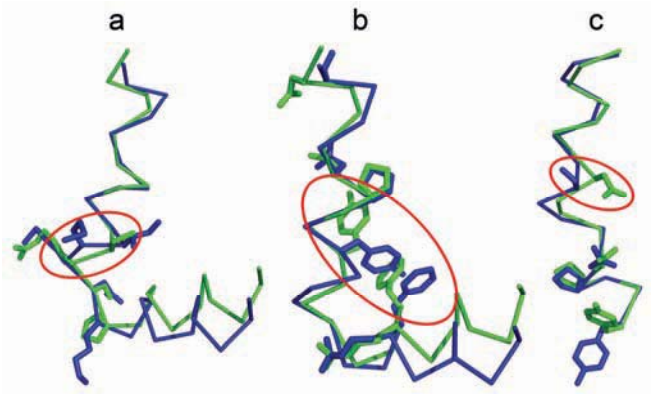
Adenosine A_{2B} receptor model

As shown in figure 3A, the loop that connects helix 7 and 8 is different between the two templates of bovine rhodopsin and the β_2-adrenergic receptor. There are six residues between the conserved NPxxY motifs and F in bovine rhodopsin while there are only five in the β_2 adrenergic receptor (Figure 3A).

Starting with the 3-D structures of bovine rhodopsin (Berman et al., 2000) and the β_2-adrenergic receptor (Cherezov et al., 2007) as templates, Insight II (San Diego, CA, USA) was applied to build homology models for the 3-D structure of the human adenosine A_{2B} receptor. There are several differences between the A_{2B} models based on these two templates. Firstly, a few differences were found in helix 7 and the link between helix 7 and helix 8 (Figure 3B). At the end of helix 7 in the β_2 adrenergic receptor model, S329 and P330 together lead to a sharp turn. These residues are absent in the A_{2B} receptor and as a result such a sharp turn is absent and instead a set of 3 amino acids constitutes a bigger turn (Figure 3Ba). Thus we introduced a gap in the β_2 adrenergic receptor-based A_{2B} receptor model to compensate for this structural difference. Residue Y(Y7.53) in the NPxxY motif forms a pi-pi stacking interaction with residue F in helix 8 in both models, but different rotamers are used by these two interacting aromatic residues (Figure 3Bb). The first N (N7.45) in the NxxxNPxxY motif points to the core of the receptor according to the bovine rhodopsin-based model while the same residue is pointing outwards according to the β_2 adrenergic receptor-based model (Figure 3Bc). Secondly, according to the β_2-adrenergic receptor model, the area of helix 1 close to the extracellular side of the receptor is further away from the centre of the receptor compared to bovine rhodopsin (Cherezov et al., 2007), shifting E14 to aplane more distant to residue H280. As a consequence, the formation of a salt bridge between these two residues which seems to occur according to the homology model based on the rhodopsin structure (Figure 3Cd) would no longer be feasible in the β_2 adrenergic receptor-based model (Figure 3Ce). Thirdly, the length of the helices is slightly different: helix 4 (119-143) is 3 amino acids longer than that according to the bovine rhodopsin model and helix 6 is 4 amino acids shorter (228-259).



A



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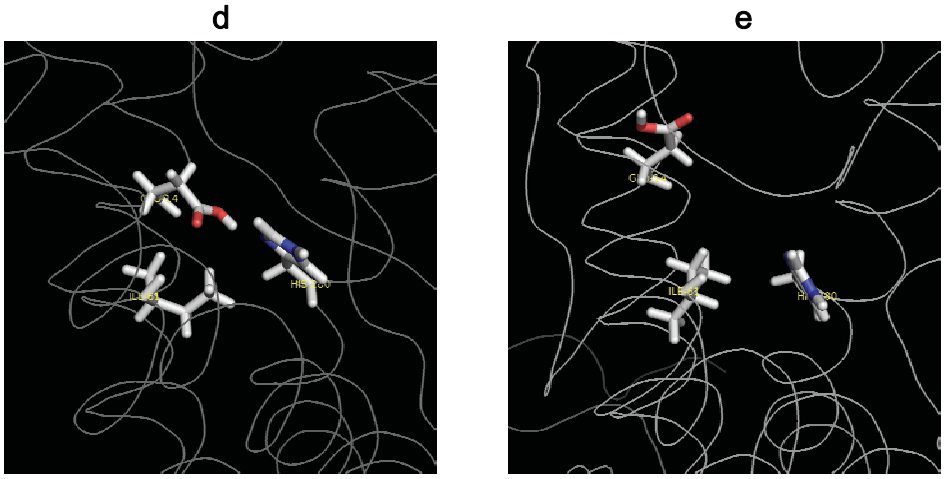

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Figure 3. A) Multiple sequence alignment of helix 7 and 8 for the human adenosine A_1, A_{2A}, A_{2B}, A_3, human β_2 adrenergic receptor and bovine rhodopsin. Conserved residues marked in black. In bovine rhodopsin six residues separate F from the conserved NPxxY motif, in the β_2 adrenergic receptor five residues do so, see red box. B) Structural overlay of helix 7 and helix 8 of bovine rhodopsin (blue) and the β_2 adrenergic receptor (green). a) the β_2 adrenergic receptor uses one residue less to finish the turn between helix 7 and helix 8 compared to bovine rhodopsin; b) The pi-pi stack is conserved in both bovine rhodopsin and the β_2 adrenergic receptor but different rotamers are adapted; c) difference in position 7.44 between bovine rhodopsin and β_2 adrenergic receptor. C) different interactions between E14, H280 and I61 in A_{2B} homology models based on bovine rhodopsin (d) and β_2 adrenergic receptor (e). d) In the model based on the bovine rhodopsin template, E14 and H280 are close enough to form a salt bridge, I61 is the vicinity. e) In the model based on the β_2 adrenergic receptor template, E14 and H280 are not close enough to form a salt bridge.

Role of 282 286 290 and 294 in ligand binding

The software package Autodock was used to investigate the interaction between the prototypic adenosine receptor agonist NECA and the NxxxNPxxY motif of the adenosine A_{2B} receptor model built based on the bovine rhodopsin crystal structure (1GZM) (see Figure 4). The residue in closest proximity to NECA is N282 in the neighbourhood of the ribose moiety of NECA. However, this N282 (7.45) is rather conserved among all class A GPCRs (60.1% N, 18% S, 9% H). Thus N282 is not expected to contribute to ligand-specific interactions. The other mutated residues of this motif, N286, Y290 and N294 are far away from NECA and unlikely to be directly involved in ligand binding (Figure 4).

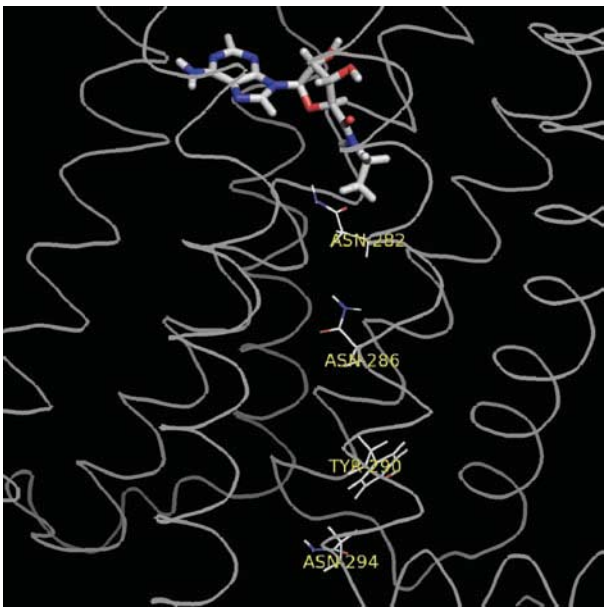

Figure 4. Docking of NECA in a ribbon representation of the 3-D human adenosine A_{2B} receptor model based on the bovine rhodopsin template with N282, N286, Y290 and N294 highlighted.

Role of xxx Pxx motif in receptor acti ation

The NxxxNPxxY motif in TM7 has been reported to be involved in the agonist-induced and constitutive activation of many GPCRs (Prioleau et al., 2002; Govaerts et al., 2001; Urizar et al., 2005). As mentioned before, our random mutagenesis screen did not reveal any mutations in this motif (Beukers et al., 2004a). To investigate whether this motif is important for receptor activation of the adenosine A_{2B} receptor, we created the following mutants: N282Q, N282R, N286A, N286Q, N286R, Y290F, and Y290N. Activation of these mutants by 5 agonists, NECA, CPA, CGS21680, IBMECA and LUF5833, was determined. Among them, NECA is a non-selective agonist for adenosine receptors (de Zwart et al., 1998), whereas CPA, CGS21680 and IBMECA are selective agonists for adenosine A_1, A_{2A} and A_3 receptors, respectively (Klotz et al., 1998). LUF5833 is a non-selective, non-ribose agonist with high affinity for the adenosine A_{2B} receptor, although it had highest affinity for the adenosine A_1 receptor (Beukers et al., 2004b). These agonists fully elicit the growth of yeast cells transfected with the wild-type human adenosine A_{2B} receptor, with an EC_{50} value of 104 nM, 3 µM, 20 µM, 6 µM and 4 nM, for NECA, CPA, CGS21680, IBMECA and LUF5833, respectively (Figure 5). The potency of the agonists for the wild-type and mutant adenosine A_{2B} receptors is presented in Table 2, their efficacy in Table 3.

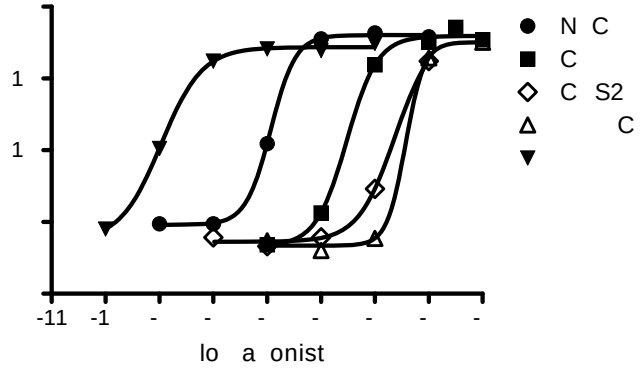

Figure 5. Concentration-effect curves for the five agonists: NECA, CPA, CGS21680, IBMECA and LUF5833 on the wild-type receptor.

None of the mutants showed any spontaneous activity. Replacement of N282 with other hydrophilic residues, Q and R, led to impaired receptor activation. The EC_{50} values of the ribose agonist NECA increased 22-fold and 12-fold for the N282Q and N282R mutant receptors, respectively. Slightly less pronounced effects were seen with the other ribose agonists CPA, CGS21680 and IBMECA; interestingly the potency of the non-ribose agonist LUF5833 seemed least affected by N282Q.

Table 2. EC_{50} values of the ribose agonists NECA, CPA, CGS21680 and IBMECA and the non-ribose agonist LUF5833 determined on the wild-type and on mutant adenosine A_{2B} receptors. The EC_{50} values on the wild-type receptor as shown in the table were used to calculate the fold difference of the mutant receptor versus the wild-type receptor.

Fold to WT	NECA	CPA	CGS21680	IBMECA	LUF5833
WT	1	1	1	1	1
N282Q	22	21	26	18	3
N282R	12	9	5	4	6
N286A	66	14	-	4	-
N286Q	81	27	-	-	-
N286R	3	2	1	1	2
Y290F	12	7	1	3	4
Y290N	-	-	-	-	-
N294I	1	1	1	1	1
E14H	-	-	-	-	-
H280E	-	-	-	-	-
E14H/H280E	-	-	-	-	-
I61A	-	nd	nd	nd	-
I61D	-	nd	nd	nd	-
I61K	-	nd	nd	nd	-
WT EC_{50}	104 20 nM	3 1 µM	20 (11-28) µM	6 (4-8) µM	4 3 nM

nd: not determined
-: no activation

The N286R receptor behaved similar to the wild-type (wt) receptor with respect to agonist activation. The greatest effect was seen with NECA which had an almost 3-fold reduced potency despite the fact that the receptor expression level of the N286R receptor was increased 20% compared to wild-type receptor (Figure 6). Apparently, the introduction of the positively charged arginine at N286 is well tolerated.

Table 3. The E_{max} values of the ribose agonists NECA, CPA, CGS21680 and IBMECA and the non-ribose agonist LUF5833 determined on the wild-type and on mutant adenosine A_{2B} receptors. The E_{max} values were calculated with respect to the E_{max} value for the respective compound on the wild-type receptor. The E_{max} values of the compounds for the wild-type receptor were set at 100%.

% to WT	NECA	CPA	CGS21680	IBMECA	LUF5833
WT	100	100	100	100	100
N282Q	96	89	67	94	85
N282R	106	111	81	112	93
N286A	99	39	9	18	14
N286Q	19	10	-	-	-
N286R	95	105	87	99	68
Y290F	97	95	74	70	62
Y290N	-	-	-	-	-
N294I	94	109	99	101	93
E14H	-	-	-	-	-
H280E	-	-	-	-	-
E14H/H280E	-	-	-	-	-
I61A	-	nd	nd	nd	-
I61D	-	nd	nd	nd	-
I61K	-	nd	nd	nd	-

nd: not determined
-: no activation

Instead, introduction of an alanine or glutamine residue at position 286 (N286A and N286Q) drastically reduced the ability of the agonists to activate the receptor. For example, the potency of NECA was decreased 81-fold on the N286Q and 66-fold on the N286A mutant.

Moreover, although NECA was still able to fully activate the N286A receptor it was no longer able to fully activate the N286Q mutant. CGS21680, CPA, IBMECA and LUF5833 did not activate these mutant receptors to any considerable extent. The responsiveness of the N286A mutant receptor could be at least partially ascribed to reduced receptor expression levels. The N286Q mutation on the other hand rendered the receptor less responsive without affecting receptor expression levels (Figure 6).

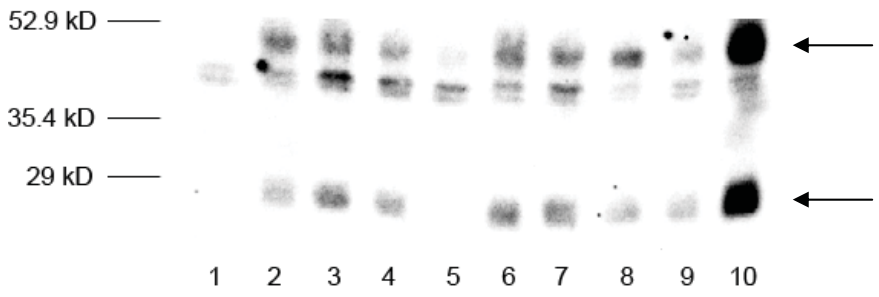

Figure 6. Western blot analysis of A_{2B} receptors expressed in yeast cells. Lane 1, yeasts carrying empty vector; lane 2 yeast cells expressing wild-type receptor; lane 3-10, mutants N282Q, N282R, N286A, N286Q, N286R, Y290F, Y290N and N294I. Two bands with an apparent molecular mass around 29 kD and bigger than 50 kD (indicated by arrows) were detected as A_{2B} receptor specific bands.

Mutation of tyrosine 290 to a hydrophilic asparagine residue (Y290N) revealed a locked off mutant, which could no longer be activated by any of the agonists tested. A more conserved replacement of tyrosine 290 with phenylalanine, Y290F, could be activated albeit with reduced potency of especially NECA (12-fold) and CPA (7-fold). Both Y290 mutants were expressed at levels similar to the wild-type receptor (Figure 6).

The N294I receptor behaved more or less similar to the wild-type receptor for all agonists tested, which suggested that the presence of a hydrophobic (I) or hydrophilic (N) amino acid at this position does not affect receptor activation. Interestingly, the expression level of the N294I mutant receptor is much higher than the wild-type receptor, which suggests that this amino acid is involved in receptor expression and or stabilization (Figure 6).

Mutations in the NxxxNPxxxY motif affect the efficacy as well as the potency of the ligands. On the wild-type receptor all agonists behave as full agonists (Figure 5). They acted as full agonists on the N294I mutant receptor as well, while partial agonism was revealed upon characterization of these compounds on several mutant receptors. While its potency was reduced most significantly, NECA exhibited the greatest efficacy among the agonists tested. Except for the N286Q mutant, all the responsive mutants were fully activated by NECA. Besides the N286Q mutant, CPA showed dramatically reduced maximal responses on the N286A mutant, while it was able to fully activate the other responsive mutants. The other ligands, CGS21680, IBMECA and LUF5833 could not activate the N286Q mutant, slightly activated the N286A mutant and partially activated the Y290F mutant. Based on these findings, the following rank order for intrinsic activity was obtained: NECA>CPA>IBMECA CGS21680 LUF5833.

Role of salt bridge in receptor acti ation

According to the models based on the crystal structure of bovine rhodopsin, adenosine receptors share a salt bridge between a glutamate, E14, in TM1 (1.39) and a histidine, H280, in TM7 (7.43). This potential salt bridge is a common feature in adenosine receptors but is not present in rhodopsin and the β_2 adrenergic receptor. Thus we investigated what would happen when these two amino acids are swapped. In the same model, I61 is located in close proximity to the potential salt bridge. If the potential salt bridge is critical for receptor activation, then one would expect an I61 mutation to affect receptor activation. Based on these assumptions, the following series of point mutants were constructed to investigate the potential salt bridge region, I61A, I61D, I61K, E14H, H280E and the double mutant E14H/ H280E.

Point mutant H280E resulted in a locked off receptor, which could not be activated by any of the tested agonists. Inversion of the potential salt bridge by swapping both residues yielded the double mutant E14H/H280E, which did not respond to any of the tested agonists either. Thus simply swapping these two residues could not restore the wild-type receptor phenotype. Characterization of both point mutations individually revealed that the H280E mutant was not properly expressed as was the E14H/H280E double mutant receptor, which finding might explain that both receptor mutants were locked off . The E14H mutant, on the other hand, showed enhanced expression levels, about 150% compared to the wild-type receptor (Figure 7). However this mutant was also not able to respond to any of the agonists, indicating that this residue is essential for agonist-induced receptor activation.

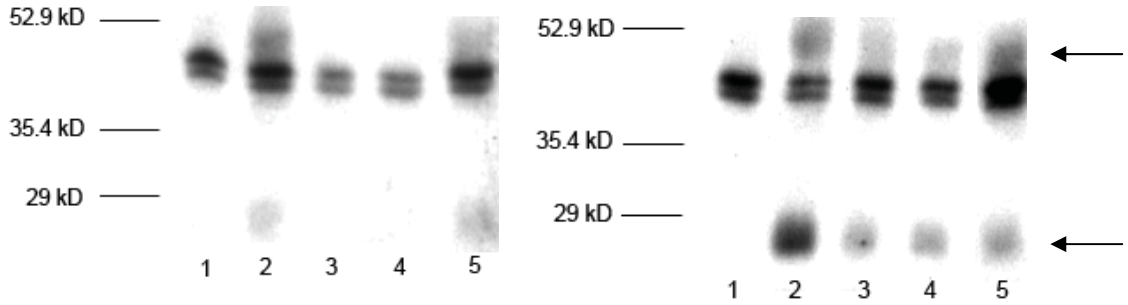

Figure 7. Western blot of mutant A_{2B} receptors. A) Lane 1 yeast carrying the empty vector, lane 2-4, yeast cells expressing mutant receptors E14H, H280E, E14H/H280E, lane 5 yeast cells expressing wild-type receptor. B) Lane 1 yeast carrying the empty vector; lane 2-4 yeast cells expressing mutant receptors I61A, I61D, I61K, lane 5 yeast cells expressing wild-type receptor.
Two bands with an apparent molecular mass around 29 kD and > 50 kD (indicated by arrows) were detected as A_{2B} receptor specific bands. None of these bands were observed for the H280E single mutant and E14H/H280E double mutant receptor.

Interestingly, the I61 mutants exhibited different expression levels: I61A showed a 50% enhanced level of expression relative to the wild-type receptor while replacement of isoleucine to the charged amino acids aspartate and lysine reduced the receptor expression to 59% and 45%, respectively (Figure 7). All these three mutants no longer responded to either the ribose agonist NECA or the non-ribose agonist LUF5833, indicating that the I61 mutation not only affects receptor expression levels but also receptor function.

iscussion

mportant motifs in GPCRs

motif

The NPxxY motif in TM7 is located close to the cytosolic interface, and is regarded as an important motif in class A GPCRs. Based on previous reports in literature this motif was expected to be of critical structural and/or functional importance and therefore subjected to mutagenesis. Mutations in this motif have been reported to affect receptor expression (Urizar et al., 2005; Lu et al., 2001), ligand binding (Gales et al., 2000; Lu et al., 2001), signaling (Urizar, 2005; Lu et al., 2001; Ferrand et al., 2006) and internalization (Kalatskaya et al., 2004) in the various GPCRs that were studied.

In this study, we constructed 7 point mutants with a mutation in the NxxxNPxxY motif, N282Q, N282R, N286A, N286Q, N286R, Y290F, and Y290N, determined their expression levels and their response to five different agonists of different selectivity and chemical nature.

Among these mutants, only the N286A mutant receptor was expressed at a greatly reduced level compared to the wild-type receptor, suggesting a role for N286 (7.49) in the human adenosine A_{2B} receptor in expression and/or receptor stabilization. There are reports on distinct effects of different receptors carrying a N7.49 mutation. Angelova et al. mutated the corresponding amino acid N597 in the rat LH receptor yielding the N597A, N597D and N597K mutants. Both receptor expression levels and receptor activation of all three mutants were compromised (Angelova et al., 2000). In the CCR5 receptor, the N293A mutant

exhibited poor expression as well (Dragic et al., 2000). These findings are in good agreement with our assumption that N of the NPxxY motif plays a critical role in receptor expression, in that mutating this residue results in lower receptor levels. However, there are also reports revealing that a mutation at this position did not significantly affect the receptor expression level, for example, the N391A cholecystokinin B receptor (CCKBR) (Gal s et al., 2000) and the N376D human serotonin 5-HT_{2B} receptor (Manivet et al., 2002). Evidently, the important amino acids determining receptor expression and/or receptor stabilization are receptor-dependent.

To provide information on the role of N of the NPxxY motif in receptor activation, 3 replacements were investigated. All three mutants behaved differently to each other. While N286R behaved rather similar to the wild-type receptor, both the N286A and N286Q mutant showed dramatically reduced activation. N286Q could not be activated by CGS21680, IBMECA or LUF5833 whereas the N286A mutant was only slightly activated which might suggest that these agonists have a smaller intrinsic efficacy than the other agonists. Substitution of this residue N7.49 in other GPCRs resulted in various phenotypes of mutant receptors. The N619R human LH receptor showed impaired efficacy towards the endogenous hormone hCG (Zhang et al., 2005). The equivalent mutation in the rat LH receptor, N597R, yielded a loss-of-function mutant while the N597Q mutant was constitutively active (Angelova et al., 2000). The N391A CCK-B receptor could not stimulate PLC and MAPK pathways in transiently transfected COS cells (Gal s et al., 2000). The N376D 5-HT_{2B} receptor had a decreased affinity for the endogenous ligand 5-HT and could not be fully activated with respect to IP_3 production (Manivet et al., 2002). In another report, the N308D 5-HT_4 receptor displayed increased, around 2-fold, constitutive activity compared to the wild-type receptor (Joubert et al., 2002). Mutation of N316D in the thyrotropin-releasing hormone receptor resulted in a 6-fold higher affinity for methyl-thyrotropin compared to the wild-type receptor without affecting the maximal activity. The N316A mutant thyrotropin-releasing receptor showed decreased maximum activity as well as reduced affinity for thyrotropin-releasing hormone (Perlman et al., 1997).

To provide information on Y in the NPxxY motif, Y290 was substituted with phenylalanine and asparagine. Despite normal expression levels, the Y290N mutant did not respond to the agonists tested. Y290F was responsive, but LUF5833, CGS21680 and IBMECA had a reduced efficacy and the potency of NECA and CPA was significantly impaired. Both the rhodopsin and the β_2 adrenergic receptor structure show that the Y and F in the conserved NPxxY$x_{(5,6)}$F motif form a pi-pi interaction. Several reports reveal the importance of this tyrosine residue in receptor activation, as receptor function is impaired by its mutation. For example, the Y297A CCR5 receptor was found to be functionally impaired in its ability to inhibit forskolin-stimulated cAMP formation in transiently transfected HEK293 cells (Aramori et al., 1997). Similarly, the rat Y305A neurokinin-1 receptor showed impaired signaling (B hm et al., 1997). A 160-fold decreased efficacy of acetylcholine was found at the Y418A M_1 muscarinic acetylcholine receptor (Lu et al., 2001). A possible explanation for this loss in signaling might be that substitution of tyrosine with alanine affected the coupling to the G protein as shown for the Y293A platelet-activating factor receptor mutant (Le Gouill et al., 1997).

Compared to the tyrosine to alanine substitution, more conserved substitutions appear to have less striking effects on the function of the receptor, as was found for the rat Y305F

neurokinin-1 receptor receptor (B hm et al., 1997), the Y305F bradykinin B_2 receptor (Kalatskaya et al., 2004) and the Y293F platelet-activating receptor (Le Gouill et al., 1997). However, in several cases, these conserved' substitutions of Y7.53 did significantly affect receptor function. For example, the Y282F adenosine A_3 receptor showed a significant decrease of maximal response and its expression level was 60% compared to the wild-type receptor (Chen et al., 2001). The Y325F mutant V_2 vasopressin receptor induced cAMP accumulation with reduced efficiency (Bouley et al., 2003). The Y7.53F 5-HT_{2C} receptor was able to bind 5-HT with high affinity but could not be activated by it (Prioleau et al., 2002). On the other hand, the Y7.53N mutant 5-HT_{2C} receptor exhibited very high constitutive activity which could be neither increased by agonists nor decreased by inverse agonists (Prioleau et al., 2002).

Since Y7.53 is located at the cytosolic interface, it is expected to affect signaling and internalization. However, this residue was also reported to affect ligand binding which might be explained either by a direct ligand interaction or indirect conformational changes. Both the Y299A cannabinoid CB2 receptor and the human Y304A SST5 receptor completely lost their ability of agonist binding (Feng and Song, 2001; Hukovic et al., 1998). In addition, the Y282F adenosine A_3 receptor had also a significantly decreased affinity for agonists, next to its reduced maximal response (Chen et al., 2001). Increases in agonist binding affinity, 10-fold, have also been reported, e.g., for the Y543H M_3 muscarinic acetylcholine receptor (Schmidt et al., 2003). Decreased receptor expression levels were also obtained for the Y7.53K, Y7.53D, and Y7.53E 5-HT_{2C} receptors.

Despite their normal expression levels, mutants N282Q and N282R significantly reduced the potency of all agonists tested whereas N282R also reduced the efficacy of IBMECA and LUF5833. This result is different from the N7.45 mutations in the LH receptor. The N593Q mutant was expressed at levels comparable to the wild-type receptor, but the responsiveness to hCG was lost while the N593R mutant showed slight constitutive activity and behaved similar to the wild-type receptor upon stimulation with hCG (Angelova et al., 2000). Mutation of N7.45 in the human AT_1 receptor, N294A, showed severely attenuated function although ligand binding was unaffected, while N294M behaved similar to the wild-type receptor (Hunyady et al., 1998; P rodin et al., 2002). In the human D_5 dopamine receptor, the N351D mutant resulted in an approximately 10-fold decrease in dopamine binding affinity (Cravchik and Gejman, 1999). In contrast, mutation of N410A in the M_1 muscarinic acetylcholine receptor caused a slightly increased affinity while the E_{max} of acetylcholine was decreased (Lu et al., 2001).

Taken together, these data suggest that the roles of the conserved residues N7.45, N7.49 and Y7.53 in the NxxxNPxxY motif are receptor-specific. An example is given below to compare the difference induced by the same substitutions in the rat LH receptor, the human 5-HT_{2C} receptor and the human adenosine A_{2B} receptor (Table 4).

Residue N294 (7.57) located 4 residues downstream of the NPxxY motif is characteristic for the adenosine A_{2B} receptor, because all other adenosine receptors share an isoleucine at this position. Evaluation of the point mutant N294I revealed that this residue is involved in receptor expression, i.e. the N294I mutant A_{2B} receptor was expressed at 3-fold higher levels than the wild-type receptor. The mutation did not affect the activation of the receptor, which suggests that this mutation stabilizes receptor integrity. To our knowledge this residue has not been subjected to mutation in other receptors.

Table 4. Effect of NxxxNPxxY mutations in the current study compared to literature data on the LH receptor (Angelova et al., 2000) and 5-HT_{2C} receptor (Prioleau et al., 2002).

Mutation	A_{2B} receptor	LH receptor	5-HT_{2C}
N7.45Q	Less sensitive to NECA	Dramatically reduced response to hCG	
N7.45R	Less sensitive to NECA	Constitutively active	
N7.49A	Reduced potency of NECA	Reduced maximal response to hCG	
N7.49R	Similar to wild-type, slightly reduced potency of NECA	Impaired efficacy to hCG	
N7.49Q	Loss of function	Constitutively active	
Y7.53F	No response to agonists tested		Loss of function
Y7.53N	Loss of function		Constitutively active

Salt ridge

Salt bridges have been suggested to form an intramolecular constraint to keep receptors in an inactive conformation or alternatively to be involved in ligand binding. For example, E113 in TM3 and K296 in TM7 form a salt bridge in rhodopsin and mutation of these two residues results in constitutive activation of the protein (Robinson, 1992). In the α_{1b} adrenergic receptor D125 and K331 are assumed to form a salt bridge. The single point mutants D125K and K331D gained constitutive activity and the D125K/K331D double mutant regained the wild-type phenotype suggesting that the salt bridge was recovered and emphasizing the strong interaction between D125 and K331 (Porter et al., 1996; Porter and Perez, 1999). However, similar experiments with the 5-HT_{2A} receptor (Kristiansen et al., 2000) demonstrated that mutation of aspartate in TM3 decreased the constitutive activity of the 5-HT_{2A} receptor, opposite to the effect obtained with the of α_{1b} adrenergic receptor.

The adenosine receptors share a glutamic acid in TM1 (1.39) and a histidine in TM7 (7.43) which are highly conserved among all subtypes and seem to be close enough to form a salt bridge. These two residues were reported to be involved in ligand binding. The E16A A_1 human adenosine receptor resulted in dramatically reduced affinity of the agonist CCPA (Barbhaiya et al., 1996). Mutation of H278 to L in bovine A_1 receptors almost abolished both agonist and antagonist binding (Olah et al., 1992). In the A_{2A} human adenosine receptor, two mutants E13Q and H278Y were investigated. Both displayed significantly reduced affinity for adenosine-derived agonists, and H278Y also had reduced affinity (20-fold) for the antagonist theophylline (Gao et al., 2000). In another study, substitution of H278 with alanine abolished the binding of radiolabeled CGS 21680 and XAC (Kim et al., 1995). The H272E mutant human adenosine A_3 receptor displayed a different profile of reduced affinity for most of the uncharged agonists and antagonists tested (Jacobson et al., 2001). Taken together, these data indicate that the presence of both E1.39 and H7.43 in adenosine receptors facilitates ligand binding, especially for agonists.

The presence of charged residues in the otherwise hydrophobic transmembrane region suggests that these residues play important roles. Mutation of ten out of the fourteen charged residues in the transmembrane region of the human prostacyclin receptor to alanine resulted in defective binding and/or activation, suggesting that these charged residues are indeed important in maintaining the binding pocket and ensuring normal activation (Stitham et al.,

2007). In our study, the two residues forming the potential salt bridge also affected receptor activation. H280E eliminated receptor activation, while the reason why the E14H mutant receptor is non-responsive to any of the agonists tested requires further investigation.

Residue I61 (2.58) was investigated in this study because it was predicted to be in the vicinity of the potential salt bridge formed by E1.39 and H7.42 according to the rhodopsin-based computer homology model. This residue is not conserved in class A GPCRs but is conserved among the A_1, A_{2A} and A_{2B} receptors, whereas the A_3 receptor contains a methionine at this position. Assuming that the salt bridge is crucial for agonist-induced activation one would expect that mutation of the nearby I61 residue is poorly tolerated. Indeed mutation of this residue to alanine, aspartate or lysine results in a receptor that can no longer respond to agonists although the receptor is well expressed.

Taken together, the potential salt bridge is apparently very sensitive to single amino acid substitutions in this area as displayed for the E1.39, I2.58 and H7.43 mutations. The conserved residues in the NxxxNPxxY motif also seem to be very sensitive to mutation considering the behavior of the point mutants and our previous random mutagenesis results that did not identify any gain-of-function mutant by mutations in this region (Beukers et al., 2004a).

omology models

Typically, homology models are based on the alignment of entire helical regions between rhodopsin and receptors with unknown structure. Loop areas are normally ignored or modeled based on databases of loop conformations. These models have been used to design experiments as well as to explain experimental data. We used the structure of bovine rhodopsin as a template to build a homology model of the human adenosine A_{2B} receptor to guide the selection of amino acids for site-directed mutagenesis. The recent disclosure of the 3-D structure of the human β_2 adrenergic receptor enabled us to make a homology model based on this template too.

A structural overlay was made to investigate the differences between the two models with respect to the regions of interest in this study. The following differences were identified, i) the angle between helix 7 and 8, ii) the number of residues to link helix 7 and 8, iii) the location and side chain orientation of residues in the NxxxNPxxY motif, and iv) the nature of the salt bridge between helix 1 and helix 7. These findings suggest that care should be taken to interpret experimental data based on a single model.

Cherezov already pointed to the shortcoming of homology models generated from a single structural template, as the structural divergence between two receptors would be quite difficult to predict accurately using only one receptor as a template (Cherezov et al., 2007). His conclusion was drawn from a similarity comparison between the β_2 adrenergic receptor crystal structure and homology models of the β_2 adrenergic receptor based on the structure of bovine rhodopsin and which were substantiated with biochemical data (Bissantz et al., 2003; Furse and Lybrand, 2003; Gouldson et al., 2004). Since the latter models used bovine rhodopsin as a template, they are more similar to the rhodopsin rather than the real β_2 adrenergic receptor structure.

Although all class A receptors share a similar backbone consisting of 7 transmembrane domains linked by extracellular and intracellular loops, each receptor has its own characteristics. Slight differences in the structure may result in significant differences in

intramolecular interactions. For example, in rhodopsin, E6.30 forms an ionic bond with R3.50 of the conserved D(E)RY motif (Palczewski et al., 2000). This interaction is postulated to be important for maintaining rhodopsin in the inactive state, but the charged groups of the two residues R3.50 and E6.30 are too far apart to form an ionic bond in the structure of the β_2 adrenergic receptor-T_4 lysozyme fusion protein (Cherezov et al., 2007). In the present study, mutations in the human adenosine A_{2B} receptor NxxxNPxxY motif were made equivalent to LH receptor and 5-HT_{2C} receptor mutations, however with strikingly different results. Apparently, the NxxxNPxxY network in the A_{2B} receptor functions in a different manner compared to the network in the LH and the 5-HT_{2C} receptors (Table 4). The potential salt bridge is also atypical with respect to other adenosine receptors since the E1.39H mutant is irresponsive to agonists whereas the H7.42E mutant is not expressed at all. These results suggest that the human adenosine A_{2B} receptor NxxxNPxxY network and the potential salt bridge are optimized for receptor function of this receptor subtype and every receptor may be slightly different in this respect.

Conclusion

Although GPCRs share several conserved motifs, the functional role of these motifs seem to differ among individual receptors. Site-directed mutagenesis of the NxxxNPxxY motif of the adenosine A_{2B} receptor indicated that this motif is involved in the receptor activation due to the fact that most mutations in this study led to impaired receptor activation. Similarly, the role of the characteristic adenosine receptor salt bridge between E1.39 and H7.42 differs among the various subtypes of adenosine receptors. Mutagenesis experiments on the adenosine A_{2B} receptor show that the H7.42E receptor is no longer expressed suggesting a role for this residue in receptor stability and/or expression of this adenosine receptor subtype. The recently revealed structure of the β_2-adrenergic receptor sheds light on these findings as slight structural changes have been identified between the crystal structures of this receptor and bovine rhodopsin which may result in significant differences in intramolecular interactions.

When homology receptor models are constructed, we must therefore bear in mind that each receptor has its own characteristics and that the use of a structurally unrelated receptor as a template will yield inaccurate receptor models. The challenge will be to identify the optimal receptor structure for each homology model.

c no led ements

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References

Alewijnse AE, Timmerman H, Jacobs EH, Smit MJ, Roovers E, Cotecchia S and Leurs R (2000) The effect of mutations in the DRY motif on the constitutive activity and structural instability of the histamine H_2 receptor. *Mol Pharmacol* 57:890-898.

Angelova K, Narayan P, Simon JP and Puett D (2000) Functional role of transmembrane helix 7 in the activation of the heptahelical lutropin receptor. *Mol Endocrinol* 14:459-471.

Aramori I, Ferguson SS, Bieniasz PD, Zhang J, Cullen B and Cullen MG (1997) Molecular mechanism of desensitization of the chemokine receptor CCR-5: receptor signaling and internalization are dissociable from its role as an HIV-1 co-receptor. *Embo J* 16:4606-4616.

Ballesteros JA, Jensen AD, Liapakis G, Rasmussen SG, Shi L, Gether U and Javitch JA (2001) Activation of the $_2$-adrenergic receptor involves disruption of an ionic lock between the cytoplasmic ends of transmembrane segments 3 and 6. *J Biol Chem* 276:29171-29177.

Ballesteros JA and Weinstein H (1995) Integrated methods for the construction of three-dimensional models and computational probing of structure–function relations in G protein coupled receptors. *Methods Neurosci*, 25: 366–428.

Barbhaiya H, McClain R, IJzerman A and Rivkees SA (1996) Site-directed mutagenesis of the human A_1 adenosine receptor: influences of acidic and hydroxy residues in the first four transmembrane domains on ligand binding. *Mol Pharmacol* 50:1635-1642.

Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, Shindyalov IN, Bourne PE (2000) The Protein Data Bank. *Nucleic Acids Res* 28:235-242.

Beukers MW, van Oppenraaij J, van der Hoorn PP, Blad CC, den Dulk H, Brouwer J and IJzerman AP (2004a) Random mutagenesis of the human adenosine A_{2B} receptor followed by growth selection in yeast. Identification of constitutively active and gain of function mutations. *Mol Pharmacol* 65:702-710.

Beukers MW, Chang LC, von Frijtag Drabbe Künzel JK, Mulder-Krieger T, Spanjersberg RF, Brussee J, IJzerman AP. New, non-adenosine, high-potency agonists for the human adenosine A_{2B} receptor with an improved selectivity profile compared to the reference agonist N-ethylcarboxamidoadenosine. *J Med Chem* (2004b) 47:3707-3709.

Bissantz C, Bernard P, Hibert M and Rognan D (2003) Protein-based virtual screening of chemical databases. II. Are homology models of G-Protein Coupled Receptors suitable targets? *Proteins* 50:5-25.

B hm SK, Khitin LM, Smeekens SP, Grady EF, Payan DG and Bunnett NW (1997) Identification of potential tyrosine-containing endocytic motifs in the carboxyl-tail and seventh transmembrane domain of the neurokinin 1 receptor. *J Biol Chem* 272:2363-2372.

Bouley R, Sun TX, Chenard M, McLaughlin M, McKee M, Lin HY, Brown D and Ausiello DA (2003) Functional role of the NPxxY motif in internalization of the type 2 vasopressin receptor in LLC-PK1 cells. *Am J Physiol Cell Physiol* 285:C750-762.

Chen A, Gao ZG, Barak D, Liang BT and Jacobson KA (2001) Constitutive activation of A_3 adenosine receptors by site-directed mutagenesis. *Biochem Biophys Res Commun* 284:596-601.

Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, Higgins DG, Thompson JD (2003) Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Res* 31:3497-3500.

Cherezov V, Rosenbaum DM, Hanson MA, Rasmussen SG, Thian FS, Kobilka TS, Choi HJ, Kuhn P, Weis WI, Kobilka BK and Stevens RC (2007) High-resolution crystal structure of an engineered human $_2$-adrenergic G protein-coupled receptor. *Science* 318:1258-1265.

Cravchik A and Gejman PV (1999) Functional analysis of the human D_5 dopamine receptor missense and nonsense variants: differences in dopamine binding affinities. *Pharmacogenetics* 9:199-206.

de Zwart M, Link R, von Frijtag Drabbe Künzel JK, Cristalli G, Jacobson KA, Townsend-Nicholson A, IJzerman AP (1998) A functional screening of adenosine analogues at the adenosine A_{2B} receptor: a search for potent agonists. *Nucleosides Nucleotides* 17:969-985.

Dragic T, Trkola A, Thompson DA, Cormier EG, Kajumo FA, Maxwell E, Lin SW, Ying W, Smith SO, Sakmar TP and Moore JP (2000) A binding pocket for a small molecule inhibitor of HIV-1 entry within the transmembrane helices of CCR5. *Proc Natl Acad Sci U S A* 97:5639-5644

Fano A, Ritchie DW and Carrieri A (2006) Modeling the structural basis of human CCR5 chemokine receptor function: from homology model building and molecular dynamics validation to agonist and antagonist docking. *J Chem Inf Model* 46:1223-1235

Feng W and Song ZH (2001) Functional roles of the tyrosine within the $NP(X)_{(n)}Y$ motif and the cysteines in the C-terminal juxtamembrane region of the CB_2 cannabinoid receptor. *FEBS Lett* 501:166-170

Feoktistov I, Polosa R, Holgate ST, Biaggioni I (1998) Adenosine A_{2B} receptors: a novel therapeutic target in asthma? *Trends Pharmacol Sci* 19:148-153.

Ferrand A, Vatinel S, Kowalski-Chauvel A, Bertrand C, Escrieut C, Fourmy D, Dufresne M and Seva C (2006) Mechanism for Src activation by the CCK2 receptor: Patho-physiological functions of this receptor in pancreas. *World J Gastroenterol* 12:4498-4503.

Furse KE and Lybrand TP (2003) Three-dimensional models for beta-adrenergic receptor complexes with agonists and antagonists. *J Med Chem* 46:4450-4462.

Gal s C, Kowalski-Chauvel A, Dufour MN, Seva C, Moroder L, Pradayrol L, Vaysse N, Fourmy D and Silvente-Poirot S (2000) Mutation of Asn-391 within the conserved NPXXY motif of the cholecystokinin B receptor abolishes G_q protein activation without affecting its association with the receptor. *J Biol Chem* 275:17321-17327.

Gao ZG, Jiang Q, Jacobson KA and IJzerman AP (2000) Site-directed mutagenesis studies of human A_{2A} adenosine receptors: involvement of glu(13) and his(278) in ligand binding and sodium modulation. *Biochem Pharmacol* 60:661-668.

Gouldson PR, Kidley NJ, Bywater RP, Psaroudakis G, Brooks HD, Diaz C, Shire D and Reynolds CA (2004) Toward the active conformations of rhodopsin and the $_2$-adrenergic receptor. *Proteins* 56:67-84.

Govaerts C, Lefort A, Costagliola S, Wodak SJ, Ballesteros JA, Van Sande J, Pardo L and Vassart G (2001) A conserved Asn in transmembrane helix 7 is an on/off switch in the activation of the thyrotropin receptor. *J Biol Chem* 276:22991-22999.

Hobrath JV and Wang S (2006) Computational elucidation of the structural basis of ligand binding to the dopamine 3 receptor through docking and homology modeling. *J Med Chem* 49:4470-4476.

Holgate ST (2005) The Quintiles Prize Lecture 2004. The identification of the adenosine A_{2B} receptor as a novel therapeutic target in asthma. *Br J Pharmacol* 145:1009-1015.

Hukovic N, Panetta R, Kumar U, Rocheville M and Patel YC (1998) The cytoplasmic tail of the human somatostatin receptor type 5 is crucial for interaction with adenylyl cyclase and in mediating desensitization and internalization. *J Biol Chem* 273:21416-21422.

Hunyady L, Ji H, Jagadeesh G, Zhang M, Gaborik Z, Mihalik B and Catt KJ (1998) Dependence of AT_1 angiotensin receptor function on adjacent asparagine residues in the seventh transmembrane helix. *Mol Pharmacol* 54:427-434.

Jacobson KA, Gao ZG, Chen A, Barak D, Kim SA, Lee K, Link A, Rompaey PV, van Calenbergh S and Liang BT (2001) Neoceptor concept based on molecular complementarity in GPCRs: a mutant adenosine A_3 receptor with selectively enhanced affinity for amine-modified nucleosides. *J Med Chem* 44:4125-4136.

Joubert L, Claeysen S, Sebben M, Bessis AS, Clark RD, Martin RS, Bockaert J and Dumuis A (2002) A 5-HT_4 receptor transmembrane network implicated in the activity of inverse agonists but not agonists. *J Biol Chem* 277:25502-25511.

Kalatskaya I, Schussler S, Blaukat A, Muller-Esterl W, Jochum M, Proud D and Faussner A (2004) Mutation of tyrosine in the conserved NPXXY sequence leads to constitutive phosphorylation and internalization, but not signaling, of the human B_2 bradykinin receptor. *J Biol Chem* 279:31268-31276.

Kim J, Wess J, van Rhee AM, Schoneberg T and Jacobson KA (1995) Site-directed mutagenesis identifies residues involved in ligand recognition in the human A_{2a} adenosine receptor. *J Biol Chem* 270:13987-13997.

Kim JM, Altenbach C, Thurmond RL, Khorana HG and Hubbell WL (1997) Structure and function in rhodopsin: rhodopsin mutants with a neutral amino acid at E134 have a partially activated conformation in the dark state. *Proc Natl Acad Sci U S A* 94:14273-14278.

Klco JM, Wiegand CB, Narzinski K and Baranski TJ (2005) Essential role for the second extracellular loop in C_{5a} receptor activation. *Nat Struct Mol Biol* 12:320-326.

Klotz KN, Hessling J, Hegler J, Owman C, Kull B, Fredholm BB, Lohse MJ (1998) Comparative pharmacology of human adenosine receptor subtypes - characterization of stably transfected receptors in CHO cells. *Naunyn Schmiedebergs Arch Pharmacol* 357:1-9

Kristiansen K, Kroeze WK, Willins DL, Gelber EI, Savage JE, Glennon RA and Roth BL (2000) A highly conserved aspartic acid (Asp-155) anchors the terminal amine moiety of tryptamines and is involved in membrane targeting of the 5-HT_{2A} serotonin receptor but does not participate in activation via a salt-bridge disruption mechanism. *J Pharmacol Exp Ther* 293:735-746.

Le Gouill C, Parent JL, Rola-Pleszczynski M and Stankova J (1997) Structural and functional requirements for agonist-induced internalization of the human platelet-activating factor receptor. *J Biol Chem* 272:21289-21295.

Lu ZL, Saldanha JW and Hulme EC (2001) Transmembrane domains 4 and 7 of the M_1 muscarinic acetylcholine receptor are critical for ligand binding and the receptor activation switch. *J Biol Chem* 276:34098-34104.

Manivet P, Schneider B, Smith JC, Choi DS, Maroteaux L, Kellermann O and Launay JM (2002) The serotonin binding site of human and murine 5-HT_{2B} receptors: molecular modeling and site-directed mutagenesis. *J Biol Chem* 277:17170-17178.

Mirzadegan T, Benko G, Filipek S and Palczewski K (2003) Sequence analyses of G-protein-coupled receptors: similarities to rhodopsin. *Biochemistry* 42:2759-2767.

Nowak M, Kolaczkowski M, Pawlowski M and Bojarski AJ (2006) Homology modeling of the serotonin 5-HT_{1A} receptor using automated docking of bioactive compounds with defined geometry. *J Med Chem* 49:205-214.

Olah ME, Ren H, Ostrowski J, Jacobson KA and Stiles GL (1992) Cloning, expression, and characterization of the unique bovine A_1 adenosine receptor. Studies on the ligand binding site by site-directed mutagenesis. *J Biol Chem* 267:10764-10770.

Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, Yamamoto M and Miyano M (2000) Crystal structure of rhodopsin: A G protein-coupled receptor. *Science* 289:739-745.

Perlman JH, Colson AO, Wang W, Bence K, Osman R and Gershengorn MC (1997) Interactions between conserved residues in transmembrane helices 1, 2, and 7 of the thyrotropin-releasing hormone receptor. *J Biol Chem* 272:11937-11942.

P rodin J, Deraet M, Auger-Messier M, Boucard AA, Rihakova L, Beaulieu ME, Lavigne P, Parent JL, Guillemette G, Leduc R and Escher E (2002) Residues 293 and 294 are ligand contact points of the human angiotensin type 1 receptor. *Biochemistry* 41:14348-14356.

Porter JE, Hwa J and Perez DM (1996) Activation of the α_{1b}-adrenergic receptor is initiated by disruption of an interhelical salt bridge constraint. *J Biol Chem* 271:28318-28323.

Porter JE and Perez DM (1999) Characteristics for a salt-bridge switch mutation of the α_{1b} adrenergic receptor. Altered pharmacology and rescue of constitutive activity. *J Biol Chem* 274:34535-34538.

Prioleau C, Visiers I, Ebersole BJ, Weinstein H and Sealfon SC (2002) Conserved helix 7 tyrosine acts as a multistate conformational switch in the $5HT_{2C}$ receptor. Identification of a novel locked-on phenotype and double revertant mutations. *J Biol Chem* 277:36577-36584.

Ralevic V, Burnstock G (1998) Receptors for purines and pyrimidines. *Pharmacol Rev* 50:413-492.

Rasmussen SG, Choi HJ, Rosenbaum DM, Kobilka TS, Thian FS, Edwards PC, Burghammer M, Ratnala VR, Sanishvili R, Fischetti RF, Schertler GF, Weis WI and Kobilka BK (2007) Crystal structure of the human $_2$ adrenergic G-protein-coupled receptor. *Nature* 450:383-387.

Robinson PR, Cohen GB, Zhukovsky EA and Oprian DD (1992) Constitutively active mutants of rhodopsin. *Neuron* 9:719-725.

Rosenbaum DM, Cherezov V, Hanson MA, Rasmussen SG, Thian FS, Kobilka TS, Choi HJ, Yao XJ, Weis WI, Stevens RC and Kobilka BK (2007) GPCR engineering yields high-resolution structural insights into $_2$-adrenergic receptor function. *Science* 318:1266-1273.

Ruprecht JJ, Mielke T, Vogel R, Villa C and Schertler GF (2004) Electron crystallography reveals the structure of metarhodopsin I. *Embo J* 23:3609-3620.

Schmidt C, Li B, Bloodworth L, Erlenbach I, Zeng FY and Wess J (2003) Random mutagenesis of the M_3 muscarinic acetylcholine receptor expressed in yeast. Identification of point mutations that silence a constitutively active mutant M_3 receptor and greatly impair receptor/G protein coupling. *J Biol Chem* 278:30248-30260.

Shi L, Liapakis G, Xu R, Guarnieri F, Ballesteros JA and Javitch JA (2002) $_2$ adrenergic receptor activation. Modulation of the proline kink in transmembrane 6 by a rotamer toggle switch. *J Biol Chem* 277:40989-40996.

Stitham J, Arehart E, Gleim SR, Li N, Douville K and Hwa J (2007) New insights into human prostacyclin receptor structure and function through natural and synthetic mutations of transmembrane charged residues. *Br J Pharmacol* 152:513-522.

Urizar E, Claeysen S, Deupi X, Govaerts C, Costagliola S, Vassart G and Pardo L (2005) An activation switch in the rhodopsin family of G protein-coupled receptors: the thyrotropin receptor. *J Biol Chem* 280:17135-17141.

Yaar R, Jones MR, Chen JF, Ravid K (2005) Animal models for the study of adenosine receptor function. *J Cell Physiol* 202:9-20

Zhang M, Mizrachi D, Fanelli F and Segaloff DL (2005) The formation of a salt bridge between helices 3 and 6 is responsible for the constitutive activity and lack of hormone responsiveness of the naturally occurring L457R mutation of the human lutropin receptor. *J Biol Chem* 280:26169-26176.

Chapter

Addressing constituti e acti ity of disease-related GPCR mutants with allosteric modulators

ummar

G protein-coupled receptors (GPCRs) are the target for many of the body's endogenous hormones and neurotransmitters, and thus play a major role in physiology. Not surprisingly, somatic mutations in GPCRs may disturb normal physiology, and indeed several of these have been implied in disease. Drugs acting on GPCRs have been developed for wild-type, not mutated, GPCRs only. So far, the majority of clinically used ligands for GPCRs are orthosteric ligands, i.e. they occupy the binding site that is also home for the endogenous ligands. They are able to improve a disease state by induction or suppression of the activation of receptors. It is questionable, however, whether the use of currently available orthosteric ligands can restore the mutant to the wild-type phenotype. An ideal drug for mutant receptors should be able to fully restore the mutant receptor function back to the wild-type function, bringing the disease phenotype back to a normal physiological state.

Compared to orthosteric ligands, allosteric ligands display a more complicated interaction with a receptor/endogenous ligand pair. They are able to cooperatively modify receptor binding and function unlike the orthosteric ligands that only compete with endogenous ligands. Hence a proper allosteric ligand, i.e. a ligand with appropriate binding cooperativity and functional cooperativity, may be able to reverse the mutant receptor phenotype into the wild-type phenotype.

In this Chapter we address, lay the theoretical framework for, and present examples of the potential therapeutic benefits of allosteric over orthosteric ligands in mutation-related diseases.

ntroduction

G protein-coupled receptors (GPCRs) constitute a large family of transmembrane proteins, mediating the communication between the outside and inside of cells. GPCRs are expressed in organisms varying from yeasts to plants and animals and function as sensitive sensors to detect a wide array of so called first messengers including light (photons), cations, small molecules such as amines, sugars, lipids and peptides up to large proteins. GPCRs take up more than 1% of the human genome and are the molecular targets for approximately 30% of currently marketed drugs (Miller et al, 2004).

Quite a few diseases are caused by somatic mutations in GPCRs. These mutations may influence expression levels of receptors, their localization, their interaction with endogenous ligands and, very prominently, their basal activity (Table 1). Therefore we will focus on disease-related mutations that affect this basal or constitutive activity of the receptor. Well known receptor mutations that cause increased constitutive activity are the ones in rhodopsin that are responsible for night blindness (Rao et al, 1994). Other examples of constitutively

active mutations leading to disease include autosomal dominant hypocalcemia induced by constitutively active Ca^{2+} receptor (Zhao et al, 1999).

Table 1 Overview of naturally occurring mutant GPCRs with increased or decreased constitutive activity

GPCR	Constitutive activity change	Effect on effector system activity	Clinical phenotype/disease	References
rhodopsin	increase	enhanced basal activity of cGMP-degrading PDE	night blindness retinitis pigmentosa	Rao et al., 1994
Ca^{2+} receptor	increase	enhanced basal activity of PLC	autosomal dominant hypocalcemia	Zhao et al., 1999
MC2R	increase	enhanced basal activity of AC	in ACTH-independent Cushing's syndrome	Swords et al., 2002
MC4R	decrease	decreased basal activity of AC	obesity	Vaisse et al., 2000
FSHR	increase	enhanced basal activity of AC	male fertility	Gromoll et al., 1996
TSHR	increase	enhanced basal activity of AC	hyperfunctioning thyroid adenoma; familial hyperthyroidism	Parma et al., 1993
LHR	increase	enhanced basal activity of AC	male-limited precocious puberty	Wu et al., 1998
TRHR	decrease	impaired IP production	isolated central hypothyroidism	Collu at al., 1997
PTH-PTHrPR	increase	enhanced basal activity of AC, but impaired agonist stimulation of AC	Jansen-type metaphyseal chondrodysplasia	Schipani et al., 1995
V2R	increase	decreased basal activity of AC	nephrogenic diabetes insipidus	Sadeghi et al., 1997

In addition, several receptor families seem to be prone to disease-causing mutations. A fine example is the melanocortin receptor family where gain-of-function mutations in the melanocortin 2 receptor (MC2R) were found to be involved in ACTH-independent Cushing's syndrome (Swords F, 2002), whereas loss-of-function mutations in the MC4R resulting in obesity (Vaisse et al, 2000).

Mutations affecting constitutive activity and thereby causing disease are also frequently reported for GPCRs recognizing endocrine hormones. Gain-of-function mutations of the follicle-stimulating hormone receptor (FSHR) caused male fertility after hypophysectomy (Gromoll et al, 1996), whereas mutations of the PTH receptor resulted in short-limb dwarfism and skeletal deformities (Schipani et al, 1995). In addition, quite a few mutations of the thyrotropin receptor (TSHR) leading to constitutive activation of the cAMP signaling pathway have been implied in hyperfunctioning thyroid adenoma or familial hyperthyroidism (Parma et al, 1993). Finally, male precocious puberty is caused by constitutively activating mutations of the LH receptor (Laue et al, 1994; Shenker et al, 1993).

Next to increase or decrease constitutively active mutations mentioned in table 1, there are also mutant receptors with disease-related gain-of-function or loss-of-function. For example, a mutant thyrotropin-releasing hormone (TRH) receptor was found unable to bind TRH and could not be activated, which caused a rare disorder called isolated central hypothyroidism (Collu, 1997). In addition, loss-of-function mutations of the FSHR result in pure ovarian dysgenesis (Simoni, 1997), of the gonadotropin-releasing hormone (GnRH) receptor in hypogonadism (de Roux et al, 1997; Karges et al, 2003), and of the LH receptor in Leydig cell hypoplasia (Wu et al, 1998). Other loss-of-function mutants responsible for disease include mutations of the type 2 vasopressin receptor (V2R) causing nephrogenic diabetes insipidus (Sadeghi et al, 1997) and loss-of-function mutations of the thromboxane A2 (TXA2) receptor which are associated with bleeding disorder (Hirata et al, 1994).

To treat such receptor-induced diseases we face a conceptual problem: currently available drugs have been developed for wild-type, healthy' receptors. Such drugs almost invariably interact with the orthosteric binding site on a GPCR where the endogenous ligand binds too.

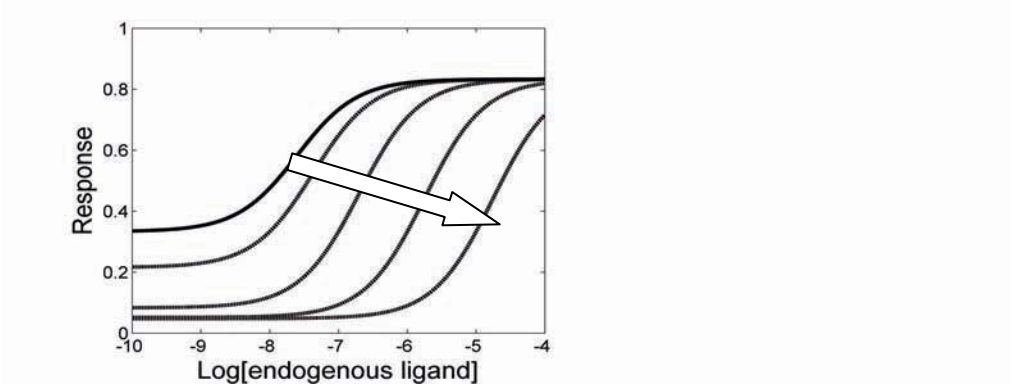


Figure 1. Inverse agonism on a constitutively active receptor.
A constitutively active mutant receptor (L 0.5; with L as the isomerization constant determining the equilibrium between the active state R* and inactive state R in the absence of ligands) was treated with an inverse agonist (K_A 100 nM, α 0.1). Upon increasing the concentrations of the inverse agonist from 100 nM, to 1μM, 10 μM and 100 μM, the dose-response curve of the endogenous ligand (K_A 100 nM, α 10) is shifted right-wards. See the appendix for all equations that we derived from pharmacological modeling.

These drugs however are unable to adjust the basal activity without affecting the EC_{50} value of the endogenous ligand. For example, using an inverse agonist to treat a constitutively active mutant GPCR can dramatically affect the sensitivity of the receptor to the endogenous ligand due to the competition for the same binding site (Figure 1).

There are arguments suggesting allosteric ligands may provide therapeutic advantages over orthosteric ligands under certain circumstances (Hall, 2000; May et al, 2007). Allosteric modulators are defined as ligands that bind to an allosteric site on a GPCR thereby modulating the binding and/or signaling properties generated via interactions with the orthosteric site (May et al, 2007). The difference between an allosteric ligand and an orthosteric ligand lies in the fact that the former can exert its function through both its own

interaction with the receptor and by influencing the effects of an orthosteric ligand. Hence allosteric ligands modulate the receptors' behavior in a more intricate way. In fact, this realization has led to a renewed emphasis on GPCRs as drug targets with many allosteric modulators in clinical trials now, and at least one, an allosteric modulator of the Ca^{2+}-sensing receptor, having reached the market.

This chapter focuses on the potential therapeutic benefit of allosteric ligands in treating mutation-related diseases that affect constitutive activity.

ow can orthosteric ligands affect the beha iour of a mutant receptor to its endogenous ligand

As stated above, orthosteric ligands are commonly used to address disease-related GPCR mutations. The effects orthosteric ligands can have on endogenous ligands to counteract the diseased phenotype are shown below. For a detailed mathematical framework and the equations used for the pharmacological modeling in the Figures we refer to the appendix to this Chapter. These effects encompass normalization of the basal activity, of the potency and of the maximal response. A one-site competition model is proposed to explicitly include the effects of two competitive ligands. This model uses the two-state model of receptor activation as its basis, improving a drug competition model proposed in 1983 (Gero, 1983) in that both association and the intrinsic efficacy of the ligands are taken into consideration. According to the one-site competition model, the effect of an orthosteric ligand is defined by its parameters β, N and [B] (See supplementary data). Three situations can be identified, in which ligand B is either an agonist, a neutral antagonist or an inverse agonist.

The ability of an agonist B to enhance the stimulation of the endogenous ligand A is displayed in Figure 2A, B. Exposure of the receptor to the agonist produces an initially active receptor in a new system, in which the initial activity of the receptor is governed by the intrinsic efficacy of the agonist. We refer here to initial activity as the term basal activity is used to describe the activity achieved in the absence of any ligand. The initial activity on the other hand is the result of the presence of a concentration of ligand B. The initial activity of the receptor is directly proportional to the value of β, a greater β value will yield a bigger initial activity of the receptor. If the agonist B has an intrinsic efficacy smaller or equal to the intrinsic efficacy of the endogenous ligand A, the maximal receptor activation upon combined addition of the endogenous ligand and the agonist B will be the same.

The effect of a variation of the β value of ligand B being an agonist is presented in Figure 2A. Increasing values of β (5, 10, 50 and 500, dashed lines) result in increased initial activities for the receptor with an L value of 0.1. The concentration for agonist B is set at 100 nM.

The effect of a variation of the concentration of orthosteric ligand B being an agonist is presented in Figure 2B. A competitive interaction results in a theoretically limitless rightward shift of the sigmoidal curve for the endogenous ligand in the presence of increasing concentrations (10 nM, 100 nM, 1 μM, 10 μM and 100 μM, dashed lines) of the agonist B with a β value of 10. The effect of ligand B being an antagonist is presented in Figure 2C. A competitive interaction results in a theoretically limitless rightward shift of the concentration-

activation curve for the endogenous ligand A in the presence of increasing concentrations (10 nM, 100 nM, 1 µM, 10 µM and 100 µM, dashed lines) of the antagonist B. The intrinsic activity of ligand B being an antagonist equals 1.

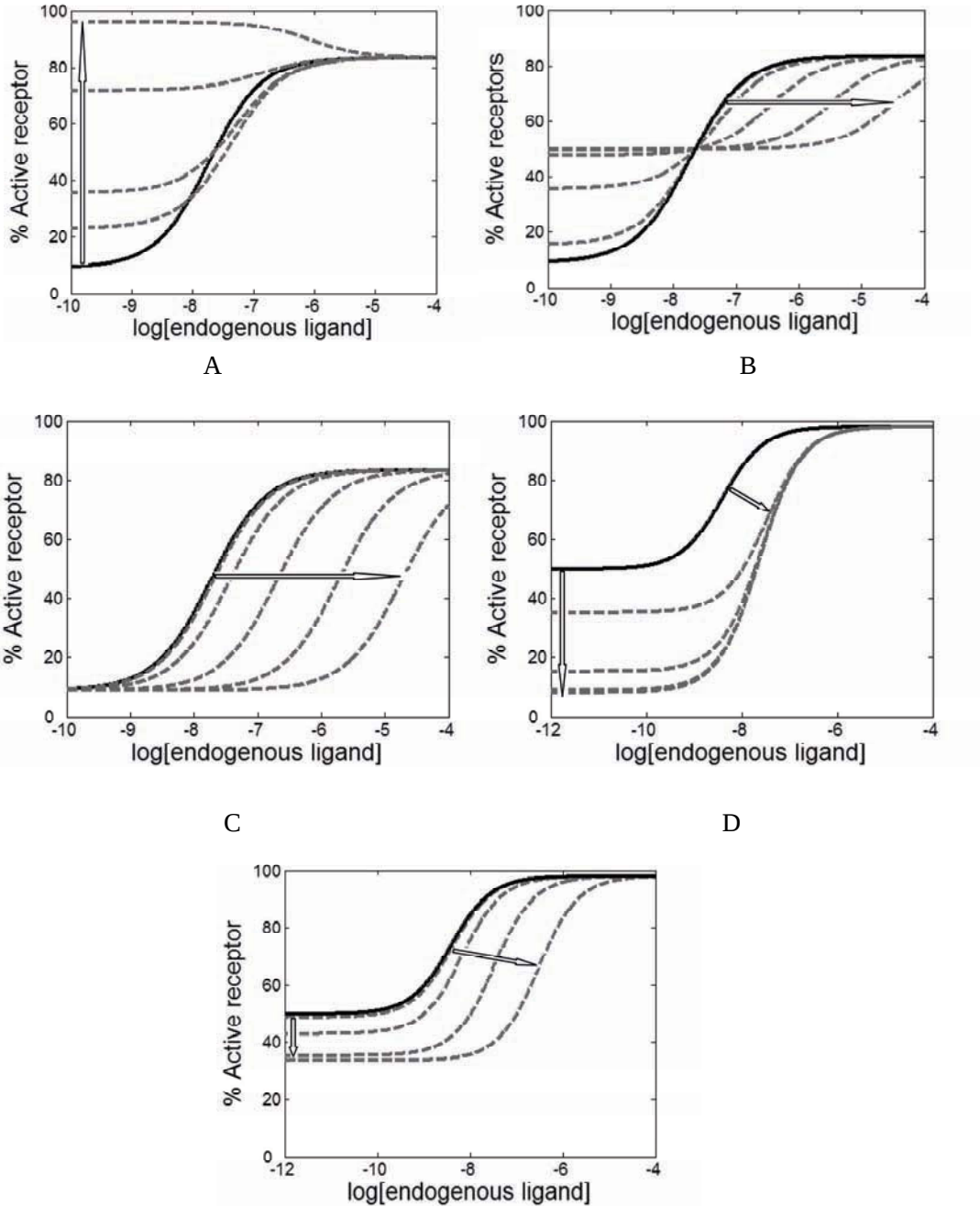


Figure 2. Competition between an orthosteric ligand (B) and an endogenous ligand (A) and its effects on receptor activation. The parameters for the endogenous ligand (continuous lines) are consistent throughout figures 2A-2E: α 50, K 10^7. The association constant of ligand B is set at 10^7. In addition the L value of the receptor is set at 0.1 in figures 2A-2C and to 1 in figure 2D-E.

The effect of a variation of the β value of ligand B being an inverse agonist is shown in Figure 2D. With an increase in the intrinsic efficacies (0.5, 0.1, 0.01, 0.001, dashed lines) at a concentration of 1 µM of the inverse agonist B the initial activity of the receptor is decreased while at the same time the sigmoidal curve is shifted to the right.
The effect of the variation of the concentration of the applied inverse agonist B is shown in Figure 2E. With an increase of the concentration (100 nM, 1 µM, 10 µM and 100 µM) and an intrinsic efficacy of 0.5, again the initial activity of the receptor is decreased and the endogenous ligand-induced sigmoidal curve is shifted to the right.
In the mean time, the potency of the endogenous ligand will be impaired due to the competitive binding of agonist B to the orthosteric site. If B has an β value that is bigger than the α value of the endogenous ligand A, the initial activation of the receptor will be even greater than the activation obtained in the presence of a saturating concentration of A. This is actually a rather interesting situation, because in this case an increasing concentration of ligand A will competitively bring down the initial activity induced by ligand B until the maximal activation ligand A can achieve is reached.

The second situation predicts the effects of the addition of a competitive antagonist B to the endogenous ligand A (see Figure 2C). Ligand B causes a rightward shift of the sigmoidal curve induced by ligand A without affecting either the basal activity of the receptor or the maximal receptor activation to ligand A.

Finally, the effect of ligand B being an inverse agonist is presented in Figure 2D, 2E. Similar to what was observed for ligand B as a competitive antagonist, the concentration-effect curves for ligand A are rightwardly shifted in the presence of ligand B. This shift depends mainly on N and the concentration of ligand B. Similar to ligand B being an agonist, the inverse agonistic ligand B is able to modulate the initial activity of the receptor but cannot affect the maximal receptor activation reached by the endogenous ligand A. The ability to reduce the initial activity is governed by the intrinsic efficacy β of ligand B. The smaller β is, the less initial activity of the receptor will be present. Thus the effect of the inverse agonist B on the endogenous ligand A is a combination of a decreased initial activity and a decreased potency of ligand A.

The simulated curves in Figure 2 reveal that all three categories of orthosteric ligands impair the potency of the endogenous ligand and none of them can influence maximal receptor activation. Thus, by simply changing parameters β, N and the concentration of orthosteric ligands it is not possible to modify all potential changes receptor mutations may cause for the receptor's behaviour. Although agonists and inverse agonists can be used to treat changes in the basal activity induced by a receptor mutation, a significant price is paid because orthosteric ligands hinder the endogenous ligand from properly interacting with the receptor.

Comparison of allosteric ligands with orthosteric ligands

A few examples that show how a saturating concentration of an allosteric ligand, or rather its parameters β, γ and δ, affect the behavior of a receptor/endogenous ligand complex, are given in figure 3. An increased intrinsic efficacy, β, results in an enhanced initial activity of the receptor and an increased maximal receptor activation (Figure 3A). When αδ > 1, an increased β value will increase the potency of the endogenous ligand (Figure 3A) while αδ 1 would result in a decreased potency of the endogenous ligand. An increase in the activation

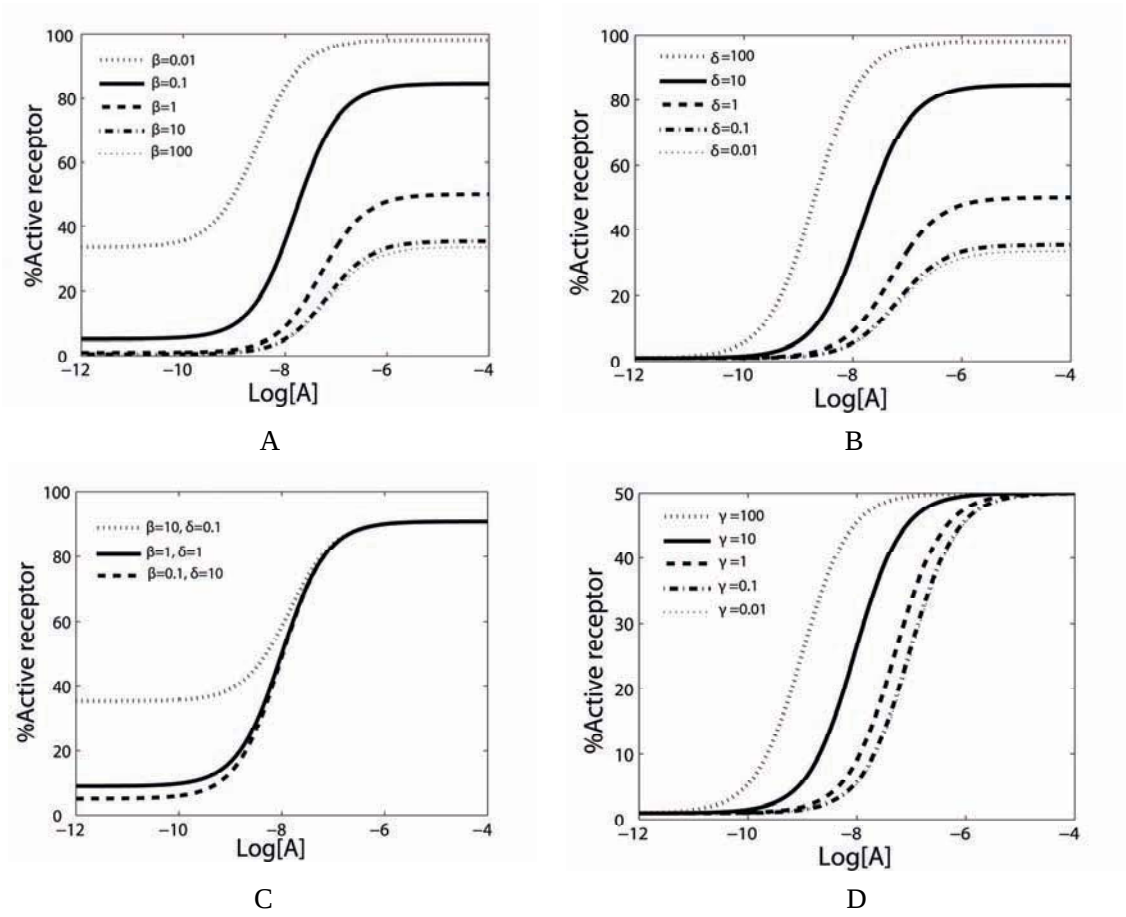


Figure 3. Simulation of the allosteric effect caused by variation of the parameters of an allosteric modulator. The following parameters are consistent throughout K 100 nM, M 100 nM, α 100, and [C] 100 nM). In addition the L value of the receptor is set at 0.1 in figures 3A, 3B, 3D and 1 in figure 3C.

A) Variation of parameter β from 0.01, 0.1, 1, 10 to 100 leads to an increased initial activity of the receptor and increased maximal receptor activation as well as an increased potency of the endogenous ligand (the other parameters are fixed: γ 1, δ 1). B) Variation of parameter δ from 0.01, 0.1, 1, 10, 100 leads to an increased maximal response while the potency of the endogenous ligand is increased (the other parameters are fixed: β 1, γ 1). C) Variation of parameter β and δ at the same time under the condition that their product equals 1. The pairs of β and δ are set as β 1, δ 1 (continuous line), β 10, δ 0.1 (dotted line) and β 0.1, δ 10 (dashed line). D) Variation of parameter γ from 0.01, 0.1, 1, 10 and 100 leads to a leftward shift of the dose-response curves (the other parameters are fixed: β 1, δ 1).

cooperativity value δ results in an enhanced maximal receptor activation by the endogenous ligand (Figure 3B). In addition, changing δ results in an alteration of the potency of the endogenous ligand. If αβ > 1, an increased δ value results in an increased potency of the endogenous ligand (Figure 3B). If β and δ vary at the same time under the condition that their product equals 1, then the maximal receptor activation by ligand A will not change (Figure 3C). On the contrary, if αβ 1, the potency of the endogenous ligand will decrease when the δ value is increased. The binding cooperativity parameter γ only affects the potency of the endogenous ligand. Increased γ values lead to a leftward shift of the concentration-effect curves of the endogenous ligand (Figure 3D).

Comparing the effects of orthosteric ligands with allosteric ligands: from theory to practice

As discussed above, allosteric ligands have a larger number of intervening mechanisms at their disposal than orthosteric ligands (Table 2). The differences relate to the greater potential of allosteric, but not orthosteric, ligands to interfere with the potency and the maximal response achieved by the endogenous orthosteric ligand. Whereas the application of an orthosteric ligand will always reduce the potency of the endogenous ligand resulting in a rightward shift of its concentration-effect curve, the characteristics of a particular allosteric ligand may enable it to increase or decrease the potency of the endogenous ligand or leave it unaffected. In addition, the application of an orthosteric ligand will not affect the maximal response, whereas an allosteric ligand may increase or decrease the maximal response or, again, leave it unaffected. Thus, whereas orthosteric ligands can only modulate the initial activity and will always decrease the potency of the endogenous ligand, allosteric ligands can modulate all three parameters, initial activity, potency and maximal response at will based on its particular characteristics. A few examples describing the practical consequences of these added properties of allosteric ligands are addressed below.

Table 2. Comparing potential interfering mechanisms of orthosteric ligands with those of allosteric ligands.

Effect on	Orthosteric ligands	Allosteric ligands
initial activity	increase, decrease or no effect	increase, decrease or no effect
potency	decrease	increase, decrease or no effect
maximal response	no effect	increase, decrease or no effect

The theoretical examples provided above are also reflected in several publications showing effects of allosteric ligands on the initial receptor activity, on the maximal response and on the sensitivity to orthosteric ligands. Concerning constitutive activity, the allosteric modulator MPEP inhibited the constitutive activity of the human mGluR5 receptor while concentration-dependently decreasing the agonist efficacy of L-glutamate without affecting its potency (Pagano et al, 2000). A second example in which the constitutive activity was decreased with an allosteric modulator is represented by BAY36-7620. This allosteric modulator of the mGluR1 receptor inhibited its constitutive activity and decreased the maximal effect as well as the potency of L-glutamate (Carroll et al, 2001).

Other examples where allosteric modulators affect the maximal effect of the orthosteric agonist include ORG27569, which allosterically increased the agonist affinity for the cannabinoid CB_1 receptor while at the same time the E_{max} values for the agonists were significantly reduced (Price et al, 2005). Opposite effects on E_{max} values have been reported for benzodiazepines which were shown to increase the maximal response of partial agonists on the α_1-adrenergic receptors with only a slight change of their potency (Waugh et al, 1999).

Two more examples have been reported in the literature where an allosteric modulator had little or no effect on the potency of the endogenous orthosteric ligand whereas the efficacy was increased. On the adenosine A_3 receptor a newly synthesized compound, N-(3,4-Dichloro-phenyl)-2-cyclohexyl-1H-imidazo [4,5-c]quinolin-4amine, was shown to increase agonist efficacy without influencing agonist potency (Goblyos, et al, 2006). The second example is taken from the Ca^{2+} receptor where both NPS467 and NPS568 caused a leftward shift of the Ca^{2+} concentration-response curve without any effect on the maximal effect achieved by Ca^{2+} (Hammerland et al, 1998).

The two next and last examples are cases where the efficacy of the agonist is increased but where opposite effects on agonist potency are achieved. DU124183 concentration-dependently increased agonist efficacy on the adenosine A_3 receptor but decreased agonist potency (Goblyos et al, 2006), whereas GS39783 increased both the potency and efficacy of GABA on its receptor (Urwyler et al, 2001).

Comparison of the properties of allosteric ligands versus orthosteric ligands in treating pathophysiology caused by mutant receptors

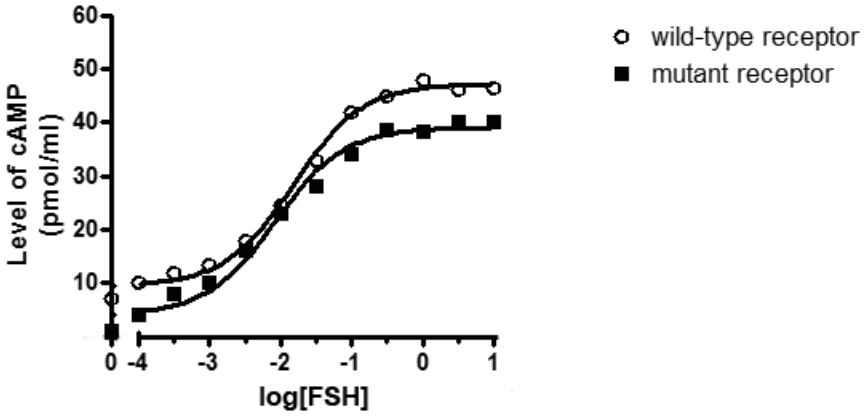
Since allosteric ligands do not compete for the binding site of endogenous ligands, and cooperatively affect a receptor's behavior both through binding and through function, they have added features to also influence GPCRs that suffer from somatic mutations. Three examples where allosteric ligands may provide advantages over orthosteric ligands are presented below.

Counteracting increased basal activity and increased maximal response without affecting the potency of the endogenous orthosteric ligand

A mutation in helix VI of the follicle-stimulating hormone receptor (D567N) was studied by Smits et al. (2003). This mutation leads to ovarian hyperstimulation syndrome. Functional characterization of this receptor revealed that the D567N mutation increased basal activity in transiently transfected COS-7 cells and caused an increased maximal response while the sensitivity to the endogenous ligand follicle-stimulating hormone (FSH) was unchanged. The constitutive activity and the greater maximal response of the receptor were not caused by increased receptor expression levels, as the amount of mutant receptor expression was 69% of that of the wild type receptor.

To revert the mutated receptor back to the normal state, a decrease in initial activity can be achieved by treating the mutant receptor with either an inverse agonist or an allosteric

modulator. However, as discussed before inverse agonists cannot reduce the receptor's maximal response, and cause a decreased potency of follicle-stimulating hormone as shown in figure 4 (dotted line). To decrease the initial activity as well as the maximal response while at the same time keeping the potency of the endogenous ligand intact, an allosteric modulator is required (Figure 4 dashed line).



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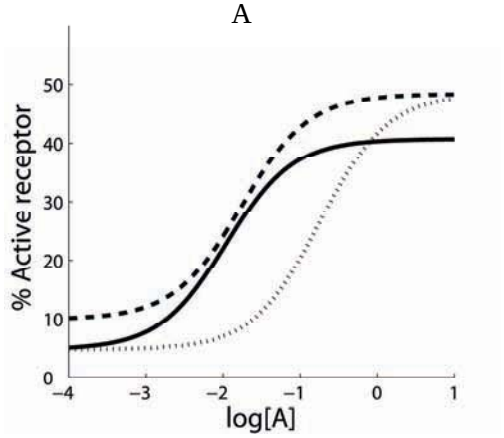

B

Figure 4. Different effects on D567N mutant follicle-stimulating hormone receptor.
A) cAMP production in both wild type and D567N mutant receptor upon stimulation with FSH (reproduced from Smits, 2003). B) Simulation of the treatment of the mutant receptor (L 0.11, continuous line) with an inverse agonist (β 0.4, N 10^7, [B] 1 μM; dotted line) and an allosteric modulator (β 0.4, γ 1.8, δ 2, M 10^7, [C] 1 μM; dashed line). The inverse agonist decreases the initial activity of the receptor and impairs the potency of the endogenous ligand A. The maximal response cannot be reduced by the inverse agonist. The allosteric modulator decreases the initial activity of the receptor as well as the maximal response to follicle-stimulating hormone and does not change the sensitivity of the receptor to follicle-stimulating hormone.

ounteracting increased asal activity and decreased ma imal response without affecting the potency of the endogenous orthosteric ligand

A number of GPCR mutants that display a rather complicated effect have been described in the literature; while their basal activity is higher than the wild-type receptor, their maximal response to the endogenous ligand is decreased. Three cases were reported. Both the D564G and H578Y mutant human luteinizing hormone (LH) receptor were found in patients suffering from familial male-limited precocious puberty (Laue et al, 1995). These two mutant receptors were characterized by their high basal cAMP production which could be further stimulated by hCG. However, the maximal response to hCG of these mutant receptors was much lower than that of the wild-type receptor. Another example concerns mutant GPR54 receptors. Similar to mutant LH receptors, the L148S mutant GPR54 receptor also displayed higher basal activity combined with a reduced maximal response to the endogenous kisspeptin ligand (Seminara et al, 2003). The last example is the H223R mutant of the PTH-PTHrP receptor (Schipani et al, 1995) (Figure 5A). Orthosteric ligands are not able to decrease the initial activity of these mutants and increase the maximum effect at the same time. However if an allosteric ligand could be made with a β 1 and the product of $\beta\ \delta > 1$, then the initial activity and the maximal effect can be modified in opposite ways (Figure 5B).

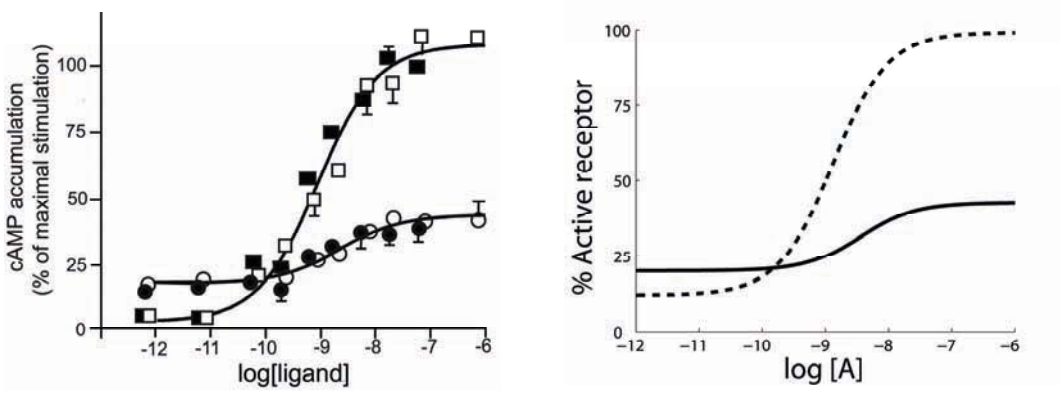

Figure 5. Mutant receptors with increased basal activity and decreased maximal response.
A) The H223R mutant PTH-PTHrP receptor (circles) has a higher basal activity level than the wild-type receptor (squares), but a reduced maximal response to ligands [$Nle^{8,18}$, Tyr^{34}] bovine PTH(1-34)amide (filled squares and circles) and [Tyr^{36}] human PTHrP (1-36) amide (open squares and circles) (reproduced from Schipani, E, 1995). B) Simulation of the treatment of the mutant receptor (continuous line) with an allosteric modulator (dashed line) to bring it back to wild-type phenotype. The parameters were set at: L 0.25, α 3, β 0.5, δ 1000, γ 0.05, K $5*10^9$, M 10^7, the concentration of the allosteric ligand was 100 nM.

ounteracting reduced asal activity and decreased ma imal response without affecting the potency of the endogenous orthosteric ligand

Loss of function mutations of the type 2 vasopressin receptor (V2R) result in recessive nephrogenic diabetes insipidus. One well-characterized mutant displaying this phenotype is the D85N receptor. This mutant has normal expression, maturation and transportation to the

cell membrane while the binding affinity and efficacy for the endogenous peptide arginine-vasopressin are reduced (Sadeghi et al, 1997). The D85N mutant receptor also showed reduced basal activity compared to the wild-type receptor. (Orthosteric) agonists can compensate for the reduced initial activity of the mutant receptor but cannot increase the maximal response to arginine-vasopressin. Such an agonist will also shift the concentration-effect curve of the endogenous ligand to the right. The same holds for another V2R mutant, G201D. This mutant displays a decreased basal activity and a decreased response to the endogenous agonist. Due to the competitive nature of an agonist for the endogenous binding site, treatment with an agonist would decrease rather than increase the potency of arginine-vasopressin. However, by adjusting the cooperativity parameters δ and γ, an allosteric ligand may increase the basal activity of the receptor as well as the potency of the endogenous ligand (Figure 6).

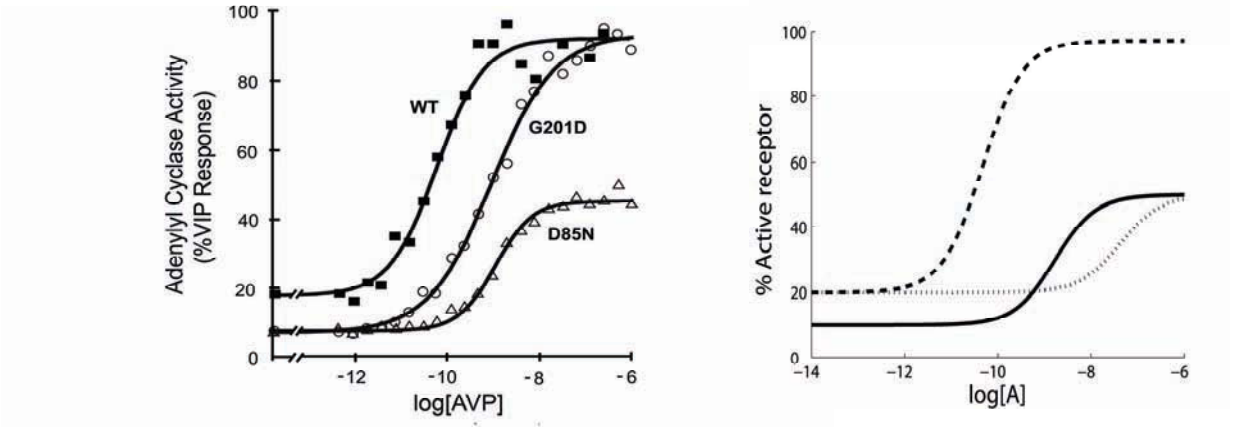

Figure 6. Mutant receptors with decreased basal activity and decreased sensitivity to the endogenous agonist. A) D85N and G201D mutant human type 2 vasopressin receptors display lower basal activity levels and reduced sensitivity to arginine vasopressin compared to the wild-type receptor (reproduced from Sadeghi H, 1997). B) Simulation of the treatment of the mutant D85N receptor (continuous line) with an allosteric modulator to restore the wild-type phenotype (dashed line). The parameters are set as: L 0.11, α 9, β 3.5, δ 10, γ 50, K $3*10^9$, M 10^7, the concentration of allosteric ligand is 100 nM. Upon application of 100 nM of an agonist (β 3.5, N 10^7) the initial activity is increased, the maximal response is unaltered and arginine-vasopressin shows a decreased potency on the mutant receptor (dotted line).

Additional examples of mutant receptors displaying a decreased basal activity compared with a decreased maximal response include several loss-of-function mutant melanocortin-4 receptors (MC4R) which cause obesity. Again, treatment of these mutant receptors with agonists cannot restore the wild-type phenotype because these mutants require an increased response as well as an increased sensitivity to α-MSH (Vaisse et al, 2000; Biebermann et al,

2003; Farooqi et al, 2003). The same is true for the loss-of-function A204E mutant growth hormone secretagogue receptor (GHSR), which has a lower basal activity and a lower maximal response to ghrelin compared to the wild-type receptor (Pantel et al, 2006).

ounteracting decreased ma imal response and restoring the potency of the endogenous orthosteric ligand

The S390R mutant endothelin B receptor responsible for Hirschsprung's disease displayed a reduced, around 90%, ET-1-induced intracellular Ca^{2+} transient compared to the wild-type receptor while at the same time the EC_{50} value of ET-1 was 30 fold increased (Tanaka et al, 1998). Again, allosteric ligands would be needed to increase the maximal response. In addition, two mutant GnRH receptors were found in a family with idiopathic hypogonadotropic hypogonadism. One of the mutants leads to decreased affinity for GnRH while another did not modify the binding of the hormone but decreased the activation by GnRH (de Roux et al, 1997). Restoration of the wild-type phenotype of both mutants requires again allosteric ligands, one with the property to enhance the binding of the receptor and GnRH and another with the ability to enhance the stimulation by GnRH. This is yet another situation where allosteric ligands only can revert the aberration to normal physiology. In theory an allosteric ligand can treat' mutant receptors with impaired signal transduction ability, since the functional cooperativity of allosteric ligands can modify the stimulation of a receptor by its endogenous ligand. Examples of such mutant receptors include the partial loss-of-function mutant FSH receptor, which causes ovarian dysgenesis due to impairment of FSH function and reduced cAMP generation when expressed in COS-7 cells (Beau et al, 1998). In addition, a loss-of-function TRH receptor has been described which is resistant to the action of thyrotropin and results in hypothyroidism or euthyroidism with increased thyrotropin secretion (Sunthornthepvarakui, 1994). Finally, also a loss-of-function thromboxane A2 (TXA2) receptor has been reported which resulted in a bleeding disorder due to a defective platelet response to TXA2 (Hirata et al, 1994).

Additional theoretical ad antages of allosteric ligands o er orthosteric ligands

In theory, allosteric sites should be more divergent than orthosteric sites as they are less subject to evolutionary pressure. Therefore, in principle greater selectivity may be achieved with allosteric ligands compared to orthosteric ligands (May et al, 2007). Not only do the allosteric ligands allow more selective binding to the receptors due to more divergent binding sites, the cooperativity between allosteric ligands and orthosteric sites further enhances the selectivity. An example is provided by the small molecule thiochrome. Thiochrome selectively increases the affinity of acetylcholine for the M_4 muscarinic acetylcholine receptor (mAchR) over the other receptor subtypes although it does bind to allosteric sites on all five mAchRs (Lazareno et al, 2004). This example demonstrates selective cooperativity of an allosteric ligand which interacts with closely related receptors.

A second advantage of allosteric modulators is that allosteric modulators can enhance or attenuate the effect of an orthosteric ligand without displaying an effect on its own. In other

words, the allosteric effect is dependent on or effectuated by the endogenous ligand. An overdose of an allosteric modulator will therefore cause less side effects than an overdose of an orthosteric ligand as the effective window of the allosteric modulator is bounded on one hand by the basal activity of the receptor and by the effect of the endogenous agonist on the other (Gao et al, 2006; Gilchrist et al, 2007).

Traditional orthosteric ligands have been shown to cause drug tolerance. That is, these ligands induce a dramatic increase or reduction of the receptor number due to their up- or downregulation by using inverse agonists or agonists, respectively (Dohlman et al, 1991; Collins et al, 1992; Milligan and Bond, 1997; MacEwan and Milligan, 1996, Smit et al, 1996). However, no effects of allosteric modulators on receptor expression levels have been reported so far. In fact, there is evidence that allosteric modulators do not change receptor expression levels. An example is the allosteric agonist ASLW which induced increased CXCR4 chemokine receptor activation compared to the agonist SDF-1α but did not cause receptor internalization (Sachpatzidis et al, 2003).

Collateral efficacy of allosteric ligands and its therapeutic benefit

There is growing evidence that orthosteric ligands may cause collateral efficacy also referred to as ligand-directed signaling or differential receptor trafficking (for a review, see Kenakin, 2005). These differences may be explained as ligands binding to receptors preferably stabilize certain conformations of a receptor, thereby triggering various receptor activation pathways. Since allosteric ligands also have a certain affinity to receptors it is reasonable to assume that they also exhibit collateral efficacy like orthosteric ligands. In other words, allosteric ligands can also stabilize certain receptor conformations resulting in a biased affinity of the orthosteric ligand thereby displaying preference for certain signaling pathways. A few reports revealed such a collateral efficacy of allosteric ligands. One of the examples is the allosteric modulator eburnamonine, which enhanced the potency of the muscarinic agonist arecoline while, in contrast, the compound decreased the potency of another agonist, pilocarpine (Jakub k et al, 1997). A second example is the gadolinium ion (Gd^{3+}). This allosteric enhancer potentiates the glutamine-mediated Ca^{2+} mobilization through $G_{q/11}$ while it inhibits cAMP accumulation through G_s proteins (Abe et al, 2003; Tateyama and Kubo, 2006). A third, even more interesting example is aplaviroc, an anti-HIV drug. It allosterically modulates the chemokine CCR5 receptor by blocking the binding of ^{125}I-MIP-1α (macrophage inflammatory protein-1α) but not that of ^{125}I-RANTES (regulated upon activation, normal T-cell expressed and secreted). Interestingly, aplaviroc selectively blocks the binding of MIP-1a and not of RANTES. As a result, aplaviroc may therefore elicit a unique viral resistance profile (Watson et al, 2005).

The ability of allosteric ligands to display collateral efficacy may provide a therapeutic benefit. For example, an FSH receptor mutation in the transmembrane domain of this receptor results in recurrent spontaneous ovarian hyperstimulation syndrome. Functional assays revealed that this mutant receptor displays wild-type behaviour with respect to constitutive activity and the response to follicle stimulating hormone. However, the mutation rendered the receptor

sensitive to human chorionic gonadotropin (Vasseur et al, 2003). To treat this disease, an allosteric ligand might be able to discriminate between the unwanted activation by chorionic gonadotropin versus the desired stimulation by follicle stimulating hormone.

Conclusions

Allosteric ligands display certain unique features which may be used advantageously in the treatment of diseases caused by somatic mutations in GPCRs. Unlike orthosteric ligands these allosteric compounds are able to modulate independently the initial activity and the maximal receptor response without affecting the potency of the endogenous ligands. In addition, they may provide added selectivity, including collateral selectivity, and may lack drug tolerance inducing activity.

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According to the two-state receptor model (Leff, 1995; Kenakin, 2003), receptors coexist in an equilibrium between an active state and an inactive state. Three parameters determine the ratio between both states of a receptor in a given system where only 1 ligand (A) is present or absent. The isomerization constant (L) of a receptor determines the equilibrium between the active state (R*) and the inactive state (R) in the absence of ligands; α is the intrinsic efficacy of ligand A (where A represents the endogenous ligand), or in other words α is the ratio of the affinity of A for the active state receptor versus the inactive state receptor; K is the association constant of A. If only the ratio of active state receptors in the whole receptor pool (R^*/R_T) is taken as a measure for activation and downstream effects are neglected, this model can be used to simulate or predict the effect of an endogenous ligand on a receptor species. Such a simplified model can be successfully applied for instance to predict the intrinsic efficacy of inverse agonists on constitutively active mutant receptors expressed in yeast (Li, 2007).

When an orthosteric ligand (B) is added to the system, which may be an agonist, antagonist or inverse agonist, and which competes for the orthosteric binding site without having a cooperative effect on ligand A, there will be two additional factors, β and the product of N[B], that may affect the equilibrium between the active and inactive receptors. In this model two intrinsic efficacies are defined: α, the intrinsic efficacy of ligand A and β, the intrinsic efficacy of ligand B. These intrinsic efficacies indicate whether the ligands A and B have a preference for R or for R*. The intrinsic efficacy may be larger than 1 (agonists) identifying a preference for R*, equal to 1 (antagonist) and no preference for R or R*, or between 0 and 1 (inverse agonist) with a preference for R. Similar to parameter K identifying the association constant of ligand A, N is the association constant of ligand B. An allosteric modulator (ligand C) is able to modulate the binding and/or function of the receptor/endogenous ligand pair. To account for this cooperative effect parameters γ and δ were introduced (Hall, 2000). Parameter γ denotes the binding cooperativity between ligand C and ligand A, recorded by a ratio of affinity of ligand C for ligand A/receptor complex AR and receptor R. Positive binding cooperativity (γ > 1) indicates an increased level of binding of ligand A in the presence of ligand C and negative binding cooperativity (γ 1) suggests a decreased level of

binding. Parameter δ represents the activation cooperativity between ligand C and ligand A, that is the ratio of affinity of ligand C for ligand A/active state receptor complex (AR*) and ligand A/inactive state receptor complex (AR). Similar to the binding cooperativity, also for the activation cooperativity we can identify a positive activation cooperativity (δ > 1) indicating an increased level of activation in the presence of ligand C and a negative activation cooperativity (δ 1), indicating a decreased level of activation. In addition to parameters γ and δ describing the cooperativity effects of ligand C to ligand A, the intrinsic efficacy of ligand C is described by β. When β 1, ligand C does not affect the equilibrium between R and R*. When β > 1, however, ligand C shifts the equilibrium towards R* and when β 1 ligand C shifts the equilibrium towards R. The association constant for ligand C is described by M. Thus four factors: β, γ, δ and the product of M[C] of an allosteric ligand are able to modify the equilibrium states for an endogenous ligand/receptor pair. The reasoning in this appendix so far is based on the following pharmacological modeling.

According to the two-state receptor model, the proportion of receptors in the active state is (Kenakin, 2003):

$$\frac{[R_{act}]}{[R_T]} = \frac{[R^*]+[AR^*]}{[R]+[R^*]+[AR]+[AR^*]} \qquad \text{equation 1}$$

Equation 1 can be restated as

$$\frac{[R_{act}]}{[R_T]} = \frac{\alpha \quad L[A]+L}{(1+\alpha L)[A]+L+1} \qquad \text{equation 2}$$

In the absence of ligand ([A] 0), the proportion of receptors in the active state represents the basal activity of the receptor

$$\frac{[R_{act}]_{basal}}{[R_T]} = \frac{L}{L+1} \qquad \text{equation 3}$$

When a theoretically infinite concentration of ligand A is present ($[A]\rightarrow\infty$), the proportion of receptors in the active state, or the maximal receptor activation of the receptor to ligand A is:

$$\frac{[R_{act}]_{max}}{[R_T]} = \frac{\alpha L}{1+\alpha L} \qquad \text{equation 4}$$

When half of the maximal receptor activation is achieved, the proportion of receptors in the active state can be stated as

$$\frac{[R_{act}]_{1/2}}{[R_T]} = \frac{1+L}{(1+\alpha L)} \qquad \text{equation 5}$$

Two-state model can be extended to the one site competition model (for detail, see supplementary data). In this model, ligand B competes with endogenous ligand A to bind to both forms of receptors (R and R*). Similar to ligand A which can form AR and AR* with different forms of receptors, ligand B can form BR or BR*. According to this model, the following equation can be derived describing the proportion of receptors in the active state when two ligands (endogenous ligand A and an orthosteric ligand B) compete for the same binding site of a receptor:

$$\frac{[R_{act}]}{[R_T]} = \frac{[R^*]+[AR^*]+[BR^*]}{[R]+[R^*]+[AR]+[AR^*]+[BR]+[BR^*]} \qquad \text{equation 6}$$

Equation 6 can be restated as

$$\frac{[R_{act}]}{[R_T]} = \frac{L(1+\alpha \quad [A]+\beta N[B])}{1+L+\quad [A](1+\alpha L)+N[B](1+\beta L)} \qquad \text{equation 7}$$

It can be seen from this expression that in the absence of ligand A ([A] 0, [B] $\neq$ 0), the proportion of receptors in the active state or the initial activity (to discriminate from the basal activity of the receptor, the activity induced by other ligands in the absence of the endogenous ligand were referred to as initial activity in this study) of the receptor obeys the following equation:

$$\frac{[R_{act}]}{[R_T]} = \frac{L(1+\beta N[B])}{1+L+N[B](1+\beta L)} \qquad \text{equation 8}$$

When a theoretically infinite concentration of ligand B is present ([B] $\rightarrow \infty$), equation 8 can be simplified into

$$\frac{[R_{act}]}{[R_T]} = \frac{\beta L}{1+\beta L} \qquad \text{equation 9}$$

Equation 9 shows that the addition of an agonistic ligand B ($\beta > 1$) will enhance, whereas an inverse agonistic ligand B (0 β 1) will reduce the initial activity of the receptor as shown in figures 2a and 2c. If β 1, in other words when ligand B is a neutral antagonist, equation 8 is identical to equation 2, indicating that an antagonist cannot modify the initial activity of the receptor.

When a theoretically infinite concentration of ligand A is present ([A] $\rightarrow \infty$), the proportion of receptors in the active state, or the maximal receptor activation of the receptor by ligand A is:

$$\frac{[R_{act}]_{max}}{[R_T]} = \frac{\alpha L}{1+\alpha L}$$

This equation is identical to equation 4, which reveals that the addition of an orthosteric ligand cannot modify the maximal receptor activation of the receptor by the endogenous ligand A.

When half of the maximal receptor activation is achieved, the proportion of receptors in the active state can be stated as

$$\frac{[R_{act}]_{1/2}}{[R_T]} = \frac{1 + L + N[B](1+\beta L)}{(1+\alpha L)} \qquad \text{equation 10}$$

Comparing equation 10 with equation 5 leads to the conclusion that the additional presence of an orthosteric ligand results in a rightward shift of the sigmoidal concentration-effect curve of the endogenous ligand.

Using the same approach as above, it is possible to predict the effect of the addition of an allosteric modulator C to the concentration-effect curve of the endogenous ligand A. We refer to the two-state allosteric model (Hall, 2000), which describes the allosteric interactions at receptors and especially accounts for the effect of the allosteric ligand itself on receptor activation. According to this model, the proportion of receptors in the active state is:

$$\frac{[R_{act}]}{[R_T]} = \frac{[R^*] + [AR^*] + [CR^*] + [AR^*C]}{[R] + [R^*] + [AR] + [AR^*] + [CR] + [CR^*] + [ARC] + [AR^*C]} \qquad \text{equation 11}$$

Equation 11 can be restated as

$$\frac{[R_{act}]}{[R_T]} = \frac{L(1+\alpha \quad [A] + \beta M[C](1+\alpha\gamma\delta \quad [A])}{1 + L + M[B](1+\beta L) + \quad [A](1+\alpha L + \gamma M[C](1+\alpha\beta\delta L)} \qquad \text{equation 12}$$

In the absence of ligand A, the initial activity of the receptor can be stated as

$$\frac{[R_{act}]_{basal}}{[R_T]} = \frac{L(1+\beta M[C])}{1 + L + M[C](1+\beta L)} \qquad \text{equation 13}$$

This equation is identical to equation 8 except that M and [C] are the parameters for an allosteric ligand while N and [B] are the parameters for an orthosteric ligand. This equation reveals that the initial activity is governed by both the receptor (L) and the allosteric ligand (β and product of M[C]). Analogous to the orthosteric ligand, an allosteric ligand is also able to modify the initial activity of the receptor/endogenous ligand complex if $\beta \neq 1$, i.e. when the allosteric ligand has a preference for either the active or the inactive state of the receptor. In the presence of a saturating concentration of ligand C, equation 13 can be restated as

$$\frac{[R_{act}]_{basal}}{[R_T]} = \frac{\beta L}{1+\beta L} \qquad \text{equation 14}$$

When a theoretically infinite concentration of ligand C is present ($[C] \to \infty$), the maximal activation of the receptor can be stated as

$$\frac{[R_{act}]_{max}}{[R_T]} = \frac{\alpha L(1+\beta\gamma\delta M[C])}{1+\alpha L+\gamma M[C](1+\alpha\beta\delta L)} \qquad \text{equation 15}$$

This equation is more complicated than the equation for maximal receptor activation in the presence of an orthosteric ligand only (see equation 4). Equation 15 reveals that the maximum response of a receptor to its endogenous ligand is governed by the receptor (L), the endogenous ligand (α) and the allosteric ligand (β, γ, δ, and product of M[C]). Thus an allosteric ligand is able to modify the maximum response of the receptor to its endogenous ligand.

When half of the maximal receptor activation is achieved, the proportion of receptors in the active state can be stated as

$$\frac{[R_{act}]_{1/2}}{[R_T]} = \frac{1+L+M[C](1+\beta L)}{(1+\alpha L+\gamma M[C](1+\alpha\beta\delta L))} \qquad \text{equation 16}$$

Comparing equation 16 with equation 10, we learn that binding cooperativity γ, functional cooperativity δ, as well as the intrinsic efficacy and the product of M[C] affect the sensitivity of the receptor to its endogenous ligand.

It is interesting to note that in the presence of a saturating concentration of ligand C, equation 16 can be restated as

$$\frac{[R_{act}]_{1/2}}{[R_T]} = \frac{(1+\beta L)}{\gamma(1+\alpha\beta\delta L)} \qquad \text{equation 17}$$

This constant reveals the ceiling effect of an allosteric ligand because the right part of equation 17 is a constant and is thus independent from the concentration of ligand C.

Effect of **β γ δ** on a receptor endogenous ligand pair

By investigating the effect of β, γ and δ on a receptor/endogenous ligand pair, the initial activity, E_{max} and EC_{50}/IC_{50} deduced from the two-state receptor model and the two-state allosteric receptor model can be compared.

Equation 3 defines the basal activity of a receptor in the absence of any ligand and equation 14 describes the initial activity of a receptor in a new equilibrium when an allosteric ligand is present in the absence of an endogenous ligand. If the two equations are identical, a condition is revealed whereby the allosteric ligand is not able to change the basal activity of a receptor. Thus, a new equation is obtained

$$\frac{\beta L}{1+\beta L} = \frac{L}{1+L}$$ equation 18

This equation only holds when β 1. In this case, the allosteric ligand has no preference for either the active state or the inactive state of the receptor. In other words, the allosteric ligand cannot affect receptor activation or inactivation by itself. If the allosteric ligand increases the initial activity of a receptor, that is, the allosteric ligand has an agonistic nature, a new expression is obtained

$$\frac{\beta L}{1+\beta L} > \frac{L}{1+L}$$

This equation only holds when β > 1. On the contrary, if β 1, the allosteric ligand reduces the initial activity and this allosteric ligand has inverse agonist characteristics. Allosteric ligands with a preference for either the active or the inactive state of the receptor are able to modify the initial activity of a receptor (Figure 3a). Since this property is only related to the intrinsic efficacy β of the ligand, the ability to modify the initial activity of a receptor of the allosteric ligand is indistinguishable from an orthosteric ligand.

Similarly, it is possible to examine the condition in which allosteric ligands do not affect the maximal activation induced by an endogenous ligand, that is

$$\frac{\alpha L}{1+\alpha L} = \frac{\alpha L(1+\beta\gamma\delta M[C])}{1+\alpha L+\gamma M[C](1+\alpha\beta\delta L)}$$ equation 19

Simplifying this equation yields βδ 1, which reveals that only the intrinsic efficacy β and the activation cooperativity δ of an allosteric ligand affect the maximal receptor activation induced by its endogenous ligand. If βδ 1, a receptor's maximal activation will decrease in the presence of allosteric ligands; if βδ > 1, a receptor's maximal activation will increase when an allosteric ligand is present (Figure 3b). For a pure allosteric ligand which cannot activate or inactivate a receptor by itself (β 1), δ alone determines its effect on maximal receptor activation. In this case, δ > 1 results in an increased maximal receptor activation and vice versa (Figure 3c). Since orthosteric ligands have no cooperative effect on the endogenous ligand, these ligands are unable to modify the maximal receptor activation.

We also investigated under which circumstances an allosteric ligand does affect the potency of an endogenous ligand. When equation 5 (the proportion of receptors in the active state when half of the maximal receptor activation is achieved in the presence of ligand A) is identical to equation 16 (the proportion of receptors in the active state when half of the maximal receptor activation is achieved in the presence of both ligand A and ligand C), an allosteric ligand does not affect the potency of an endogenous ligand to a receptor, thus a new expression is obtained

$$\frac{1+L}{(1+\alpha L)} = \frac{1+L+M[C](1+\beta L)}{(1+\alpha L+\gamma M[C](1+\alpha\beta\delta L))} \qquad \text{equation 20}$$

Manipulation of this equation yields

$$\gamma = \frac{(1+\alpha L)(1+\beta L)}{(1+L)(1+\alpha\beta\delta L)} \qquad \text{equation 21}$$

If the left part of equation 20 is smaller than the right part, or γ being smaller than the right part of equation 21, the allosteric ligand is able to reduce the potency of the endogenous ligand, thus a rightward shift of the sigmoidal curve of the endogenous ligand will be observed and vice versa (Figure 3d). Equation 21 reveals that β, γ and δ of an allosteric modulator can affect the potency of an endogenous ligand on a receptor. Increasing γ and δ both results in an increased potency of the endogenous ligand.

Reference

Abe H, Tateyama M and Kubo Y (2003) Functional identification of Gd^{3+} binding site of metabotropic glutamate receptor 1α. *FEBS Lett* 545: 233–238.

Beau I, Touraine P, Meduri G, Gougeon A, Desroches A, Matuchansky C, Milgrom E, Kuttenn F and Misrahi M (1998) A novel phenotype related to partial loss of function mutations of the follicle stimulating hormone receptor. *J Clin Invest* 102: 1352-1359.

Biebermann H, Krude H, Elsner A, Chubanov V, Gudermann T and Gruters A (2003) Autosomal-dominant mode of inheritance of a melanocortin-4 receptor mutation in a patient with severe early-onset obesity is due to a dominant-negative effect caused by receptor dimerization. *Diabetes* 52: 2984-2988.

Carroll FY, Stolle A, Beart PM, Voerste A, Brabet I, Mauler F, Joly C, Antonicek H, Bockaert J, Muller T (2001) BAY36-7620: a potent non-competitive mGlu1 receptor antagonist with inverse agonist activity. *Mol Pharmacol* 59: 965-973.

Collins S, Caron MG and Lefkowitz RJ (1992) From ligand binding to gene expression: new insights into the regulation of G-protein-coupled receptors. *Trends Biochem Sci* 17: 37-39.

Collu R, Tang J, Castagne J, Lagace G, Masson N, Huot C, Deal C, Delvin E, Faccenda E, Eidne KA (1997) A novel mechanism for isolated central hypothyroidism: inactivating mutations in the thyrotropin-releasing hormone receptor gene. *J Clin Endocrinol Metab* 82: 1561-1565.

de Roux N, Young J, Misrahi M, Genet R, Chanson P, Schaison G and Milgrom E (1997) A family with hypogonadotropic hypogonadism and mutations in the gonadotropin-releasing hormone receptor. *N Engl J Med* 337: 1597-1602.

Dohlman HG, Thorner J, Caron MG and Lefkowitz RJ (1991) Model systems for the study of seven-transmembrane-segment receptors. *Annu Rev Biochem* 60: 653-688.

Farooqi IS, Keogh JM, Yeo GS, Lank EJ, Cheetham T and O'Rahilly S (2003) Clinical spectrum of obesity and mutations in the melanocortin 4 receptor gene. *N Engl J Med* 348: 1085-1095.

Gao ZG and Jacobson KA (2006) Keynote review: allosterism in membrane receptors. *Drug Discov Today* 11: 191-202.

Gero A (1983) Desensitization, two-state receptors and pharmacological parameters. *J Theor Biol*. 103:137-161.

Gilchrist A (2007) Modulating G-protein-coupled receptors: from traditional pharmacology to allosterics. *Trends Pharmacol Sci* 28: 431-437.

Goblyos A, Gao ZG, Brussee J, Connestari R, Santiago SN, Ye K, IJzerman AP and Jacobson KA (2006) Structure-activity relationships of new 1H-imidazo[4,5-c]quinolin-4-amine derivatives as allosteric enhancers of the A_3 adenosine receptor. *J Med Chem* 49: 3354-3361.

Gromoll J, Simoni M and Nieschlag E (1996) An activating mutation of the follicle-stimulating hormone receptor autonomously sustains spermatogenesis in a hypophysectomized man. *J Clin Endocrinol Metab* 81: 1367-1370.

Hall DA (2000) Modeling the functional effects of allosteric modulators at pharmacological receptors: an extension of the two-state model of receptor activation. *Mol Pharmacol* 58: 1412-1423.

Hammerland LG, Garrett JE, Hung BC, Levinthal C and Nemeth EF (1998) Allosteric activation of the Ca^{2+} receptor expressed in Xenopus laevis oocytes by NPS 467 or NPS 568. *Mol Pharmacol* 53: 1083-1088.

Hirata T, Kakizuka A, Ushikubi F, Fuse I, Okuma M and Narumiya S (1994) Arg60 to Leu mutation of the human thromboxane A2 receptor in a dominantly inherited bleeding disorder. *J Clin Invest* 94: 1662-1667.

Jakub k J, Bac kov L, El-Fakahany EE. and Tucek S (1997) Positive cooperativity of acetylcholine and other agonists with allosteric ligands on muscarinic acetylcholine receptors. *Mol Pharmacol* 52:172-179.

Karges B, Karges W, Mine M, Ludwig L, Kuhne R, Milgrom E and de Roux N (2003) Mutation Ala(171)Thr stabilizes the gonadotropin-releasing hormone receptor in its inactive conformation, causing familial hypogonadotropic hypogonadism. *J Clin Endocrinol Metab* 88: 1873-1879.

Kenakin T (2003) *A Pharmacology Primer*. Elsevier Academic Press, California and London.

Kenakin T (2005) New concepts in drug discovery: collateral efficacy and permissive antagonism. *Nat Rev Drug Discov* 4: 919-927.

Laue L, Chan WY, Hsueh AJ, Kudo M, Hsu SY, Wu SM, Blomberg L and Cutler GB (1995) Genetic heterogeneity of constitutively activating mutations of the human luteinizing hormone receptor in familial male-limited precocious puberty. *Proc Natl Acad Sci U S A* 92: 1906-1910.

Lazareno S, Dolezal V, Popham A and Birdsall NJ (2004) Thiochrome enhances acetylcholine affinity at muscarinic M_4 receptors: receptor subtype selectivity via cooperativity rather than affinity. *Mol Pharmacol* 65: 257-266.

Li Q, Ye K, Blad CC, den Dulk H, Brouwer J, IJzerman AP and Beukers MW (2007) ZM241385, DPCPX, MRS1706 are inverse agonists with different relative intrinsic efficacies on constitutively active mutants of the human adenosine A_{2B} receptor. *J Pharmacol Exp Ther* 320: 637-645.

Liu G, Duranteau L, Carel JC, Monroe J, Doyle DA and Shenker A (1999) Leydig-cell tumors caused by an activating mutation of the gene encoding the luteinizing hormone receptor. *N Engl J Med* 341: 1731-1736.

MacEwan DJ and Milligan G (1996) Up-regulation of a constitutively active form of the β_2-adrenoceptor by sustained treatment with inverse agonists but not antagonists. *FEBS Lett* 399: 108-112.

May LT, Leach K, Sexton PM and Christopoulos A (2007) Allosteric modulation of G protein-coupled receptors. *Annu Rev Pharmacol Toxicol* 47: 1-51.

Miller KJ, Murphy BJ and Pelleymounter MA (2004) Central G-Protein Coupled Receptors (GPCR)s as molecular targets for the treatment of obesity: assets, liabilities and development status. *Curr Drug Targets CNS Neurol Disord* 3: 357-377.

Milligan G and Bond RA (1997) Inverse agonism and the regulation of receptor number. *Trends Pharmacol Sci* 18: 468-474.

Pagano A, Ruegg D, Litschig S, Stoehr N, Stierlin C, Heinrich M, Floersheim P, Prezeau L, Carroll F, Pin JP (2000) The non-competitive antagonists 2-methyl-6-(phenylethynyl) pyridine and 7-hydroxyiminocyclopropan[b]chromen-1a-carboxylic acid ethyl ester interact with overlapping binding pockets in the transmembrane region of group I metabotropic glutamate receptors. *J Biol Chem* 275: 33750-33758.

Pantel J, Legendre M, Cabrol S, Hilal L, Hajaji Y, Morisset S, Nivot S, Vie-Luton MP, Grouselle D, de Kerdanet M (2006) Loss of constitutive activity of the growth hormone secretagogue receptor in familial short stature. *J Clin Invest* 116: 760-768.

Parma J, Duprez L, Van Sande J, Cochaux P, Gervy C, Mockel J, Dumont J and Vassart G (1993) Somatic mutations in the thyrotropin receptor gene cause hyperfunctioning thyroid adenomas. *Nature* 365: 649-651.

Price MR, Baillie GL, Thomas A, Stevenson LA, Easson M, Goodwin R, McLean A, McIntosh L, Goodwin G, Walker G (2005) Allosteric modulation of the cannabinoid CB1 receptor. *Mol Pharmacol* 68: 1484-1495.

Rao VR, Cohen GB and Oprian DD (1994) Rhodopsin mutation G90D and a molecular mechanism for congenital night blindness. *Nature* 367: 639-642.

Sachpatzidis A, Benton BK, Manfredi JP, Wang H, Hamilton A, Dohlman HG and Lolis E (2003) Identification of allosteric peptide agonists of CXCR4. *J Biol Chem* 278: 896-907.

Sadeghi H, Robertson GL, Bichet DG, Innamorati G and Birnbaumer M (1997) Biochemical basis of partial nephrogenic diabetes insipidus phenotypes. *Mol Endocrinol* 11: 1806-1813.

Schipani E, Kruse K and Juppner H (1995) A constitutively active mutant PTH-PTHrP receptor in Jansen-type metaphyseal chondrodysplasia. *Science* 268: 98-100.

Seminara SB, Messager S, Chatzidaki EE, Thresher RR, Acierno JS, Shagoury JK, Bo-Abbas Y, Kuohung W, Schwinof KM, Hendrick AG (2003) The GPR54 gene as a regulator of puberty. *N Engl J Med* 349: 1614-1627.

Shenker A, Laue L, Kosugi S, Merendino JJ, Minegishi T and Cutler GB (1993) A constitutively activating mutation of the luteinizing hormone receptor in familial male precocious puberty. *Nature* 365: 652-654.

Simoni M, Gromoll J and Nieschlag E (1997) The follicle-stimulating hormone receptor: biochemistry, molecular biology, physiology, and pathophysiology. *Endocr Rev* 18: 739-773.

Smit MJ, Leurs R, Alewijnse AE, Blauw J, Van Nieuw Amerongen GP, Van De Vrede Y, Roovers E, Timmerman H. (1996) Inverse agonism of histamine H_2 antagonist accounts for upregulation of spontaneously active histamine H_2 receptors. *Proc Natl Acad Sci U S A* 93: 6802-6807.

Smits G, Olatunbosun O, Delbaere A, Pierson R, Vassart G and Costagliola S (2003) Ovarian hyperstimulation syndrome due to a mutation in the follicle-stimulating hormone receptor. *N Engl J Med* 349: 760-766.

Sunthornthepvarakui T, Gottschalk ME, Hayashi Y and Refetoff S (1995) Brief report: resistance to thyrotropin caused by mutations in the thyrotropin-receptor gene. *N Engl J Med* 332: 155-160.

Swords FM, Baig A, Malchoff DM, Malchoff CD, Thorner MO, King PJ, Hunyady L and Clark AJ (2002) Impaired desensitization of a mutant adrenocorticotropin receptor associated with apparent constitutive activity. *Mol Endocrinol* 16: 2746-2753.

Tanaka H, Moroi K, Iwai J, Takahashi H, Ohnuma N, Hori S, Takimoto M, Nishiyama M, Masaki T and Yanagisawa M (1998) Novel mutations of the endothelin B receptor gene in patients with Hirschsprung's disease and their characterization. *J Biol Chem* 273: 11378-11383.

Tateyama M and Kubo Y (2006) Dual signaling is differentially activated by different active states of the metabotropic glutamate receptor 1α. *Proc Natl Acad Sci U S A*. 103: 1124–1128.

Urwyler S, Mosbacher J, Lingenhoehl K, Heid J, Hofstetter K, Froestl W, Bettler B and Kaupmann K (2001) Positive allosteric modulation of native and recombinant gamma-aminobutyric acid(B) receptors by 2,6-Di-tert-butyl-4-(3-hydroxy-2,2-dimethyl-propyl)-phenol (CGP7930) and its aldehyde analog CGP13501. *Mol Pharmacol* 60: 963-971.

Vaisse C, Clement K, Durand E, Hercberg S, Guy-Grand B and Froguel P (2000) Melanocortin-4 receptor mutations are a frequent and heterogeneous cause of morbid obesity. *J Clin Invest* 106 : 253-262.

Vasseur C, Rodien P, Beau I, Desroches A, Gerard C, de Poncheville L, Chaplot S, Savagner F, Croue A, Mathieu E (2003) A chorionic gonadotropin-sensitive mutation in the follicle-stimulating hormone receptor as a cause of familial gestational spontaneous ovarian hyperstimulation syndrome. *N Engl J Med* 349: 753-759.

Watson C, Jenkinson S, Kazmierski W and Kenakin TP (2005) The CCR5 receptor-based mechanism of action of 873140, a potent allosteric non-competitive HIV entry-inhibitor. *Mol. Pharmacol* 67: 1268–1282.

Waugh DJ, Gaivin RJ, Damron DS, Murray PA and Perez DM (1999) Binding, partial agonism, and potentiation of alpha(1)-adrenergic receptor function by benzodiazepines: A potential site of allosteric modulation. *J Pharmacol Exp Ther* 291: 1164-1171.

Wu SM, Hallermeier KM, Laue L, Brain C, Berry AC, Grant DB, Griffin JE, Wilson JD, Cutler GB, and Chan WY (1998) Inactivation of the luteinizing hormone/chorionic gonadotropin receptor by an insertional mutation in Leydig cell hypoplasia. *Mol Endocrinol* 12: 1651-1660.

Zhao XM, Hauache O, Goldsmith PK, Collins R, Spiegel AM (1999) A missense mutation in the seventh transmembrane domain constitutively activates the human Ca^{2+} receptor. *FEBS Lett* 448: 180-184.

Chapter

General Conclusions and Perspecti es

General Conclusions

By the research described in this thesis, new insights were gained concerning the identification of inverse agonists for the human adenosine A_{2B} receptor and their intrinsic efficacy. The antagonists ZM241385, DPCPX and MRS1706 exhibited intrinsic efficacy values (α) of 0.14, 0.35 and 0.31, respectively, on 7 constitutively active mutant (CAM) human adenosine A_{2B} receptors as determined with a yeast growth assay. These compounds were therefore classified as inverse agonists (α 1) for the human adenosine A_{2B} receptor.

Subsequently, a series of 9 CAM receptors with varying levels of constitutive activity were used to quantify the effects of the three inverse agonists. These experiments showed that mutated adenosine A_{2B} receptors with various levels of constitutive activity responded differently to these three inverse agonists. Two high level CAM receptors were locked in an active state and were insensitive to the inverse agonists. The three intermediate level CAM receptors were partially inhibited, whereas the four low level CAM receptors were almost completely inhibited.

Next, the two-state receptor model was used to theoretically explain these experimental observations. The response of an inverse agonist and the experimental window were shown to depend not only on the intrinsic efficacy of the inverse agonist, but also on the receptor isomerization constant (L) value which defines the level of constitutive activity of a receptor.

A further study investigated the optimal conditions for the screening of inverse agonists. The data revealed that the optimal window for measurements is achieved when the value of the isomerization constant (L, being the ratio between the active (R*) and inactive (R) states of the receptor thereby determining the constitutive activity of the receptor) equals the reciprocal square root of the intrinsic efficacy (α) of the inverse agonist. When L is smaller than this value, the window will increase with an increase of the constitutive activity of the receptor. On the other hand, when L is bigger than the reciprocal square root of the intrinsic efficacy (α) of the inverse agonist, the window will decrease with an increase of the constitutive activity. In other words, receptors with intermediate levels of constitutive activity should be the most sensitive screening tools to detect inverse agonists.

Concerning receptor activation, site-directed mutant A_{2B} receptors were constructed to investigate the role of the NxxxNPxxY motif and the potential salt bridge between TM1 (E14) and TM7 (H280) in receptor activation. In total 12 mutant receptors were constructed and tested with 5 agonists: NECA, CPA, CGS21680, IBMECA and LUF5833. N282, N286 and Y290 of the NxxxNPxxY motif were subjected to mutation yielding seven point mutants N282Q, N282R, N286A, N286Q, N286R, Y290N and Y290F. Except for the N286A mutant receptor which was expressed at lower levels all constructed mutants were expressed at levels comparable to the wild-type receptor. With respect to the NxxxNPxxY motif, the N282R and N286R receptors behaved more or less similar to the wild-type receptor for all agonists tested. Interestingly, the introduction of a glutamine at position 282 was reasonably well tolerated (N282Q), but the introduction of an alanine or glutamine residue at position 286 (N286A and N286Q) drastically reduced the ability of the agonists to activate the receptor. Whereas the responsiveness of the N286A mutant receptor could at least partially be ascribed to reduced receptor expression levels, the N286Q was expressed at wild-type levels. Mutation of Y290 to N and F revealed that this residue is very sensitive to mutagenesis also. Whereas the conservative Y290F mutant still responded quite well to the agonists, the Y290N receptor could no longer be activated despite the fact that this receptor was expressed at normal levels.

To study the role of the potential salt bridge, three mutants were made. Mutation of H280E and E14H/H280E abolished receptor expression. Apparently the salt bridge in the adenosine A_{2B} receptor can not be swapped as the H280E mutation interferes with receptor expression. Moreover, although the E14H mutant receptor was normally expressed no activation by any of the tested agonists could be identified. Interestingly, based on a rhodopsin-based homology model of the adenosine A_{2B} receptor, the proximity of I61 to the salt bridge suggested a potential role for this amino acid. To evaluate the role of this residue I61A, I61D and I61K mutant receptors were made. None of these mutants responded to any of the tested agonists despite normal expression levels.

Apparently, both the potential salt bridge and the NxxxNPxxY motif play an important role in the process of receptor activation explaining why most changes at this region impair receptor activation. This result is consistent with our previous findings that none of the gain-of-function mutant receptors identified from the random mutation bank (Beukers et al., 2004) contained mutations in the conserved NPxxY motif or the NxxxNPxxY motif. Based on the experiments performed, we are not able to indicate whether effects on receptor activation are also reflected in ligand binding. The location of the mutated residues would suggest however that ligand binding would not be altered for the mutants that were properly expressed.

Based on the crystal structures of the human β_2 adrenergic receptor and bovine rhodopsin, two homology models of the adenosine A_{2B} receptor were made. Analysis of these two models revealed subtle differences in the length and angle of helixes, the turn between helix 7 and helix 8 and rotamers of interacting aromatic residues at the cytosolic side of helix 7 and in helix 8. These differences actually account for changes in the intramolecular interactions within the two models of the adenosine A_{2B} receptor, despite the fact that GPCRs share a common overall structure. Thus we conclude that receptor activation is a receptor-specific phenomenon as shown by site directed mutagenesis experiments addressing the conserved NPxxY motif and the characteristic adenosine receptor salt bridge.

A comparison of inverse agonists and allosteric modulators to treat disease-related receptor mutations caused by constitutive activity indicated that allosteric ligands display a more complicated interaction with a receptor/endogenous ligand pair and are able to cooperatively modify receptor binding and function. Unlike the orthosteric ligands that only compete with endogenous ligands, a proper allosteric ligand, i.e. a ligand with appropriate binding cooperativity and functional cooperativity, may be able to reverse the mutant receptor phenotype into the wild-type phenotype. Thus allosteric modulators but not inverse agonists are able to reverse constitutive activity without affecting the EC_{50} value of the endogenous agonist thereby providing in theory a more physiological treatment to diseases stemming from constitutively active GPCR mutations.

Perspecti es

irect read-out for human adenosine A_{2B} receptors

As a model, we use the adenosine A_{2B} receptors (wild-type and mutants) expressed in yeast and measure cell growth as a read-out for GPCR activity. This system is ideally suited to select mutant receptors of interest from a large pool of mutant receptors. However, strictly speaking, yeast growth is not a direct reflection of GPCR activity. Activation of A_{2B} receptors expressed on the yeast membrane results in the activation of the *Fus1* promoter and induces

the synthesis of imidazole glycerolphosphate dehydratase, a key enzyme in the synthesis of histidine. Thus yeast growth in histidine-deficient medium is positively correlated rather than proportional to the activation of A_{2B} receptors. Supplementary data may therefore be obtained by expression in a mammalian system, by investigating more proximal read-outs such as GTP-dependent adenylyl cyclase activity or, even better, guanine nucleotide exchange.

easure receptor acti ation in mammalian cells

Compared to mammalian cells, yeast cells are more convenient in both random and site-directed mutagenesis studies as these cells take up a single plasmid. The combination of such a mutagenesis approach and a robust screening assay make yeast cells a great tool to investigate receptor activation in an indirect way. However, some differences between yeast and mammalian cells, for example post-translational modifications, may need to be taken into consideration. It is known that yeast *S. cerevisiae* cells can only synthesize core or high mannose oligosaccharides and do not have the ability to synthesize mature or complex oligosaccharides (Miret et al., 2002). As a result rhodopsin expressed in yeast *S. cerevisiae* has a glycosylation pattern different from natively purified bovine rhodopsin as revealed with SDS/PAGE and in an endoglycosidase H sensitivity assay (Mollaaghababa et al., 1996).

Another difference between yeast and mammalian cells is the lipid composition of the membrane which may affect the behavior of heterologously expressed receptors in yeasts. Lagane and his colleges demonstrated that the loss of agonist affinity of the human μ opioid receptor expressed in *S. cerevisiae* could be simply restored by replacing ergosterol from yeast with cholesterol which is normally found in mammalian rather than yeast plasma membranes (Lagane et al., 2000).

Depending on the receptor of interest and the research questions at hand these differences between yeast and mammalian expression systems should be taken into consideration. In some cases it may be useful to supplement experiments in yeast with experiments in mammalian cells. Alternatively, a direct translation from *in vitro* cells to for example brain slices or *in vivo* research may be considered. An example where substantiation of yeast experiments with experiments in mammalian cells was useful has been described for the muscarinic M_3 receptor. At first a random mutagenesis study of the rat M_3 muscarinic acetylcholine receptor was performed in *S. cerevisiae* yielding 174 mutants with the desired phenotype were identified. To confirm this result, mutants were transfected in COS7 cells and 159 out of 174 mutants showed consistent behavior in yeast and mammalian cells (Li et al., 2007).

In our study of the adenosine A_{2B} receptor, the phenotype with regard to the interaction with ligands of several mutants identified from the random mutagenesis screen in yeast were confirmed in mammalian cells. These findings suggest that the translation from yeast to mammalian cells for this receptor subtype seems to be straightforward. It would be interesting nevertheless to extend findings obtained in yeast to mammalian cells and preferably *in vivo* test systems. Such extended investigations will enable the identification of ligand-selective down-stream signaling pathways and of interactions with other protein partners.

Conser ed GPCR motifs

There has been a sustained effort to elucidate the functional mechanisms of GPCRs, including their ability to undergo conformational changes and activate G proteins (Schwartz et al.,

2006). The functional role of the two Ns and Y in the conserved NxxxNPxxY motif of the human adenosine A_{2B} receptor was investigated in our study (Chapter 5). There are other conserved GPCR motifs, for example, the E/DRY motif. This triplet of amino acids located at the boundary between transmembrane domain (TM) III and intracellular loop 2 of class A GPCRs, has been suggested to play a pivotal role in regulating GPCR conformational states (Rovati et al., 2006). Mutation studies of charged residues in the E/DRY motif of class A GPCRs revealed a role for this motif in G protein coupling, receptor expression, ligand binding, ligand-depend receptor activation and constitutive receptor activation (for reviews, see Rovati et al., 2006 and Flanagan 2005). In his review, Rovati pointed out that there is no rule for the function of this motif despite its high conservation. If every receptor has its own story, it would be interesting to investigate the role the E/DRY motif plays in the adenosine A_{2B} receptor.

The cytosolic part of helix 7 and helix 8 also deserves more detailed investigation. It is well known that π-π interactions exist between Y290 in helix 7 and F297 in helix 8. Mutations which disturb this interaction result in receptors with impaired signaling ability, as has been demonstrated for the CCR5 receptor, neurokinin-1 receptor and M_1 muscarinic acetylcholine receptor (Aramori et al., 1997; B hm et al., 1997; Lu et al., 2001). In our study (see Chapter 5), two mutants were constructed; Y290F showed less activation than the wild-type receptor and Y290N could not be activated at all. To investigate whether π-π interactions exist between Y290 and F297, a swapped Y290F/F297Y mutant receptor should be constructed to see if the phenotype of the wild-type receptor is regained. An important control would be to make single point F297 mutations also to rule out any unforeseen effects like we encountered while investigating the salt bridge between E14 and H280. In that case mutation of H280 was detrimental to receptor expression.

Helix 8 is an important helix although it has been studied much less extensively. It is located on the cytosolic side of the membrane, and it is suggested to be involved in cell membrane location, binding with cell skeleton, internalization and G protein coupling (Bermak et al., 2001; Feng et al., 2003). It is interesting to note that adenosine receptors possess a cluster of aromatic amino acids at the end of helix 7 and helix 8. In the A_{2B} receptor, Y290, F297 and F301 may interact in pairs. A hydrophobic residue (I) was found 4 amino acids downstream of this cluster. Such a pattern is not only conserved among adenosine receptors, but also present in many other receptors, for example rhodopsin and the β_2 adrenergic receptor (figure 1). If conservation is a measure for importance, then this aromatic cluster would be interesting to be investigated. Residue F (in grey box in figure 1) located in helix 8 is one of the residues thought to form π-π interactions with residues in helix 7. In addition, the residues 4 and 8 amino acids downstream of this F residue are also hydrophobic (see black box in figure 1).

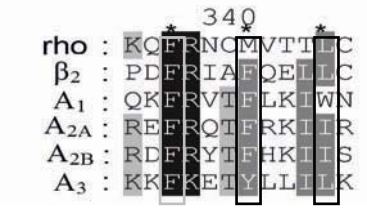

Figure 1. Alignment of helix 8 of human adenosine A_1, A_{2A}, A_{2B}, A_3, human β_2 adrenergic receptors and bovine rhodopsin. Conserved residues are marked in black. Residue F located in helix 8 is located in the grey box, whereas the black boxes highlight the hydrophobic residues present 4 and 8 amino acids downstream of this F residue.

Allosteric modulators versus inverse agonists

Constitutive activity, the spontaneous basal signalling activity of GPCRs in the absence of agonists, has been identified for a considerable number of GPCRs, mutant as well as wild-type receptors (de Ligt et al., 2000). Quite a few constitutively active mutant receptors are disease-related. For example, night blindness, diabetes insipidus, and male precocious puberty are caused by constitutively active mutations of rhodopsin, the V_2 vasopressin receptor and the LH receptor, respectively (Rao et al., 1994; Morello et al., 2000; Shenker et al., 1993). Inverse agonists have therapeutic benefits for the treatment of constitutive activity-related diseases because these compounds are able to reduce the constitutive activity of the receptors. However, as demonstrated in Chapter 6, inverse agonists will also reduce the sensitivity of the receptor to its endogenous ligand due to competition.

Besides inverse agonists, allosteric modulators may be a great supplement to the available ligand repertoire due to their ability to selectively increase or decrease receptor activity without affecting the EC_{50} value of the endogenous ligand. In fact, the tightly controlled release and synthesis of endogenous ligands is retained while the allosteric modulator itself only potentiates or dampens this response rather than having an effect by itself. The potential to use allosteric modulators to treat particular diseases stemming from increased constitutive receptor activity warrants further exploration.

In this thesis, a combined approach was described to investigate the constitutive activity of G protein coupled receptors (GPCRs) using human adenosine A_{2B} receptors as a prototype and to evaluate disease-related constitutive GPCR activity as a target for treatment. A yeast expression system proved to be a very versatile experimental tool to easily follow receptor activation. Integration of the results of these studies with pharmacological and theoretical receptor models laid the foundation for a deeper and detailed understanding of a receptor's constitutive activity. In future research, a combination of more physiological expression systems together with such receptor models should be investigated to validate the therapeutic application of inverse agonists and allosteric modulators, and to discriminate between these two ligand categories.

References

Aramori I, Ferguson SS, Bieniasz PD, Zhang J, Cullen B and Cullen MG (1997) Molecular mechanism of desensitization of the chemokine receptor CCR-5: receptor signaling and internalization are dissociable from its role as an HIV-1 co-receptor. *Embo J* 16: 4606-4616.

Bermak JC, Li M, Bullock C and Zhou QY (2001) Regulation of transport of the dopamine D_1 receptor by a new membrane-associated ER protein. *Nat Cell Biol* 3:492-498.

Bohm SK, Khitin LM, Smeekens SP, Grady EF, Payan DG and Bunnett NW (1997) Identification of potential tyrosine-containing endocytic motifs in the carboxyl-tail and seventh transmembrane domain of the neurokinin 1 receptor *J Biol Chem* 272: 2363-2372.

de Ligt RA, Kourounakis AP and IJzerman AP (2000) Inverse agonism at G protein-coupled receptors: (patho)physiological relevance and implications for drug discovery. *Br J Pharmacol* 130:1-12.

Feng GJ, Kellett E, Scorer CA, Wilde J, White JH, Milligan G (2003) Selective interactions between helix VIII of the human mu-opioid receptors and the C terminus of periplakin disrupt G protein activation. *J Biol Chem* 278: 33400-33407.

Flanagan, CA (2005) A GPCR that is not DRY . *Mol Pharmacol* 68:1-3.

Kenakin T (2005) New concepts in drug discovery: collateral efficacy and permissive antagonism. *Nat Rev Drug Discov* 4:919-927.

Lagane B, Gaibelet G, Meilhoc E, Masson JM, Cezanne L and Lopez A (2000) Role of sterols in modulating the human mu-opioid receptor function in Saccharomyces cerevisiae. *J Biol Chem* 275: 33197-33200.

Li B, Scarselli M, Knudsen CD, Kim SK, Jacobson KA, McMillin SM and Wess J (2007a) Rapid identification of functionally critical amino acids in a G protein-coupled receptor. *Nat Methods* 4:169-174.

Lu ZL, Saldanha JW and Hulme EC (2001) Transmembrane domains 4 and 7 of the M_1 muscarinic acetylcholine receptor are critical for ligand binding and the receptor activation switch. *J Biol Chem* 276: 34098-34104.

Miret JJ, Rakhilina L, Silverman L and Oehlen B (2002) Functional expression of heteromeric calcitonin gene-related peptide and adrenomedullin receptors in yeast. *J Biol Chem* 277: 6881-6887.

Mollaaghababa R, Davidson FF, Kaiser C and Khorana HG (1996) Structure and function in rhodopsin: expression of functional mammalian opsin in Saccharomyces cerevisiae. *Proc Natl Acad Sci USA* 23:11482-11486.

Morello JP, Salahpour A, Laperriere A, Bernier V, Arthus MF, Lonergan M, Petaja-Repo U, Angers S, Morin D, Bichet DG and Bouvier M (2000) Pharmacological chaperones rescue cell-surface expression and function of misfolded V_2 vasopressin receptor mutants. *J Clin Invest* 105:887-895.

Rao VR, Cohen GB and Oprian DD (1994) Rhodopsin mutation G90D and a molecular mechanism for congenital night blindness. *Nature* 367:639-642.

Rovati GE, Capra V and Neubig RR (2007) The highly conserved DRY motif of class A G protein-coupled receptors: beyond the ground state. *Mol Pharmacol* 71:959-964.

Schwartz TW, Frimurer TM, Holst B, Rosenkilde MM and Elling CE (2006) Molecular mechanism of 7TM receptor activation--a global toggle switch model. *Annu Rev Pharmacol Toxicol* 44: 481-519.

Shenker A, Laue L, Kosugi S, Merendino JJ, Minegishi T and Cutler GB (1993) A constitutively activating mutation of the luteinizing hormone receptor in familial male precocious puberty. *Nature* 365:652-654.

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In this thesis a combined approach is described to investigate the constitutive activity of G protein protein-coupled receptors (GPCRs) using human adenosine A_{2B} receptors and to evaluate disease-related constitutive GPCR activity as a target for treatment. To this end a yeast expression system together with pharmacological and theoretical receptor models have been applied.

In Chapter 2 the advantages of yeasts as tools to study GPCRs are reviewed. Adapted yeast cells able to communicate with mammalian GPCRs have become available and provide a very convenient system to express mutated receptors. A major advantage of yeast cells over mammalian cells extends from easy culturing conditions to the characteristic of yeast cells to allow entry of only a single plasmid. This latter property in combination with the robust screening assay based on yeast growth makes them an ideal test system to study randomly as well as site-directed mutated receptors. In chapters 3 to 5 this yeast growth assay is the main experimental tool to evaluate the functional properties of random and site-directed mutant receptors.

In chapters 3 and 4 the yeast system is exploited to study inverse agonism of the human adenosine A_{2B} receptor. At first, constitutively active mutant (CAM) human adenosine A_{2B} receptors have been used to discriminate inverse agonists of the adenosine A_{2B} receptor from A_{2B} receptor antagonists. As a result, three inverse agonists ZM241385, DPCPX and MRS1706 were identified and their rank order of efficacy determined. Moreover, an interesting system-dependent phenomenon was noticed, that is the intrinsic activities of the inverse agonists were affected by the level of constitutive activity. It was demonstrated that inverse agonists show the greatest intrinsic activity on receptors displaying a medium level of constitutive activity.

To further investigate the relationship between the effectiveness of an inverse agonist and the level of constitutive activity of the receptor, the two-state receptor model was introduced in both Chapter 3 and Chapter 4. According to this two-state model, both the receptor isomerization constant (L) and the intrinsic efficacy (α) of the inverse agonist determines the sensitivity to detect the intrinsic activity of an inverse agonist which is reflected by the observed experimental window. The biggest experimental window can be achieved on receptors with an L value equaling the reciprocal square root of α. Our experiments show that mutant A_{2B} receptors with an intermediate level of constitutive activity possess the greatest experimental window, whereas mutants with a low level of constitutive activity showed small experimental windows and highly constitutively active mutants did not respond to our tested inverse agonists. Based on these findings we conclude that receptors with intermediate levels of constitutive activity should be the most sensitive screening tools for detecting inverse agonists.

In Chapter 5 the activation of the human adenosine A_{2B} receptor was investigated. To investigate the role of the NxxxNPxxY motif and the potential salt bridge between TM1 (E14) and TM7 (H280) in receptor activation, site-directed mutagenesis was applied to yield 15 mutant A_{2B} receptors. The mutations were selected based on an adenosine A_{2B} receptor model using the structure of bovine rhodopsin as a template. The expression levels of these mutants were determined by western blot analysis and the activation of the receptors was measured in the presence or absence of the following agonists NECA, CPA, CGS21680, IBMECA and

LUF5833. None of the mutant receptors displayed constitutive activity. On the contrary, most mutants had a reduced potency and/or efficacy, e.g. mutants N282Q, N282R, N286A, N286Q, N286R, and Y290F showed impaired activation and mutants Y290N, E14H, H280E, E14H/H280E, I61A, I61D and I61K could not be activated by any of the agonists tested. Among all the mutants constructed, only N282R and N286R receptors behaved similarly to the wild-type receptor. Moreover, mutant N286A reduced receptor expression and H280E and E14H/H280E abolished receptor expression. These results suggest an important role for the NxxxNPxxY motif and the potential salt bridge in receptor expression and activation.

The recent publication of the β_2-adrenergic receptor structure enabled us to construct a second model of the adenosine A_{2B} receptor. Comparison of the two A_{2B} receptor models based on these two different templates is also described in Chapter 5. The various effects caused by mutations in the NxxxNPxxY motif and the potential salt bridge of different receptors in both our experiments and from literature do suggest that receptor activation is a receptor-specific phenomenon.

In Chapter 6 we provide a theoretical investigation of the treatment of disease-related constitutively active receptor mutations. Comparison of the characteristics of allosteric ligands with traditional orthosteric ligands using a two-state allosteric model predicts that allosteric ligands display a more complicated interaction with a receptor/endogenous ligand pair and are able to cooperatively modify receptor binding and function. As a result allosteric modulators may affect the level of constitutive activity without changing the potency of the endogenous ligand. Thus allosteric modulators may provide advantages over orthosteric ligands in the treatment of diseases caused by constitutively active GPCR mutations.

Finally in Chapter 7, general conclusions about the research described in this thesis are drawn. This is also supplemented by an outlook on some potential aspects of research to be pursued, based upon the application of receptor models, pharmacology models and functional receptor assays.

amen attin

In dit proefschrift wordt een gecombineerde aanpak besproken om aan de hand van de adenosine A_{2B} receptor de constitutieve activiteit van aan G-eiwitten gekoppelde receptoren (GPCRs) te bestuderen en daarnaast de mogelijkheden te evalueren om ziekte-gerelateerde constitutief actieve receptoren te gebruiken als aangrijpingspunt voor behandeling. Daartoe is gebruik gemaakt van een expressiesysteem in gist tezamen met farmacologische en theoretische receptormodellen.

In hoofdstuk 2 wordt een overzicht gegeven van de voordelen van het gebruik van gist als hulpmiddel om GPCRs te onderzoeken. Gemodificeerde gistcellen die kunnen communiceren met GPCRs afkomstig van zoogdieren zijn beschikbaar gekomen en bieden een heel geschikt systeem om gemuteerde receptoren tot expressie te brengen. Grote voordelen van gistcellen ten opzichte van zoogdiercellen zijn onder andere de eenvoudige kweek en hun karakteristieke eigenschap om maar n enkel plasmide op te nemen. Deze laatste eigenschap in combinatie met de robuuste meetmethode gebaseerd op gistgroei maakt deze gistcellen een ideaal testsysteem om de gemuteerde receptoren te bestuderen, of ze nu willekeurige dan wel plaatsspecifieke mutaties bevatten. In de hoofdstukken 3 t/m 5 is deze meetmethode gebaseerd op de groei van gist het voornaamste middel om de functionele eigenschappen van de willekeurig en plaatsspecifiek gemuteerde receptoren te onderzoeken.

In de hoofdstukken 3 en 4 wordt dit gistsysteem gebruikt om invers agonisme van de adenosine A_{2B} receptor van de mens te bestuderen. Allereerst zijn de constitutief actief gemuteerde (CAM) adenosine A_{2B} receptoren van de mens gebruikt om onderscheid te maken tussen inverse agonisten en antagonisten voor de A_{2B} receptor. Op basis hiervan werden de inverse agonisten ZM241385, DPCPX en MRS1706 ge dentificeerd en werd de volgorde van werkzaamheid bepaald. Bovendien werd een interessant systeemafhankelijk fenomeen opgemerkt, namelijk dat de intrinsieke activiteit van de inverse agonisten be nvloed wordt door de mate van constitutieve activiteit. De inverse agonisten bleken de grootste intrinsieke activiteit te vertonen op receptoren met een gemiddeld niveau van constitutieve activiteit.

Om de relatie tussen de effectiviteit van een inverse agonist en de mate van constitutieve activiteit van de receptor verder te onderzoeken hebben we het two-state' receptormodel ge ntroduceerd in hoofdstuk 3 en 4. Volgens dit two-state' receptormodel bepalen zowel de receptor isomerisatieconstante (L) als de intrinsieke activiteit (α) van de inverse agonist de gevoeligheid waarmee de intrinsieke activiteit van de inverse agonist bepaald kan worden; dit wordt weerspiegeld in het waargenomen experimentele meetbereik. Het grootste experimentele meetbereik wordt verkregen met receptoren die een L waarde bezitten die gelijk is aan de omgekeerde wortel van α. Onze experimenten laten zien dat gemuteerde receptoren met een gemiddeld niveau van constitutieve activiteit het grootste experimentele meetbereik hebben, terwijl mutanten met een lage constitutieve activiteit kleine experimentele meetbereiken hebben en mutanten met een hoge constitutieve activiteit niet reageren op de door ons geteste inverse agonisten. Op grond van deze bevindingen concluderen we dat receptoren met een gemiddeld niveau van constitutieve activiteit het meest gevoelige meetinstrument zullen zijn voor de detectie van inverse agonisten.

In hoofdstuk 5 is de activering van de adenosine A_{2B} receptor van de mens onderzocht. Plaatspecifieke mutaties zijn gebruikt om 15 gemuteerde A_{2B} receptoren te maken waarmee

de rol van het NxxxNPxxY motief en de veronderstelde zoutbrug tussen het transmembranaire domein 1 (E14) en het transmembranaire domein 7 (H280) te bestuderen. De mutanten werden geselecteerd op basis van een adenosine A_{2B} receptormodel met gebruikmaking van rhodopsine als matrijs. De expressieniveaus van deze mutanten werd bepaald met western blot analyse en de activering van de receptoren werd gemeten in de aan- en afwezigheid van de volgende agonisten: NECA, CPA, CGS21680, IBMECA en LUF5833. Geen van de gemuteerde receptoren vertoonde constitutieve activiteit. In tegendeel, de meeste mutanten hadden een verlaagde werkzaamheid en/of effectiviteit, dat wil zeggen de N282Q, N282R, N286A, N286Q, N286R en Y290F mutanten vertoonden een verminderde activering en de Y290N, E14H, H280E, E14H/H280E, I61A, I61D en I61K mutanten konden niet geactiveerd worden door de onderzochte agonisten. Van al deze gemaakte mutanten gedroegen alleen de N282R en de N286R receptoren zich vergelijkbaar aan de wild-type receptor. Bovendien vertoonde de N286A mutant een verlaagde receptorexpressie en was de expressie van de H280E en de E14H/H1280E receptor verdwenen. Deze resultaten suggereren een belangrijke rol voor het NxxxNPxxY motief en de veronderstelde zoutbrug in receptorexpressie en -activering.

De recente structuuropheldering van de β_2-adrenerge receptor heeft het ons mogelijk gemaakt om een tweede model voor de adenosine A_{2B} receptor te construeren. Een vergelijking van de twee A_{2B} receptormodellen gebaseerd op deze twee verschillende matrijzen is ook in hoofdstuk 5 beschreven. De verscheidene effecten die veroorzaakt worden door de mutaties in het NxxxNPxxY motief en de veronderstelde zoutbrug in verschillende receptoren, zowel in onze experimenten als in de literatuur, suggereren dat receptoractivering een receptor-specifiek fenomeen is.

In hoofdstuk 6 doen we een theoretisch onderzoek naar de behandeling van ziektegerelateerde constitutief actieve receptormutaties. Een vergelijking van de eigenschappen van allostere liganden ten opzichte van traditionele orthostere liganden voorspelt op basis van het two-state' allostere receptormodel dat allostere liganden een meer gecompliceerde interactie vertonen met de combinatie van receptor en endogeen ligand en dat ze in staat zijn om receptorbinding en receptorfunctie co peratief te modificeren. Daardoor kunnen allostere modulatoren de mate van constitutieve activiteit veranderen zonder dat de effectiviteit van het endogene ligand wijzigt. Met andere woorden, allostere modulatoren kunnen voordelen bieden ten opzichte van orthostere liganden in de behandeling van ziekten die veroorzaakt worden door constitutief actieve GPCR mutaties.

Tot slot worden in hoofdstuk 7 algemene conclusies getrokken over het onderzoek zoals beschreven in dit proefschrift. Op basis van de toepassing van de receptor- en farmacologische modellen en functionele receptormeetmethoden wordt vooruitgeblikt naar verder onderzoek.

ist of Pu lications

Qilan Li, Kai Ye, Hans den Dulk, Jaap Brouwer, Ad P. IJzerman, Margot W. Beukers. Measuring negative intrinsic efficacy: the role of constitutive activity levels.
Submitted

Qilan Li, Kai Ye, Clara C. Blad, Hans den Dulk, Jaap Brouwer, Ad P. IJzerman, Margot W. Beukers. ZM241385, DPCPX and MRS1706 are inverse agonists with different relative intrinsic efficacies on constitutively active mutants of the human adenosine A_{2B} receptor. *Journal of Pharmacology and Experimental Therapeutics* 2007 Feb; 320(2):637-45.

Qilan Li, Xin Liu, Rong Xiang, Xiaolin Qu, Yipeng Qi. The Apoptosis-Inducing Function of HAP Depends on Its ER Localization.
Chinese Journal of Biochemistry and Molecular Biology 2004 20(4):52 -533

Kai Ye, Qilan Li, Hong Xu, Kironde Fred Alexander S, Huiwen Ma. Immune characteristics of plasmid DNA containing Plasmodium falciparum Pf70 gene fragment.
Chinese Journal of Microbiology and Immunology 2003 23 (5):35 -363

Xiaoling Qu, Yipeng Qi, Ping Lan, Qilan Li. The novel endoplasmic reticulum (ER)-targeted protein HAP induces cell apoptosis by the depletion of the ER Ca^{2+} stores
EBS Letters 2002 52 :325-331

Curriculum Vitae

Qilan Li was born on the 15th of May 1977 in HuBei, China. In 1994, after finishing secondary school, she started biology education at Central China Normal University and obtained her bachelor degree in 1998. From then on, she chose a teaching job in New Century Primary School in Wuhan, China. In 2000, she continued her study as a master student in Microbiology department at Wuhan University. During this stage, she performed two internships: one in the group of prof Dr. Yipeng Qi (Molecular Biology), investigating the mechanism of an apoptosis inducing gene *hap*; the other is preparing Plasmodium falciparum Pf70 gene vaccine in the group of prof Dr. Huiwen Ma (pharmacology). After graduation in the end of 2003, she attended a cooperation programme between Chinese Universities and Leiden University and moved from China to the Netherlands. In the end of 2004, she obtained an Mphil (master of philosophy) degree in Leiden University. After that, she immediately continued her study with her Ph.D. project in the lab of prof. Dr. Ad IJzerman (Medicinal Chemistry, LACDR) and in collaboration with the lab of Prof. Dr. Jaap Brower (Molecular genetics, Leiden University) in the beginning of 2005. The performed work, described within this thesis, is a combined investigation of the constitutive activity of G protein protein-coupled receptors (GPCRs) using wild-type and mutant human adenosine A_{2B} receptors and to evaluate disease-related constitutive GPCR activity as a target for treatment.

ist of re iations

3-AT	3-aminotriazole
α	intrinsic efficacy
BAY 60-6583	(2-[6-Amino-3,5-dicyano-4-(4-cyclopropylmethoxy-phenyl)–pyridin -2-ylsulfanyl]-acetamide)
CAM	constitutively active mutant
CGS15943	9-chloro-2-(2-furanyl)-[1,2,4]triazolo[1,5-c]quinazolin-5-amine
CGS21680	2-[4-(2-carboxyethyl)phenethylamino]-5'-*N*- ethylcarboxamidoadenosine
CPA	N^6-cyclopentyladenosine
DPCPX	1,3-dipropyl-8-cyclopentylxanthine
EC_{50}	Effective concentration (50%)
GDP	Guanosine diphosphate
GPCR	G protein-coupled receptor
GTP	Guanosine triphosphate
IB-MECA	N^6-(3-iodobenzyl)adenosine-5'-*N*-methyluronamide
IC_{50}	Inhibitory concentration (50%)
K_A	equilibrium dissociation constant
L	isomerization constant
MRS1706	*N*-(4-acetylphenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3–dipropyl-1H -purin-8-yl)phenoxy]acetamide
MRS1754	[N-(4-cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3 -dipropyl-1H -purin-8-yl)phenoxy] acetamide]
MRE2029F20	[N-benzo[1,3]dioxol-5-yl-2-[5-(2,6-dioxo-1,3-dipropy l-2,3,6,7-tetrahydro-1*H*-purin-8-yl)-1-methyl-1*H*-pyrazol-3-yloxy]-acetamide
NECA	*5'-N*-ethylcarboxamidoadenosine
OSIP339391	N-(2- 2-Phenyl-6-[4-(3-phenylpropyl)-piperazine-1-carbonyl] -7H -pyrrolo[2,3-d]pyrimidin-4-ylamino -ethyl)-acetamide
PD81,723	(2-Amino-4,5-dimethyl-3-thienyl)-[3-(trifluoromethyl) phenyl]methanone
(S)-PHPNECA	(S)-2-phenylhydroxypropynyl-5'-N-ethylcarboxamidoadenosine
wt	wild-type
ZM 241385	(4-(2-[7-amino-2-(2-furyl 1,2,4 -triazolo 2,3-a 1,3,5 triazin-5-yl-aminoethyl)phenol

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Prof. D.D. Breimer met me 5 years ago in Wuhan University and recommended me to Prof. Ad IJzerman. This recommendation brought me a great opportunity to continue my study as a PhD student in the Medicinal Chemistry group in Leiden University, which is gratefully acknowledged. It has been a pleasure to work in such a hospitable and inspiring environment which I enjoyed for over 4 years.

Within these 4 years, I got numerous help from various people. I would like to begin with those who provided the greatest practical contributions.

Hans den Dulk in the Molecular Genetics group supervised me in all the molecular biology work since I was an Mphil student. He provided great help in making various site-directed mutant A_{2B} receptors. When I ran into trouble, I could always turn to help from him.

Clara setup most yeast assay protocols and made a detailed record of all the mutants from the random mutation bank, which is the base ground of this thesis. She also helped me a lot to become familiar with the procedure of yeast assays.

I am very grateful for the help from other colleagues as well: Tineke in the Molecular Genetics group provided me technical help on dealing with yeasts; Henk helped me with the westernblot; Thea and Jacobien helped me with the radioligand binding assays; Kai gave me theoretical support to explain my experimental data and helped me to become familiar with several software programs: Matlab, Pymol and so on.

I appreciate the help outside the working hours. Hans Brussee, Hans den Dulk, Jeroen, Jacobus, Eelke, Henk, Remon, Jurre and Marloes, Yue tao, Haiyan Liu, Junjun Shan, Xingfu Xu, Linhua Jiang, Zhi Ding There is a long list with the names of those who helped me to settle down and have an easy life in a foreign county that is far away and quite different of my home country. Thanks to them, I could focus on my work without being disturbed by trivial affairs.

Last but not the least, I would like to give my acknowledgements to my family members. Kai and I together tried our best to solve the problems we met during our stay in Holland; and he was the most important support for me in these years. I am also grateful to my parents for their understanding and supporting me to study abroad. I hope they will find this thesis to be their most precious present.

TELL GE

behorende bij het proefschrift

Constitutive receptor activation and pharmacological modeling

The adenosine A_{2B} receptor as a prototype

1. To detect inverse agonists with low efficacy or to rank compounds based on their intrinsic efficacy, receptors with intermediate levels of constitutive activity provide a better test system than receptors with either a rather high constitutive activity or negligible constitutive activity.
This thesis

2. The assumption that the inhibitory effect of an inverse agonist will increase with an increase in constitutive activity of the receptors is valid only for highly efficacious inverse agonists.
This thesis

3. Although all class A receptors share a similar backbone consisting of 7 transmembrane domains linked by extracellular and intracellular loops, each receptor has its own characteristics.
This thesis

4. Unlike orthosteric ligands, the allosteric compounds are able to modulate independently the initial activity and the maximal receptor response without affecting the potency of the endogenous ligands.
This thesis

5. It is believed that an inhibitory effect of an inverse agonist will increase with an increase in constitutive activity of the receptor it interacts with. However, this is not always true.
Seifert and Wenzel-Seifert, Naunyn-Schmiedeberg's Arch Pharmacol. 2002, 366:381-416; This thesis

6. The three available ligand-bound GPCR structures suggest that there is no generally conserved receptor binding pocket. Rather, the pocket itself can vary in position and orientation, yielding more opportunity for receptor diversity and ligand selectivity.
Jaakola et al., Science 2008

7. When compared to the β_2AR crystal structure, all β_2AR models based on the structure of rhodopsin were more similar to rhodopsin rather than β_2AR. This is not entirely surprising but highlights a general shortcoming in homology models generated from a single structural template.
Cherezov V, et al., Science. 2007, 318:1253-4

8. Receptor models can be used to simulate and fit data and yield quantitative parameters that can be used to measure drug activity, system responsiveness, and ligand behavior. However, the shortcoming of these models is that they require predefinition of the receptor species and this is not always obvious.
Kenakin T. J Recept Signal Transduct Res. 2008, 28:109-25

9. Libraries are not made; they grow.
Augustine Birrell

10. It is a capital mistake to theorize before one has data.
Sir Arthur Conan Doyle

11. He who would distinguish the true from the false must have an adequate idea of what is true and false.
Benedict

12. By indirections find directions out.
William Shakespeare

ilan Li Leiden 18th ecember 2008