The GTPase Assay as a Highly Sensitive Model System for Characterization of Human Cannabinoid Receptors and their Ligands

$G\alpha_{i2}$ Co-Expression and Fusion Studies and the Impact of RGS Proteins

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Erfahrung ist der Anfang aller Kunst und jedes Wissens.

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1 General Introduction

1.1 G protein coupled receptors

1.1.1 Preliminary remarks

G protein coupled receptors (GPCRs) are the largest known gene family of the human genome and the most versatile class of cell surface proteins. A wide range of extracellular messengers such as biogenic amides, lipids, peptides and proteins, odorants and tastants, hormones, neurotransmitters, ions and even photons exert their signals through GPCRs. With specific manipulation of GPCR signaling a diverse array of physiological and pathophysiological processes can be modified and therefore GPCRs are of high therapeutic value for existing and emerging drug therapies. All GPCRs share a common structural architecture consisting of seven transmembrane (7-TM) segments that are connected by extracellular and intracellular loops (Fredriksson et al., 2003). The 7-TM domain is framed by an extracellular N-terminus and an intracellular C-terminus. Classically, the GPCRs mediate signals by coupling to heterotrimeric G proteins, but it became increasingly apparent that they can also transduce signals through other proteins (Rajagopal et al., 2005). As a consequence of these G protein independent mechanisms, it is actually recommended to replace the term GPCR by “7-TM receptor” or “serpentine receptor”, but the GPCR terminology is more established. Nevertheless, GPCRs can generally be grouped into six main families which are Class A Rhodopsin-like receptors, Class B Secretin-like receptors, Class C Metabotropic glutamate receptors, Class D Pheromone receptors, Class E cAMP receptors and Class F Frizzled/smoothening family (Horn et al., 2003).

1.1.2 GPCR activation

Binding of an agonist to a GPCR induces a conformational change of the receptor. This conformation leads to an interaction with heterotrimeric G proteins accompanied by the release of bound GDP which is immediately replaced by GTP (see Figure 1.1). Bound GTP reduces affinity of the Gα subunit to Gβγ and provokes dissociation of the Gα-GTP-Gβγ complex into the subunits Gα-GTP and Gβγ. Both subunits can regulate specific effector systems depending on the associated Gα subunit (see below). Deactivation of the G protein is accomplished by the intrinsic GTPase activity of the Gα subunit, cleaving GTP to GDP and P_i. This step of the G protein cycle can be catalyzed by GTPase accelerating proteins (GAPs), also called

regulators of G protein signaling (RGS) proteins. Subsequently, reassociation of Gα, GDP and Gβγ completes the G protein cycle and the initial state is restored.

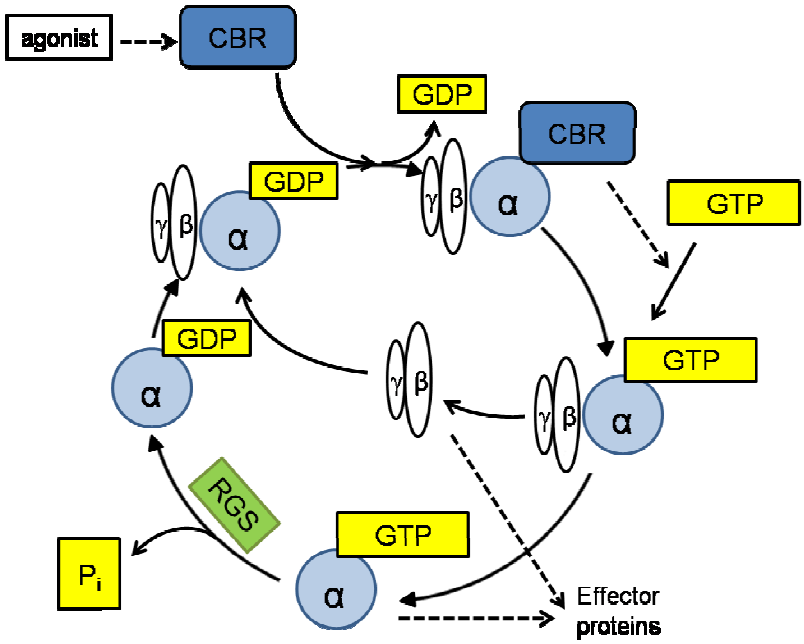

Figure 1.1: G protein activation of GPCRs - demonstrated by the example of CBR
Activation cycle of heterotrimeric G protein after stimulation by an agonist (adapted from Seifert and Wieland, 2005)

The intracellular effects of GPCR activation is mediated by four classes of heterotrimeric G proteins. This classification is based on sequence homology of the Gα subunit. $G\alpha_s$ subunits stimulate and $G\alpha_{i/o}$ subunits inhibit adenyly cyclase activity which leads to altered cyclic 3´,5´-adenosine monophosphate (cAMP) production. $G\alpha_{q/11}$ subunits activate phospholipase C (PLC) β and thereby catalyze the hydrolysis of phosphatidyl-inositol-4,5-diphosphat (PIP_2) to 1,2-diacylglycerol (DAG) and inositol-1,4,5-triphosphat (IP_3). $G\alpha_{12/13}$ subunits are shown to interact with guanine nucleotide exchange factors resulting in the regulation of RhoA activity (Worzfeld et al., 2008). All second messengers are able to cause fast responses such as modulation of intracellular ion concentration or altered enzyme activity or, as long term effect, induce regulation of gene expression by modulating transcription factors. Moreover, activated Gβγ subunits have also the ability to affect effector systems (Birnbaumer, 2007).

Summarized, the G protein signal cascade is influenced by following key issues: interaction of a ligand with the receptor, transduction of the signal to the heterotrimeric G proteins, interaction of the activated G proteins with effector systems and, finally, inactivation of G proteins.

1.1.3 The two-state activation model of GPCRs

For illustration of pharmacological properties attributed to a GPCR ligand the two-state model is often referred (see Figure 1.2). In this model a GPCR exists in an active and in an inactive state, which are equilibrated (Seifert and Wenzel-Seifert, 2003). A receptor in the active state (R*) is able to bind a G Protein and to initiate the intracellular signaling machinery. The inactive state of the receptor (R) marginally interacts with G proteins and thus hardly causes the exchange of GDP to GTP. An agonist stabilizes the active form of the receptor, whereas a compound that stabilizes the inactive state is called inverse agonist. A neutral antagonist does not change the equilibrium, but occupies the binding site at the GPCR and blocks thereby agonist and inverse agonist effects. Partial agonists or inverse agonists have, relative to a full agonist or inverse agonist, lower capabilities to activate or inhibit the receptor. Interestingly, some receptors occur in the active state without a bound agonist. This attribute is called constitutive activity and is often observed among GPCRs (Seifert and Wenzel-Seifert, 2003).

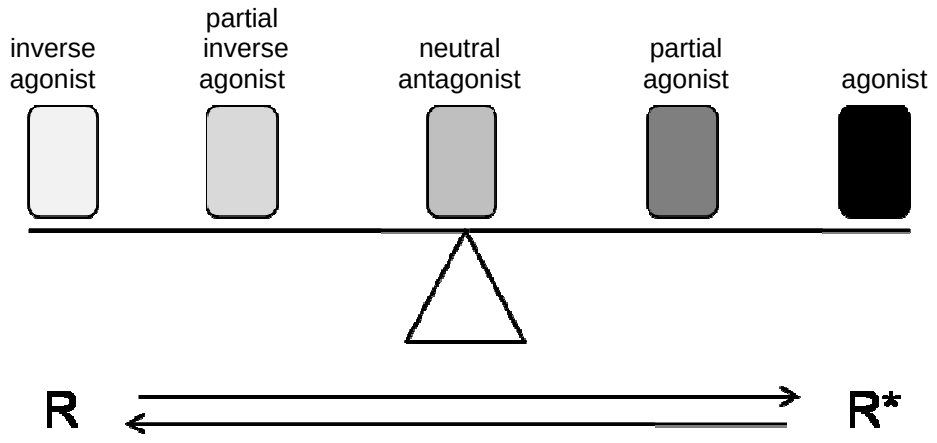

Figure 1.2: Two-state model of GPCR activation
Receptors can adopt an active (R*) and an inactive conformation (R). Ligands are characterized according to their ability to shift the equilibrium to either side of both states (Seifert and Wenzel-Seifert, 2003).

Observations of agonist-specific trafficking of a receptor stimulus refined the two-state model. In accordance with this model, each agonist is capable of stabilizing or selecting a unique receptor conformation. This phenomenon results in an unlimited number of active receptor states (Kenakin, 1995; Urban et al., 2007). Each of these conformations can interact highly selective with specific intracellular signaling complexes. Receptors with this functional selectivity likely form the basis for a new strategy of drug development. Screening for novel ligands would not only include binding affinity studies or evaluation of agonist/inverse agonist properties, but also studies on activation of the receptor connected to specific and appropriate intracellular response.

1.1.4 Fusion proteins

The first GPCR-Gα fusion protein was described in 1994, when Bertin et al. reported the successful fusion of the β adrenergic receptor with $G\alpha_s$ (Bertin et al., 1994). This construct, expressed in S49 lymphoma cells, induced a greater stimulation of cAMP with an increase in potency compared to wild-type cells. These effects were suggested to be caused by a more efficient coupling between receptor and G protein. As the efficiency of receptor–G protein interaction is influenced by the protein expression levels, a clear advantage of the fusion approach is the defined 1:1 stoichiometry together with a close proximity of the signaling partners, both firmly anchored in the plasma membrane (Seifert et al., 1999). Initially, it was hypothesized that fusion of a GPCR to a Gα leads to a functional interaction no matter whether the subunit is appropriate or not. This proximity-induced loss of G protein selectivity would have been a great opportunity to study orphan receptors or GPCRs coupled to $G\alpha_s$ or $G\alpha_q$ for which the agonist-regulated guanine nucleotide exchange is more difficult to measure. Unfortunately several studies revealed that this is not the case. Co-expression of $G\alpha_i$ with the IP prostanoid receptor, a $G\alpha_s$ coupled GPCR, did not result in activation of G protein and the use of the IP-$G\alpha_i$ fusion protein did not revealed any effect (Fong and Milligan, 1999). However, the fusion approach is an elegant way to study receptor-G protein interaction with a guaranteed lack of receptor reserve and thus an useful tool to explore the basis of ligand efficacy and to measure effects of point mutations in GPCR and G proteins (Colquhoun, 1998; Milligan, 2000).

Another area of application concerning fusion proteins is the examination of homo- and heterodimerisation of GPCRs. As it is known many GPCRs can form dimers or oligomers with co-expressed receptors in a homologous or heterologous

manner (Milligan, 2007). For these studies two fusion proteins are constructed that are both inactive when expressed individually because of specific mutations. Mutations in the highly conserved hydrophobic residue in the second intracellular loop of a GPCR to acidic residues generate receptors that do not transmit the signal to the G protein or are simply unable to bind an agonist. This receptor is fused to a wild-type Gα subunit. Another or the same wild-type receptor is fused to a G protein unable to promote GCP/GTP exchange and hence activation. To induce inactivity of G proteins, Gly^{202} is mutated to Ala. These constructs are co-transfected and by measuring reconstitution of function, conclusions can be drawn on their interactions concerning homo- and heterodimerisation (Milligan et al., 2005).

Despite some reported exceptions (Dupuis et al., 1999), the use of fusion proteins can increase signal-to-background ratio. The higher sensitivity compared to the Gα co-transfection systems makes the fusion protein approach to an interesting tool for ligand screening assays. Also, for the characterization of orphan receptors fusion proteins are applied (Guo et al., 2001; Takeda et al., 2003). As mentioned above, a set of GPCR-G protein fusions must be constructed to evaluate the effect of a ligand or to ensure the lack of effect through a specific receptor.

GPCR-G fusion proteins may not exactly reflect the physiological situation as we still do not know to how many G proteins one single receptor has access to. But as the efficacy of receptor-G protein interaction depends on their expression levels, the fixed stoichiometry and the forced proximity in the cell membrane make the fusion approach to an attractive and precious tool for the above mentioned fields of research.

1.1.5 RGS proteins

G protein signaling is determined by the intrinsic GTPase activity, hydrolyzing bound GTP in GDP and P_i, which then induces reassociation of the α and βγ subunits and the return to the basal receptor state. The activity of GTPase can be influenced by specific proteins that are consequently called regulators of G protein signaling (RGS). These RGS proteins are able to act as GTPase accelerating proteins (GAPs) and terminate signaling of Gα subunits through reducing maximal or steady-state levels of active G proteins. Among the more than 20 known RGS with GAP activity only for $Gα_s$ no RGS interaction partner has been confirmed yet, whereas all other interact with $Gα_i$ and/or $Gα_q$ class of G proteins. The ability of RGS protein to act as

GAPs is due to a highly conserved region of approximately 120 amino acids termed as the RGS domain, that can interact with Gα subunits (Popov et al., 1997).

Increasing evidence showed that RGS proteins are involved in more cellular functions beyond shortening the time in which the Gα subunit stay in its active conformation. RGS proteins can interact with additional cellular molecules such as receptors, effectors and scaffolds. These interactions are on the one hand important for GAP effects and show on the other hand that RGS proteins are involved in many cellular mechanisms playing diverse functional roles in living cells (Abramow-Newerly et al., 2006). The interactions are not exclusively served by the RGS domain but by additional domains being part of many RGS proteins (Sethakorn et al., 2010). Due to their link to other proteins and signaling pathways as well as for their ability to shorten G protein signaling, RGS proteins are interesting new pharmacological targets (Sjogren et al., 2010). In the following, two RGS proteins will be presented in more detail.

1.1.5.1 *RGS4*

RGS4 belongs to the B/R4 subfamily of RGS proteins and is a relatively small molecule which is selectively enriched in CNS and heart (Bowden et al., 2007; Cifelli et al., 2008). As described by Zeng et al. (1998) the N-terminal domain of the RGS4 protein is implicated in GPCR binding as deletion of this region reduces the ability of RGS4 to modulate GPCR signaling. This provided indication that the modulation of a G protein signal depends on the activating receptor and that the receptors regulate affinity of RGS4 proteins to the G protein.

Several studies showed that RGS4 is involved into the generation of several diseases. As an example it is thought that RGS4 is an important factor in breast cancer metastasis (Xie et al., 2009) and a genetic biological marker of schizophrenia (Bowden et al., 2007). Furthermore, RGS4 is essential for cardiac adaption (Cifelli et al., 2008) and has been shown to control critical signal events that are contributed to addictive processes such as opiate dependence (Hooks et al., 2008).

1.1.5.2 *RGS19*

Members of the A/RZ subfamily of RGS proteins are quiet similar in size to the members of the B/R4 subfamily but differ in the N-terminal regions, containing a cysteine string motif for palmitoylation and thereby anchoring RGS protein to the membrane. RGS19, or also called Gα interacting protein (GAIP), is a prominent

member of this subfamily and contains an additional C-terminal PDZ binding motif. This for RGS protein unique C-terminus seems to be crucial for the recruitment of RGS19 activity. De Vries et al. (1998), reported the isolation and characterization of a protein, named GIPC (GAIP interaction protein C-terminus), that interacts with its PDZ domain with RGS19. As it was demonstrated that GIPC can also specifically bind to GPCR, it is assumed that GIPC can act as an adapter protein between receptor and RGS19. This was recently confirmed for the dopamine D_2 receptor (Jeanneteau et al., 2004b).

Up to now, physiological functions of RGS19 are related to signal determination (Hepler et al., 1997; Jeanneteau et al., 2004a) and cell proliferation (Tso et al., 2010) but further investigations have to be done to clarify its mechanism of action.

1.2 The endocannabinoid system

1.2.1 Cannabinoid receptors, endogenous ligands and involved enzymes

The endocannabinoid system (ECS) comprises cannabinoid receptors (CBRs), various endocannabinoids and enzymes for endogenous ligand biosynthesis and inactivation. So far, two human CBRs have been identified. The CB_1R was cloned in 1990 (Matsuda et al., 1990) and is the most abundant GPCR in the central nervous system (CNS), but is also present in several peripheral tissues, such as the gastrointestinal tract, the cardiovascular and reproductive systems (Svizenska et al., 2008), as well as in liver, pancreas, adipocytes, lung and skeletal muscle (Pacher et al., 2006). The CB_2R, cloned in 1993 (Munro et al., 1993), is mostly restricted to immune tissues (Berdyshev, 2000; Cabral et al., 2008), osteoclasts and osteoblasts (Bab and Zimmer, 2008). In contrast to the CB_1R, the N-terminal domain of CB_2R is much shorter. The overall homology of the CB_1R and the CB_2R is about 44%, which increases up to about 68% in the TM domains (Lutz, 2002).

The discovery of the specific receptors initiated research on the identification of endogenous ligands, so called endocannabinoids. The first and major endocannabinoids discovered were anandamide (Devane et al., 1992), the amide of arachidonic acid and ethanolamine, and 2-arachidonoyl glycerol (2-AG) (Mechoulam et al., 1995; Sugiura et al., 1995). Further less investigated endocannabinoids are 2-arachidonyl glycerol ether (noladin ether) (Hanus et al., 2001) and N-arachidonoyl dopamine (Bisogno et al., 2000), both agonists at the CB_1R, and O-arachidonoyl ethanolamine (virodhamin) with CB_1R antagonistic properties (Porter et al., 2002). More recently, the first endogenous compound interacting with CBRs that is not derived from membrane lipids was discovered. Hemopressin was identified as a peptide ligand with inverse agonist effects at CB_1R (Heimann et al., 2007). Whether this nonapepide is an endogenous ligand has to be verified.

The metabolism pathways of anandamide and 2-AG have been largely investigated but are not yet fully understood (Di Marzo, 2009). The main biosynthetic route is through on-demand hydrolysis of precursors present in the cytoplasm membrane. The synthesis of anandamide is catalyzed by N-acylphosphatidyl ethanolamine specific phospholipase D (Okamoto et al., 2004). For 2-AG two sn-1-selective diacylglycerol lipases are confessed (Bisogno et al., 2003). The inactivation of anandamide is mostly actuated by intracellular cleavage of the amide structure by the fatty acid amide hydrolase (FAAH) (Cravatt et al., 1996). This enzyme can also

catalyze 2-AG hydrolysis, but additional hydrolases for 2-AG degradation are known such as monoaclyglycerol lipase (MAGL) (Dinh et al., 2002). Furthermore, beside the hydrolytic metabolism of endocannabinoids, an oxidative degradation via oxygenases including COX and LOX is assumed (Kozak and Marnett, 2002).

The enzymes for endocannabinoid biosynthesis and inactivation as well as cellular transporters for the release and uptakes of ligands and of course the CBRs are important pharmacological tools for the modulation of ECS activity.

1.2.2 Cannabinoid signaling

Both human CBRs are belonging to family A of GPCRs (Howlett et al., 2002) and are mostly coupled to $G\alpha_{i/o}$. This interaction consequently leads to inhibition of adenylyl cyclase with reduction in cAMP accumulation. Both receptors regulate the activation of mitogen-activated protein (MAP) kinase and stimulation of the receptors is coupled to PLC activation resulting in a subsequent release of Ca^{2+} from internal stores.

In contrast to the CB_2R, the CB_1R is able to interact with ion channels. For CB_1Rs, inhibition of voltage gated Ca^{2+} channels as well as activation of A type and inwardly rectifying K^+ channels are reported (Bosier et al., 2010; Demuth and Molleman, 2006).

Evidence suggests that agonists at both CBRs can activate one single signaling pathway over another (Shoemaker et al., 2005; Bosier et al., 2007) – a phenomenon that is already described for other GPCRs (Urban et al., 2007). This functional selectivity offers the great possibility to identify new CBR ligands that regulate a specific signaling pathway. Particularly in the case of CBRs that modulate multiple physiological functions (see Chapter 1.2.4), novel therapeutic applications without psychoactive side effects might be conceivably.

The ability of cannabinoids to modulate the activity of other receptor types or their signal transduction pathways has been demonstrated. For example, experiments revealed that the release of opioids is elevated by administration of Δ^9-THC (Parolaro et al., 2010). Also, the encephalin and dynorphin biosynthesis can be modulated by cannabinoids (Corchero et al., 1997). The synergistic effect of cannabis and the endomorphic system with respect to antinociception is an interesting target for pain therapy.

For anandamide and other CBR ligands, interactions with the orphan receptor GPR55 (Ryberg et al., 2007), the transient receptor potential vanilloid 1 receptor

$TRPV_1$ (Smart et al., 2000), as well as with serotonin $5\text{-}HT_3$ (Barann et al., 2002) and N-methyl-D-aspartate receptor NMDA (Hampson et al., 1998) were reported, showing that the behavioral effects of the (endo-)cannabinoids do not occur exclusively through the CBRs but also through interactions with other signaling systems.

1.2.3 *In-vitro* bioassay systems for CBRs

In-vitro test are often designed for screening procedures, to investigate receptor pharmacology and/or to explore the molecular mechanisms of action of a compound. Most common assays are cell based and measure downstream effects of the signal cascade. For the CBRs the most widely used *in-vitro* assays are competition binding assays, [^{35}S]GTPγS binding experiments or – at more distal points - measurement of cAMP production and inhibition of electrically evoked contractions of isolated smooth muscle preparations.

For binding assays, radiolabeled CBR ligands as [^{3}H]CP 55,940, [^{3}H]HU-243 or [^{3}H]WIN 55,212-2 are commonly used (Howlett et al., 2002). In this type of assay the radiolabeled probe competes with the test compound for binding to the CBRs and the amount of bound radioligand can be measured. This allows a conclusion on the affinity of the tested compound. However, no statements on the pharmacological properties of the tested ligand can be deducted.

The [^{35}S]GTPγS binding measures the coupling of G protein to CBRs at a very early step of the signal cascade following receptor occupation by a ligand. In case of occupation by an agonist the affinity of the G protein to GTP is increased. [^{35}S]GTPγS replaces endogenous GTP and binds to the Gα subunit. [^{35}S]GTPγS is resistant against hydrolysis by the intrinsic GTPase activity of Gα. Therefore bound [^{35}S]GTPγS to Gα can be measured. Conclusions concerning the pharmacological behavior of the tested ligand and calculation of its potency and efficacy can be drawn by quantifying the change of bound [^{35}S]GTPγS in relation to basal. Measurements of receptor-mediated G protein activation via [^{35}S]GTPγS binding assays are sensitive test systems and offer the possibility to evaluate pharmacological parameters of a ligand at a very proximal point of the signal cascade, which minimizes interfering factors (Seifert and Wieland, 2005).

Due to the ability of CBRs to modulate cAMP production, adenylyl cyclase assays are often exploited to screen potential CBR ligands. Furthermore, Rhee et al.

(1998) could show, that regulation of adenylyl cyclase isoforms 1, 3, 5, 6 or 8 by CB_1R activation resulted in an inhibition of cAMP accumulation, whereas for isoforms 2, 4, or 7 a stimulation of cAMP accumulation was computed (Rhee et al., 1998). This supported the assumption that CB_1R can also couple to $G\alpha_s$ proteins (Glass and Felder, 1997).

CB_1Rs are located on presynaptic terminals and mediate inhibition of electrically evoked transmitter release. These transmitters cause muscle contraction, which can be measured in *in-vitro* assays using isolated vas afferens from mice or small intestine muscle preparation of guinea pig. Of course, several other signal pathways can alter the contraction of these muscles and thus it is necessary to proof selective CBR dependency with a selective CB_1R antagonism.

1.2.4 The ECS as therapeutic target

The ECS is involved in many physiological functions. Investigations for targeting this system in pathophysiological conditions are up-coming since it has been realized that Marijuana and its active secondary metabolites has - beneath its risk of abuse and addiction - a huge clinical potential. The involvement of the ECS in energy metabolism and appetite regulation is proven and modulation of CBR activity can be effectively used for the treatment of metabolic diseases like obesity and anorexia. Targeting increased activity of the EC system in obese animals led to the development of the CB_1R inverse agonist rimonabant, which caused significant weight loss with beneficial effects on different metabolic parameters (Patel and Pathak, 2007). While in obese or overweight patients weight loss is a main goal, diminished appetite is an immense problem for HIV, Alzheimer or cancer patients. It was demonstrated that cannabis not only increased appetite, but was also effective in improving the mood and decreasing (chemotherapy-related) nausea and emesis (Nauck and Klaschik, 2004).

Also, the ECS figures prominently in the CNS and is associated to disorders and diseases. This is not surprising since the CB_1R is the most abundant GPCR in the brain with particularly high densities in the cerebral cortex, cerebellum, hippocampus and basal ganglia (Herkenham et al., 1991). These areas are related to motor, mood and anxiety disorders, as well as to the brain rewarding system and processes of learning and memory. Therefore, targeting the CB_1R for therapeutically purposes in pathophysiological conditions as Parkinson´s disease, Huntington´s disease, amyotrophic lateral sclerosis, epilepsy and Gilles de la Tourette´s syndrome

is under investigation. Other mental CNS disorders as depression, anxiety and insomnia seem to be sensitively modulated by the ECS and pharmacological interventions are studied. Also for the treatment of Alzheimer´s disease, beneficial effects of cannabinoids are discussed. For example, it was demonstrated that Δ^9-THC inhibits acetyl cholinesterase and prevents amyloid β-peptide aggregation (Eubanks et al., 2006).

Sativex®, a Δ^9-THC/Cannabidinol standardized *Cannabis sativa* extract, is approved as adjunctive medication for patients with multiple sclerosis (MS) in Canada since 2005 (Barnes, 2006). MS is a complex, auto-immune, inflammatory disease which leads into demyelination and axonal damage. The add-on therapy with Sativex® relieved neuropathic pain, lowered spasm frequency and increased mobility in MS patients, not responding to other drugs. In July 2010, approval has given to Sativex® in Spain and the drug manufacturer GW pharmaceuticals already submitted the application for approval in other European countries.

Another topic of investigation is the involvement of the ECS in inflammatory and neuropathic pain conditions. The precise mechanisms underlying nociception are not jet fully understood. Beside (endo-)cannabinoid induced modulation of inflammatory regulation (Pandey et al., 2009) and suppression of cellular nociceptive responses (Walker and Huang, 2002), an interplay of the ECS and the opioid system is discussed (Parolaro et al., 2010). Animal pain models offer promising results of cannabinoids blocking pain responses (Walker and Huang, 2002). Studies with knock-out mice showed that both CBRs are involved in the modulation of antinociception (Fox and Bevan, 2005) and that the peripheral CB_1Rs play a greater role than those localized in the CNS (Agarwal et al., 2007). In humans, beneficial effects of cannabis or synthetic cannabinoids in pain associated with MS, cancer, neuropathies and HIV infections are reported, but because of central side effects the widespread application is limited. Nevertheless, the antinociceptive effects, the interaction with the opioid system and the action mediated via peripheral CBRs provide a complex, but solid base for the development for cannabinoids that do not cross the brain-blood barrier. This would result in a novel class of analgetics peripherally acting against inflammatory or neuropathic pain.

As mentioned above, cannabinoids exhibited palliative effects in cancer patients. This includes appetite stimulation, inhibition of nausea and emesis during chemotherapy, pain relief and mood elevation. Furthermore, studies showed that

they might direct inhibit cancer growth through complex mechanisms that may involve apoptosis induction and anti-proliferative, anti-angiogenic and anti-metastatic effects in various cancer types (Bifulco et al., 2007). For example, CB_2R selective agonist JHW-133 inhibited tumor angiogenesis through reduction of vascular endothelial cell migration and inhibition of tumor expressed pro-angiogenic factors, such as vascular endothelial growth factor (VEGF), matrix-metalloproteinase-2 and angiopoietin (Blazquez et al., 2003). Blocking pro-angiogenic factors with a remedy that has “side benefits” may provide an interesting therapeutic approach for cancer therapy.

Other promising clinical approaches are targeting the ECS for Asthma therapy - because of the well known anti-inflammatory effects of cannabinoids and reports that endogenous cannabinoids can modulate bronchodilatation (Calignano et al., 2000) -, for the treatment of glaucoma - where some cannabinoids could effectively lower intraocular pressure, which was mainly CB_1R dependent (Porcella et al., 1998) - and for prevention of osteoporosis (Bab and Zimmer, 2008).

The ECS is a very complex and fine-tuned system and further studies and investigations are required to fully understand the physiological and pathophysiological role and possibilities of intervention. Of course, the negative side effects of cannabis such as the enhanced incidence of amotivational syndrome (Tunving, 1987) and increased risk of schizophrenia-like psychoses (Murray et al., 2007) must be monitored carefully or can, in a best-case scenario, be avoided by highly selective compounds or more selective distribution patterns. However, the above mentioned diseases, that are by no means complete, are being treated or have the potential to be treated by modulating ECS activity.

1.3 Objectives

Since the discovery of the ECS its involvement in many physiological functions and therapeutic interventions in pathophysiological conditions are investigated. As well as targeting biosynthesis and degradation of endocannabinoids for modulation of ECS signaling, the two so far known CBRs provide promising drug targets. Hence, new and preferable selective CBRs agonists, inverse agonists and antagonists are desired and test systems for ligand screening procedures are needed.

The aim of this thesis was to establish a functional and efficient assay system for the search and characterization of new CBR ligands. Therefore, different cell or tissue membranes heterogeneously or endogenously expressing CBR should be investigated on their ability to serve as an appropriate expression system. Finally, the steady-state GTPase assay should be developed and validated with known standard ligands in the most suitable expression system to characterize the pharmacological property of CBR ligands at a very proximal point of the signal transduction cascade. To examine whether the sensitivity of the test system can be enhanced, the influence of different co-expressed RGS proteins should be explored.

The second part of the thesis focused on the investigation and characterization of CBR-Gα fusion proteins. Fusion of the receptor to $G\alpha_{i2}$ subunit should be constructed and studies on their impact on receptor pharmacology was to be conducted. In addition, our interest was again to explore how RGS proteins would influence G protein signaling in the fusion approach and how this interaction is altered compared to the system where the CBRs are co-expressed with $G\alpha_{i2}$.

Finally, the assay with the highest sensitivity should be applied to examine potential ligands concerning their CBR activity. These ligands include natural compounds isolated from different *Echinacea* species as well as synthetic 2,3-disubstituted indole derivatives.

In summary, this thesis comprises the establishment of a highly sensitive assay system that is suitable for analyzing CBR pharmacology and for ligand screening procedures.

1.4 References

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2 Establishment of recombinant cannabinoid receptor assays and characterization of several natural and synthetic ligands*

2.1 Abstract

Cannabinoid receptors (CBR) are important drug targets for the treatment of various inflammatory, metabolic and neurological diseases. Therefore, sensitive test systems for the assessment of ligands are needed. In this study, a steady-state GTPase assay for human CBR subtypes 1 and 2 was developed to characterize the pharmacological property of ligands at a very proximal point of the signal transduction cascade. Establishing these *in-vitro* test systems, we studied cell or tissue membranes heterogeneously or endogenously expressing CBR, such as CBR infected Human Embryonic Kidney (HEK) 293 cells, rat cerebellum and spleen cells. The lack of effects in the GTPase assay and in [^{35}S]GTPγS binding experiments in these expression system, directed us to use *Spodoptera frugiperda* (Sf9) cells. Co-expressing CBR, different Gα subunits, Gβγ heterodimer and RGS (Regulator of G protein signaling) proteins in Sf9 cell membranes greatly improved the sensitivity of the assay, with highest GTPase activation in the CBR + $G\alpha_{i2}$ + $G\beta_1\gamma_2$ + RGS4 system. We examined exogenous and endogenous standard ligands as well as secondary metabolites as Δ^9-tetrahydrocannabinol (Δ^9-THC), dodeca-2*E*,4*E*-dienoic acid isobutylamide, an alkamide from *Echinacea purpurea,* and an *Echinacea purpurea* hexane extract according their agonistic and antagonistic properties. The suitability of the assay for screening procedures was also proven by detecting the activity of Δ^9-THC in a matrix of other less active compounds (Δ^9-THC free *Cannabis sativa* extract). In conclusion, we have developed highly sensitive test systems for the analysis of CBR ligands.

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2.2 Introduction

Cannabinoid receptors (CBRs) and their ligands have been of interest since their discovery in the early 1990's. The first CBR mainly expressed in the CNS was cloned in 1990 and named CB_1R (Howlett et al., 2002). A second cannabinoid receptor (CB_2R), mainly found in the periphery on immune cells and tissues, was discovered in 1993 (Howlett et al., 2002).

Ligands of cannabinoid receptors are of high therapeutic interest since the endocannabinoid system (ECS) is involved in the regulation of several biological functions such as immune regulation, memory, movement, appetite and pain (De Petrocellis et al., 2004; Di Marzo et al., 2004). Targeting increased activity of the ECS in obese animals led to the development of the first CB_1R antagonist/inverse agonist named rimonabant. Currently, the interrelationship between neuroinflammatory disorders such as Alzheimer's disease, amyotrophic lateral sclerosis, multiple sclerosis and Parkinson`s disease with malfunctions of the CBR system is under investigation (Centonze et al., 2007). Activation of CB_1R by agonists results in anticonvulsive and neuroprotective effects during ischemia and after traumatic brain injury (Panikashvili et al., 2001; Marsicano et al., 2003). Furthermore, the connection of the ECS and pain is still a topic of intensive investigation (Lever and Rice, 2007).

A major problem in the search for new CBR ligands is the limited number of available *in-vitro* test systems for their functional characterization and mechanistic studies, whereas several well established models for the *in-vivo* effects of CBR ligands exist (Cheng and Hitchcock, 2007). Often, competition binding assays with radioligands are applied (McPartland et al., 2007). However, these assays do not allow the differentiation of full or partial agonists, inverse agonists and antagonists. Available functional test systems measure the effects at a rather distal point of the signal cascade via second messengers, e. g. the increase of intracellular Ca^{2+} levels or the measurement of cAMP concentrations (Navarro et al., 2009; Silvestri et al., 2008). These second messengers are sometimes influenced by receptor-independent effects or cross-talk from other targets addressed by the test compound. Further information can be obtained by GTPγS binding assays kinetically determining the GDP/GTP exchange at the Gα subunit using [^{35}S]GTPγS, an assay that was recently successfully established in Sf9 cell membranes (Nickl et al., 2008).

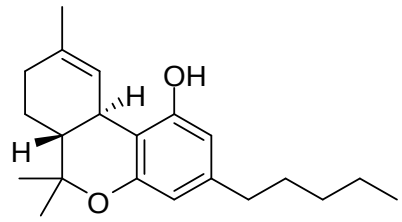
The aim of our present study was to establish a highly sensitive functional CBR assay to study CBRs and their ligands at a proximal point of the signal cascade. A guide for the establishment of such systems is provided by the corresponding models established for the β_2 adrenergic receptor (Seifert et al., 1999a) and the formyl peptide receptor (Wenzel-Seifert et al., 1999). Activation of a receptor initiates its binding to the Gα subunit accompanied with the exchange of bound GDP to GTP resulting in the dissociation of the ternary Gα-GTP-Gβγ complex into the subunits Gα-GTP and Gβγ. Deactivation of the G protein is accomplished by the intrinsic GTPase activity of the Gα subunit hydrolyzing GTP to GDP and P_i (Seifert and Wenzel-Seifert, 2002). Using [γ-^{32}P]GTP, the amount of $^{32}P_i$ released by the intrinsic GTPase activity of Gα under state conditions can be measured.

We adopted this concept and established CB_1R and CB_2R steady-state GTPase assays. As expression systems, we used membranes of rat cerebellum and spleen, endogenously expressing CBRs, as well as membranes of CBR transfected HEK 293 cells and Sf9 cells, co-expressing CBR, different Gα subunits, Gβγ heterodimer and various RGS proteins. For validation several exogenous and endogenous ligands of CBRs, i.e. anandamide, 2-AG (2-arachidonoyl glycerol), CP 55,940 ((-)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol), WIN 55,212-2 ((*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1 naphthalenylmethanone mesylate) as well as AM 251 (N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide), AM 281 (1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-4-morpholinyl-1*H*-pyrazole-3-carboxamide) and AM 630 (6-iodo-2-methyl-1-[2-4(morpholinyl)-ethyl]-[1*H*-indol-3-yl]-(4-methoxyphenyl)methanone) (Figure 2.1) were characterized.

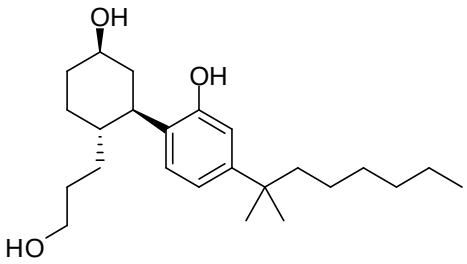
Since the discovery of Δ^9-THC (Figure 2.1), isolated from *Cannabis sativa* L. var. *indica* (Cannabaceae) as an agonist on CBRs, secondary natural products have also been addressed as promising leads for the discovery of cannabinoid ligands. Recently, other natural products like the alkamides from *Echinacea* species (Raduner et al., 2006) and β-caryophyllene (Gertsch et al., 2008) have been addressed as ligands of the CB_2R and thus, one of the main alkamides from *Echinacea purpurea*, dodeca-2*E*,4*E*-dienoic acid isobutylamide, was tested in our assay (Figure 2.1).

Due to the fact that secondary natural metabolites almost exclusively occur as mixtures, a screening test system applicable for bioactivity guided isolation of natural

compounds must be sensitive enough to detect single active compounds in a matrix of other inactive compounds. Therefore, we evaluated the potential of our assay to detect Δ^9-THC artificially added to a Δ^9-THC free cannabis extract and tested an alkamide containing *Echinacea purpurea* root extract.



Δ^9-THC



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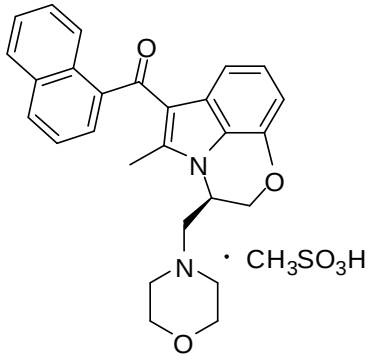

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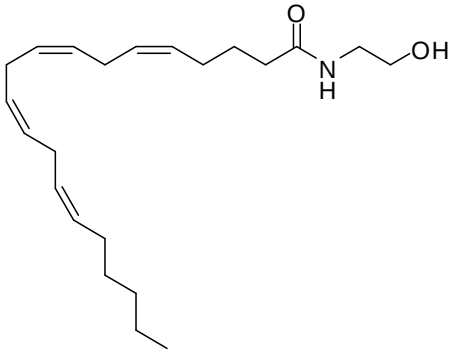
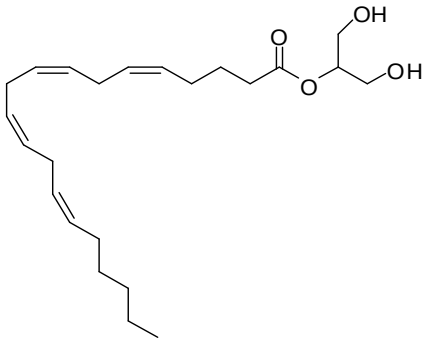
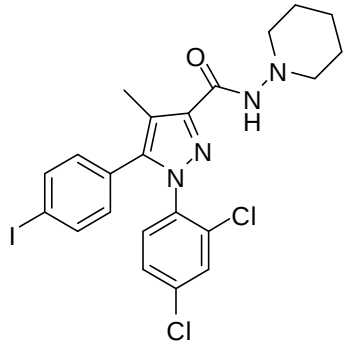


anandamide



2-AG



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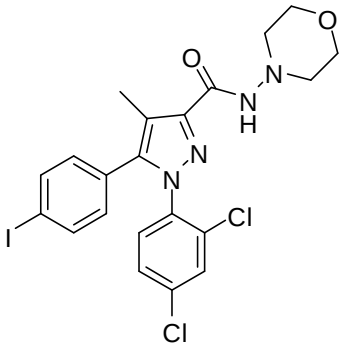

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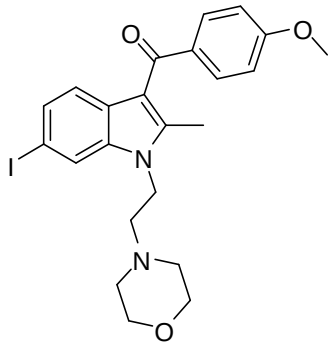
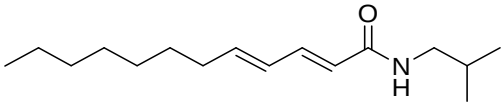


AM 630



dodeca-2*E*,4*E*-dienoic acid isobutylamide

Figure 2.1: Structures of the investigated compounds

2.3 Materials and Methods

2.3.1 Materials

The cDNA for hCB_1R and hCB_2R in pcDNA 3.1 was obtained from the cDNA bank of the University of Missouri (Rolla, MO, USA). All restriction enzymes and T4 ligase were from New England Biolabs (Beverley, MA, USA). Cloned *Pfu* DNA polymerase was from Stratagene (La Jolla, CA, USA). PCR primers were synthesized by MWG Biotech (Ebersberg, Germany), dNTP mix was obtained from Promega (Madison, WI, USA). pcDNA3.1(+) plasmid was purchased by Invitrogen (Carlsbad, CA, USA). Baculovirus encoding for $G\alpha_0$ was a kind gift of Dr. J. Garrison (University of North Carolina, Chapell Hill, NC, USA). Baculovirus encoding $G\alpha_{i2}$ was generously provided by Dr. A. G. Gilman (Department of Pharmacology, University of Texas, Southwestern Medical Center, Dallas, TX, USA). Baculovirus encoding $G\beta_1\gamma_2$ was a kind gift from Dr. P. Gierschik (Department of Pharmacology, University of Ulm, Germany). Baculoviruses encoding for RGS4 and RGS19 were a kind gift from Dr. E. Ross (University of Texas, Southwestern Medical Center, Dallas, TX, USA). Phenylmethylsulfonylfluoride and leupeptine hemisulfate were from Calbiochem (La Jolla, CA, USA). Benzamidine was from Sigma (99%, St. Louis, MO, USA). Adenylyl imidodiphosphate was obtained from Roche (Mannheim, Germany). [^{35}S]GTPγS (1,100 Ci/mmol) was purchased from PerkinElmer (Boston, MA, USA), [γ-^{32}P]GTP was synthesized through enzymatic phosphorylation of GDP and [^{32}P]orthophosphoric acid (8,000 Ci/mmol, PerkinElmer Life Sciences, Boston, MA, USA) as described previously (Walseth and Johnson, 1979).

All nucleotides, unlabeled GTPγS, creatine kinase, creatine phosphate and salts (highest purity available) were purchased either from Roche (Mannheim, Germany) or Sigma (St. Louis, MO, USA). Dimethyl sulfoxide was from Merck (Darmstadt, Germany). Tris base was purchased from USB (Cleveland, OH, USA).

GF/C filters were from Brandel (Gaithersburg, MD, USA) and Rotiszint® eco plus cocktail was from Roth Chemie (Karlsruhe, Germany). Radioactive samples were counted in a PerkinElmer Tricarb-TR liquid scintillation analyzer.

The CBR ligands CP 55,940, anandamide, 2-AG, WIN 55,212-2, AM 251 and AM 630 were purchased from Tocris Cookson (Ballwin, MO, USA).

Δ^9-THC was obtained by THC Pharm (Frankfurt/Main, Germany). Dodeca-2*E*,4*E*-dienoic acid isobutylamide (2 mM (w/v) in DMSO) was a kind gift of Dr. J. Gertsch (ETH Zurich, Switzerland). An ethanolic Δ^9-THC free *Cannabis sativa* extract

was obtained from the Bundesinstitut für Arzneimittel und Medizinprodukte (Bonn, Germany). The *Echinacea* hexane extract was prepared from the roots of *Echinacea purpurea* (kind gift of Martin Bauer GmbH, Alveslohe, Germany) through Accelerated Solvent Extraction (ASE 100, Dionex, Germering, Germany).

2.3.2 Methods

2.3.2.1 *Construction of FLAG epitope- and hexahistidine-tagged hCBR and pcDNA3.1-hCBR plasmids*

hCBR constructs were generated according to a previously described strategy using sequential overlap-extension polymerase chain reaction (PCR) (Nickl et al., 2008; Wenzel-Seifert et al., 1999; Wenzel-Seifert and Seifert., 2000). Briefly, a DNA sequence encoding the cleavable signal peptide from influenza hemagglutinin to direct the receptor protein to the cell membrane, followed by the FLAG epitope, which is recognized by the respective monoclonal antibody, was placed 5' of the start codon of the cDNA of the CBRs; 3' of the cDNA a hexahistidine (His_6) was placed to allow further purification. These DNA constructs were used for generation of recombinant baculoviruses and were also inserted between the KpnI and XbaI sites (CB_1R) and the KpnI and XhoI sites (CB_2R), respectively, of the pcDNA3.1(+) plasmid. Constructs were confirmed by extensive restriction enzyme analysis and enzymatic sequencing.

2.3.2.2 *Cell culture, transfection, membrane preparation and cell microscopy of HEK 293 cells*

HEK 293 cells were cultivated in Dulbecco´s modified eagle medium supplemented with 10% (v/v) bovine serum albumin BSA (Invitrogen, Carlsbad, CA, USA). Cells were transfected in 24 well plates with 0.5 µg of pcDNA-CBR plasmid DNA using Fugene HD transfection reagent (Roche, Mannheim, Germany).

For membrane preparation, cells were seeded in culture flasks and incubated until a density of approximately 95%. Cells were scraped off the plates and membranes were prepared by analogy to the procedure described for Sf9 membrane preparation.

For imaging, cells were seeded in BD Biosciences 8 well chamber slides (San Jose, CA, USA) at a density of 70-80% and incubated overnight. Cells were fixed for 30 min with 4% (w/v) paraformaldehyde (Sigma, St. Louis, MO, USA), permeabilized with 0.2% (w/v) Triton X-100 (Serva, Heidelberg, Germany), washed and incubated

for 1 hour with anti-FLAG polyclonal (rabbit) antibody (Sigma, St. Louis, MO, USA), 1:400 (v/v) diluted in PBS buffer containing 0.5% BSA. After washing, cells were incubated with Cy2-conjugated AffiniPure Goat Anti-Rabbit IgG (Dianova, Hamburg, Germany) 1:400 (v/v) diluted in PBS buffer containing 0.5% BSA, for 1 hour. Thereafter, preparation was mounted with Confocal Matrix (Micro Tech Lab, Graz, Austria) and hardened overnight. A Carl Zeiss Axio Observer Z1 microscope was employed for acquisition of fluorescence images.

2.3.2.3 *Animals and rat cerebellum membrane preparation*

The study was conducted in accordance with the European Communities Council Directive (86/609/EEC) and of the Local Government of the Oberpfalz (Bavaria, Germany). All efforts were made to minimize the number of rats used and their suffering. Male rats (12 weeks of age, 430 - 560 g body weight; Charles River, Sulzfeld, Germany) was kept in the animal facilities under standard laboratory conditions (12:12 light-dark cycle, lights on at 6 a.m., 22℃, 55% relative humidity) with free access to water and standard rat chow. The rat was killed with an increasing concentration of CO_2. The cerebellum and the spleen were removed, quickly frozen in prechilled *n*-methyl butane on dry ice, and stored at -20℃ until further processing. For membrane preparation, tissues was homogenized in 10 mM Tris/HCl buffer, pH 7.4, containing 1 mM EDTA, 0.2 mM phenylmethylsulfonylfluoride, 10 µg/ml benzamidine, 10 µg/ml leupeptin with an Ultra-Turrax 18-10 (IKA, Staufen, Germany). After centrifugation at 500 x g for 15 min, the supernatant fluid was decanted and the membrane pellet was obtained by spinning the supernatant at 20,000 x g for 30 min. The pellet was washed and homogenized in buffer containing 75 mM Tris/HCl, pH 7.4, 1 mM EDTA and 12.5 mM $MgCl_2$. Aliquots were stored at -80℃. Protein concentration was determined using the DC protein assay kit (Bio-Rad, Hercules, CA) according to the instructions of the manufacturer.

2.3.2.4 *Generation of recombinant baculoviruses and membrane preparation of transfected Sf9 cells*

Sf9 cells, derived from *Spodoptera frugiperda* pupal ovarian tissue, were used for the baculovirus expression. Sf9 cells were cultured in Erlenmeyer flasks at 28℃ under rotation at 125 rpm in SF 900 II medium (Invitrogen, Carlsbad, CA, USA), supplemented with fetal calf serum (Pan-Biotech, Aidenbach, Germany) to 5% (v/v) and gentamicin sulfate (BioWhittaker, Walkersville, MD, USA) to 0.1 mg/ml.

Recombinant baculoviruses encoding FLAG- and hexahistidine-tagged CB_1R and CB_2R, $G\beta_1\gamma_2$ as well as varying Gα subunits ($G\alpha_0$, $G\alpha_{i2}$) and RGS proteins (RGS4 and RGS19) were generated in Sf9 insect cells using the BaculoGOLD transfection kit (BD PharMingen, San Diego, CA) (Seifert and Wenzel-Seifert 2001; Wenzel-Seifert et al., 1999; Wenzel-Seifert and Seifert, 2000) according to the manufacturer´s instructions. After initial transfection, high-titer virus stocks were generated by two sequential virus amplifications.

For transfection, cells were seeded (cell density 3.0 x 10^6 cells/ml) and infected with a 1:100 dilution of high-titer baculovirus stocks. Cells were cultured for 48 h and Sf9 membranes were prepared as described previously (Wenzel-Seifert and Seifert, 2000). Briefly, cells were washed once by centrifugating for 10 min at 170 x g, discarding the supernatant and resuspending the cell pellet in PBS buffer. After repeating the centrifugation step, the supernatant fluid was discarded and the pellet was suspended in lysis buffer (containing 10 mM Tris/HCl pH 7.4, 1 mM EDTA, 0.2 mM phenylmethylsulfonylfluoride, 10 µg/ml benzamidine, 10 µg/ml leupeptin) and homogenized in a Dounce homogenizer with 25 strokes. After centrifugation at 40 x g for 5 min the pellet contained the nuclei and unbroken cells and the supernatant contained the membranes. Therefore, the supernatant fluid was carefully transferred to a plastic tube and spun down by 38,500 x g for 20 min. The pellet containing the membranes was resuspended in lysis buffer and again centrifuged as described above. The resultant membrane pellet was suspended in buffer containing 75 mM Tris/HCl, pH 7.4, 1 mM EDTA and 12.5 mM $MgCl_2$, homogenized by a syringe with 20 strokes and stored in aliquots at -80℃. Protein concentrations were determined using the DC protein assay kit (Bio-Rad, Hercules, CA) according to the instructions of the manufacturer. Membranes used in the assays were analyzed by SDS page and immunoblotting with specific antibodies against FLAG-tagged cannabinoid receptors, G protein subunits and RGS proteins to ensure correct transfection (Nickl et al., 2008).

2.3.2.5 *Solubility of CBR ligands*

All compounds were dissolved in DMSO 100% (v/v) or in BSA (1 mg/ml) and stored at -20° C. The final DMSO concentration in assays in all cases was 3% (v/v) and did not influence the functionality of the assay (Table 2.3).

2.3.2.6 *[^{35}S]GTPγS binding experiments*

To determine the agonist-stimulated guanine nucleotide binding to G proteins, [^{35}S]GTPγS binding experiments were performed in the presence of GDP at increasing concentrations according to a previously described protocol (Seifert et al., 1998). Briefly, membranes were thawed, sedimented by centrifugation at 18,000 x g for 10 min at 4°C, and carefully homogenized in bin ding buffer containing 75 mM Tris/HCl, pH 7.4, 1 mM EDTA and 12.5 mM $MgCl_2$. Assay tubes contained 0.25% (w/v) BSA, membranes of rat cerebellum (2.5 µg of protein) or rat spleen (5 µg of protein), 0.4 nM [^{35}S]GTPγS and varying concentrations of GDP ranging from 1 nM to 10 µM for rat cerebellum and 10 nM to 100 µM for rat spleen in 250 µl binding buffer. Binding of [^{35}S]GTPγS was examined in the absence and presence of 10 µM CP 55,940 with or without 100 mM NaCl. Non-specific binding was determined in the presence of 100 µM unlabeled GTPγS and was less than 0.4% of total binding. Incubation was conducted for 90 min at room temperature and shaking at 250 rpm on a platform shaker (Innova™ 2000, New Brunswick Scientific, Edison, NJ, USA). Assays were stopped by filtration through GF/C filters equilibrated with binding buffer. After filtration, filters were washed 3 times with cold binding buffer (4°C) and filter-bound radioactivity was determined by liquid scintillation counting in Rotiszint® eco plus cocktail after 4 hours of equilibration.

2.3.2.7 *Steady-state GTPase assay*

The GTPase assay was performed as described previously (Wenzel-Seifert et al., 1999). Briefly, membranes were thawed, sedimented by centrifugation at 18,000 x g for 10 min at 4°C, and carefully resuspended in 10 mM Tris/HCl, pH 7.4. Assay tubes contained membranes (HEK 15 µg, rat cerebellum 2.5 µg, rat spleen 5 µg, Sf9 15 µg of protein/tube), 1.0 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM ATP, 100 nM GTP, 0.1 mM adenylyl imidodiphosphate, 5 mM creatine phosphate, 40 µg of creatine kinase, 0.2% (w/v) BSA in 50 mM Tris/HCl, pH 7.4, to prevent binding of protein or ligand to the polystyrol tubes, and CB_1R and CB_2R ligands at various concentrations. To suppress constitutive activity of CB_1Rs in rat cerebellum, 150 mM NaCl was added to assay tubes (Wenzel-Seifert et al., 1998). This addition enabled us to increase the effect of an agonist on GTPase activity. Reaction mixtures (80 µl) were incubated for 2 min at 25°C before the addition of 20 µl of [γ-^{32}P]GTP (0.1 µCi/tube). All stock and work dilutions of [γ-^{32}P]GTP were prepared in 20 mM Tris/HCl, pH 7.4. Reactions were conducted for 20 min and 10 min for rat tissues, respectively, at 25°C. Reactions were terminated by the addition of 900 µl of slurry consisting of 5% (w/v) activated charcoal and 50 mM NaH_2PO_4, pH 2.0. Charcoal absorbs nucleotides but not $^{32}P_i$. Charcoal-quenched reaction mixtures were centrifuged for 7 min at room temperature at 15,000 x g. Six hundred µl supernatant fluid of reaction mixtures was removed and $^{32}P_i$ was determined by Čerenkov radiation in 3 ml water. Enzyme activities were corrected for spontaneous degradation of [γ-^{32}P]GTP. Spontaneous [γ-^{32}P]GTP degradation was determined in tubes containing all of the above described components plus a high concentration of unlabeled GTP (1 mM) that, by competition with [γ-^{32}P]GTP, prevents [γ-^{32}P]GTP hydrolysis by enzymatic activities present in Sf9 membranes. Spontaneous [γ-^{32}P]GTP degradation amounted to <1% of the total amount of radioactivity added. The experimental conditions chosen ensured that not more than 20% of the total amount of [γ-^{32}P]GTP added was converted to $^{32}P_i$. Neutral antagonism was determined in the presence of 30 nM (Table 2.4) or 10 nM CP 55,940 (Figure 2.6).

2.3.2.8 *Calculations and statistics*

Data are expressed as means ± SD and represent a minimum of 3 independent experiments, each performed in triplicate. Statistical evaluations and curve fittings were calculated using the GraphPad Prism 4 software (La Jolla, CA) and the Microsoft Excel 2007 software. K_b values were derived from the equation by Cheng and Prusoff (Cheng and Prusoff, 1973).

To compare more than two values, statistical significance was determined by the one-way ANOVA (Table 2.2), followed by the Dunnett post test. The statistical evaluation of two values was performed with the Student´s t-test. P values are given in the text (section results) and in the respective tables.

2.4 Results

2.4.1 CBR transfected HEK 293 cells

To obtain a mammalian test system, CBRs were expressed in HEK 293 cells. Expression of receptor proteins was confirmed with immunostaining (Figure 2.2). Both receptors were present at the plasma membrane, and in CB_1R transfected HEK 293 cells a fraction of the receptor was additionally localized in intracellular vesicles.

Although the CBR were clearly expressed, the use of HEK 293 cell membranes did not allow us to successfully establish the steady-state GTPase assay in mammalian cells. Despite intense efforts, with different CBR ligands no change of GTPase activity was observed (data not shown).

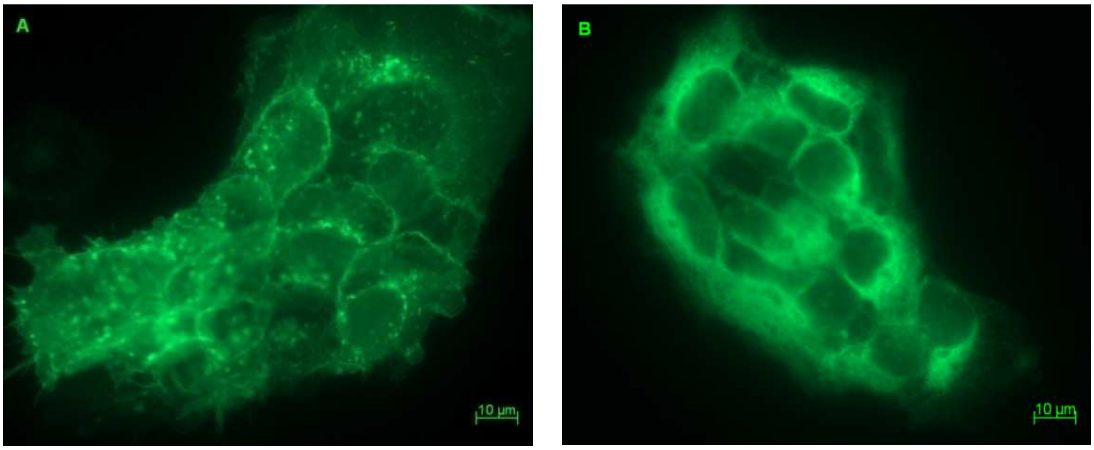


Figure 2.2: Immunostaining of CBR transfected HEK 293 cells
Cell staining was performed as described in *Material and Methods.* CB_1R transfected HEK 293 cells show localization of receptor at the plasma membrane and in intracellular vesicles (**A**), whereas in CB_2R-transfected HEK 293 cells the receptor is predominantly localized at the plasma membrane (**B**).

2.4.2 GTPγS binding experiments in rat cerebellum and rat spleen membrane

To determine the effect of agonists on guanine nucleotide exchange, [^{35}S]GTPγS bindings were conducted, in the presence of GDP at increasing concentrations (Figure 2.3). In rat cerebellum membranes, GDP inhibited [^{35}S]GTPγS binding with a logIC_{50} value of -7.16 ± 0.10. In the presence of an agonist the affinity of Gα for GDP should be decreased (Seifert et al., 1998) resulting in a right-shift of the concentration-response curve. However, the addition of 10 µM CP 55,940 did not significantly change the affinity of the G proteins for GDP (logIC_{50} value of -7.20 ± 0.09). Even the addition of 100 mM NaCl did not unmask a measurable right-shift (logIC_{50} value of -7.18 ± 0.06 and -7.21 ± 0.07 in the presence of agonist CP 55,940).

In contrast, in myeloid differentiated HL-60 membranes, NaCl was very effective at enhancing agonist effects on [^{35}S]GTPγS binding (Gierschik et al., 1991).

Analysis of rat spleen membranes revealed a similar pattern. In this system, a $logIC_{50}$ value of -6.90 ± 0.08 for the GDP competition under basal conditions was determined. In the presence of CP 55,940, a $logIC_{50}$ value of -6.86 ± 0.09 for GDP was measured. Again, NaCl did not influence the sensitivity of the system ($logIC_{50}$ value of -7.16 ± 0.07 for GDP and -7.14 ± 0.08 in the presence of agonist CP 55,940).

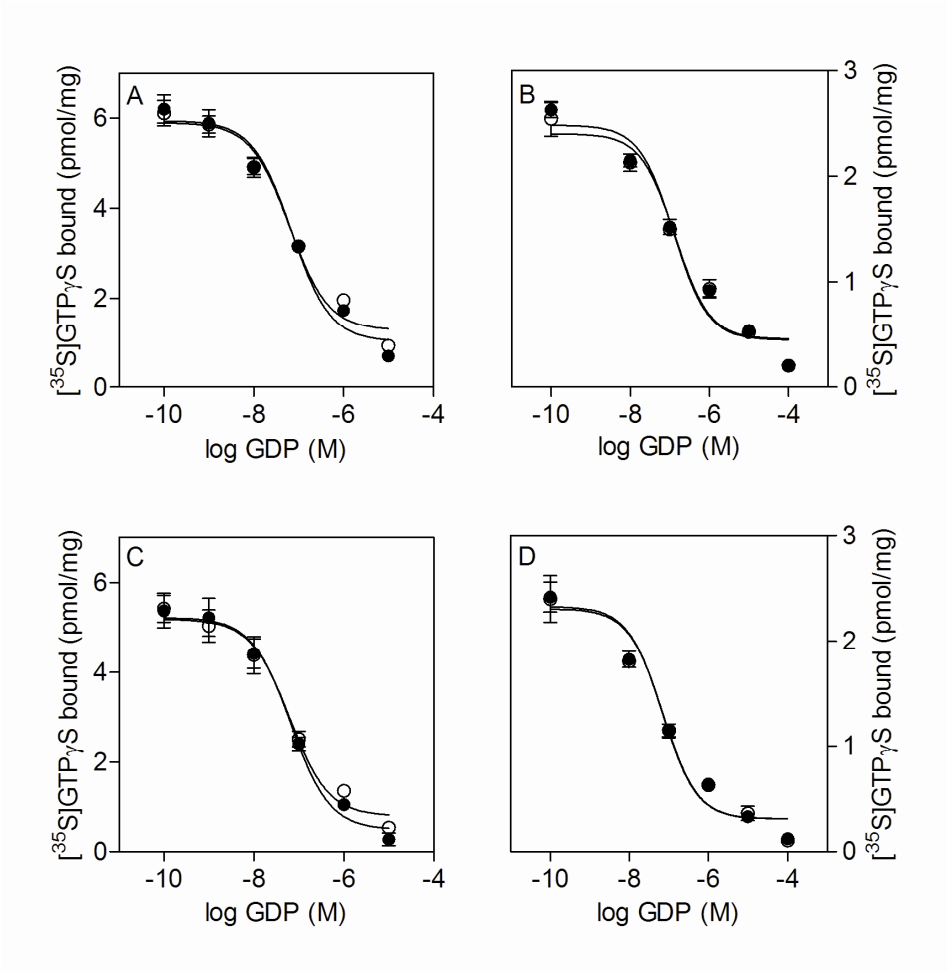


Figure 2.3: Effect of CP 55,940 on GTPγS binding in rat cerebellum and spleen membrane
GDP affinity binding was determined as described in *Materials and Methods*. Data show the merged results of 3 independent [^{35}S]GTPγS binding assays. Reaction mixtures contained 0.4 nM [^{35}S]GTPγS and GDP at the concentrations indicated on the abscissa in the absence (**A** and **B**) or presence (**C** and **D**) of 100 mM NaCl. Data were obtained for rat cerebellum membrane (**A** and **C**) and rat spleen membrane (**B** and **D**) with (○) and without (●) 10 µM CP 55,940.

2.4.3 Analysis of GTPase activity in rat tissue membrane

Rat cerebellum membranes exhibited a substantial basal GTPase activity (Table 2.1). The addition of 150 mM NaCl to the reaction mixture resulted in a decreased constitutive activity of the CB_1R in rat cerebellum membrane, resulting in an increased agonist effect (Table 2.1). In this system we could reach 15 ± 2% stimulation above basal by CP 55,940. A reduction of 19 ± 2% from basal GTPase activity was achieved with AM 251 in absence of NaCl. However, for detailed pharmacological studies, these signals were far too small.

In rat spleen membrane no change of GTPase activity was measurable with agonist CP 55,940 in concentrations from 1 nM to 10 µM. Also, the CB_2R antagonist AM 630 did not show any effect in the concentrations from 1 nM to 100 µM, whether in the normal or in the antagonist mode (data not shown).

Table 2.1: Analysis of GTPase activity of CB_1R in rat cerebellum membrane

	Basal activity	**Activity with 10 uM ligand**	
	[pmol x mg^{-1} x min^{-1}]	**[pmol x mg^{-1} x min^{-1}]**	**[% change of basal activity]**
CP 55,940	25.05 ± 2.41	27.18 ± 2.24	9 ± 4
CP 55,940 with 150 mM NaCl	15.57 ± 0.77	17.88 ± 1.08	15 ± 2
AM 251	25.00 ± 2.60	20.22 ± 1.96	-19 ± 2

GTPase activity was determined as described in *Materials and Methods*. Reaction mixture contained CP 55,940 and AM 251 at concentrations from 1 nM to 10 µM to generate sigmoidal concentration-response curves. Data were analyzed by nonlinear regression and best fit to sigmoidal concentration-response curves. Data shown are the mean values ± SD and represent 4 independent experiments performed in triplicates.

2.4.4 Analysis of six different co-expression systems of CBRs in Sf9 cells

To obtain a highly sensitive test system for the CBRs we compared the co-expression of $G\alpha_0$ and $G\alpha_{i2}$ subunits in Sf9 insect cells. $G\alpha_0$ is expressed in neuronal and neuroendocrine cells, whereas $G\alpha_{i2}$ is ubiquitously expressed (Offermanns, 2003). Furthermore, we evaluated the potential effects of GTPase activating proteins (GAPs) (Ross and Wilkie, 2000) on the GTPase activity and started our investigations with RGS4 and RGS19, using in all constructs the additional heterodimer $G\beta_1\gamma_2$. In previous studies on the human histamine H_4 receptor and chemokine receptor $CXCR_4$, RGS proteins enhanced agonist-stimulated GTP hydrolysis (Schneider and Seifert, 2009; Kleemann et al., 2008).

Expression of the $G\alpha_0$ subunit resulted in a low GTPase activation by 10 µM CP 55,940 (19 ± 9% for CB_1R and 27 ± 6% for CB_2R, Table 2.2). In contrast, the expression of $G\alpha_{i2}$ increased the GTPase stimulation to 53 ± 19% for CB_1R and 70 ± 13% for CB_2R, Table 2.2). The co-expressed RGS protein significantly influenced the GTPase activation. Co-expression with RGS4 was beneficial for all used constructs, with the highest increase in membranes with $G\alpha_{i2}$ and RGS4 (CB_1R: 158 ± 8% stimulation and CB_2R: 156 ± 15%, Table 2.2), whereas RGS19 co-expression was only beneficial for the CB_1R + $G\alpha_0$ construct. $LogEC_{50}$ values were not statistically different for the CB_1R in all constructs ($p = 0.9181$), but the increase of GTPase stimulation of CB_1R + $G\alpha_{i2}$ + $G\beta_1\gamma_2$ + RGS4 was significantly higher with a p value of < 0.001 compared to all other CB_1R constructs. Also, the p value for the comparison of GTPase stimulation of CB_2R + $G\alpha_{i2}$ + $G\beta_1\gamma_2$ + RGS4 construct to the other CB_2R constructs is ≤ 0.001, showing a very high significant difference. Interestingly, the $logEC_{50}$ of CP 55,940 on the CB_2R + $G\alpha_0$ construct is significantly different from all other constructs with a p value of 0.0136. Regarding the baseline values of the different constructs, the CB_1R + $G\alpha_{i2}$ system with RGS4 and RGS19 showed a significant increase of basal activity ($p = 0.0034$), whereas in the CB_2R + $G\alpha_{i2}$ system only RGS4 influenced basal activity positively ($p < 0.001$).

Based on these results we went on working with the CBR + $G\alpha_{i2}$ + $G\beta_1\gamma_2$ + RGS4 construct for further experiments. This system showed the highest sensitivity recognizable at the highest GTPase stimulation by CP 55,940 with no significant influence on the log EC_{50} value.

Table 2.2: Analysis of six different co-expression systems of CBRs in Sf9 cells

	hCB_1R				hCB_2R			
	Basal	**CP 55,940 10 µM**		**$logEC_{50}$**	**Basal**	**CP 55,940 10 µM**		**$logEC_{50}$**
membrane	**[pmol x mg^{-1} x min^{-1}]**	**[pmol x mg^{-1} x min^{-1}]**	**[% change of basal activity]**		**[pmol x mg^{-1} x min^{-1}]**	**[pmol x mg^{-1} x min^{-1}]**	**[% change of basal activity]**	
$G\alpha_o$	2.08 ± 0.76	2.44 ± 0.81	19 ± 9	-7.45 ± 0.90	0.59 ± 0.12	0.75 ± 0.17	27 ± 6	-8.73 ± 0.41*
$G\alpha_o$ + RGS4	3.38 ± 1.27	5.33 ± 2.04	67 ± 21	-7.81 ± 0.43	0.73 ± 0.40	1.13 ± 0.63	57 ± 17	-8.38 ± 0.49
$G\alpha_o$ + RGS19	2.91 ± 0.86	4.34 ± 1.34	49 ± 4	-7.74 ± 0.27	0.95 ± 0.25	1.26 ± 0.40	31 ± 11	-7.86 ± 0.43
$G\alpha_{i2}$	3.77 ± 0.48	5.69 ± 0.50	53 ± 19	-8.07 ± 0.07	1.07 ± 0.45	1.81 ± 0.78	70 ± 13	-8.29 ± 0.30
$G\alpha_{i2}$ + RGS4	5.40 ± 0.02**	13.95 ± 0.38	158 ± 8***	-7.86 ± 0.08	3.20 ± 0.46***	8.14 ± 0.77	156 ± 15***	-7.94 ± 0.14
$G\alpha_{i2}$ + RGS19	5.60 ± 0.92**	8.53 ± 0.86	55 ± 8	-8.02 ± 0.28	1.48 ± 0.56	2.50 ± 0.80	69 ± 11	-7.92 ± 0.65

Steady-state GTPase assay was performed as described in *Materials and Methods*. Each membrane preparation additionally contained $G\beta_1\gamma_2$ protein. Reaction mixture contained CP 55,940 concentrations from 1 nM to 10 µM to generate sigmoidal concentration-response curves. Data were analyzed by nonlinear regression and best fit to sigmoidal concentration-response curves. Data shown are the mean values ± SD of 3 - 9 independent experiments performed in triplicates with three different membrane preparations. Statistical significance was determined by the one-way ANOVA, followed by the Dunnett´s multiple comparison test. Stimulation of GTPase activity was significantly higher in the $G\alpha_{i2}$ + RGS4 system with p values ≤ 0.001 for CB_1R and CB_2R. Regarding basal values of GTPase activity, the CB_1R + $G\alpha_{i2}$ system with RGS4 and RGS19 shows a significant increase of basal activity ($p = 0.0034$), whereas in the CB_2R + $G\alpha_{i2}$ system only RGS4 influences basal activity significantly ($p < 0.001$). The $logEC_{50}$ of the CB_2R + $G\alpha_o$ differed to the $logEC_{50}$ values of the other CB_2R co-transfection systems ($p = 0.0136$).
(no symbol: not significant; *p ≤ 0.05, **p ≤0.01, ***p ≤0.001)

2.4.5 Evaluation of solvent effects in the steady-state GTPase assays

As ligands of CBRs are usually lipophilic, the use of BSA (Breivogel, 2006) or organic solvents like DMSO is often necessary to avoid solubility problems. Therefore, we evaluated the effect of either 0.1 mg/ml BSA or 3% DMSO (v/v) (final concentrations) on the outcome of the steady-state GTPase assays using the ligands anandamide, CP 55,940 and WIN 55,212-2 (Table 2.3). For these evaluations, we used the CBR + $G\alpha_{i2}$ + $G\beta_1\gamma_2$ + RGS4 co-expression system. The use of BSA solution resulted in an increase of GTPase stimulation except for CP 55,940 at the CB_1R, a finding, we do not have a plausible explanation for. A better solubilisation under modified physiochemical conditions can be discussed. The $logEC_{50}$ values were mostly not affected (no statistical significance). An exception is WIN 55,212-2 in the CB_1R system. Its BSA $logEC_{50}$ value differs significantly with a p value of 0.0482 from the DMSO $logEC_{50}$ value.

Table 2.3: Comparison of two solvents on GTPase activity of CB_1R and CB_2R co-expressed with $G\alpha_{i2}$ + $G\beta_1\gamma_2$ + RGS4 in Sf9 cells

		hCB_1R			hCB_2R		
ligand	**solvent**	**Basal [pmol x mg^{-1} x min^{-1}]**	**GTPase stimulation [%]**	**log EC_{50}**	**Basal [pmol x mg^{-1}x min^{-1}]**	**GTPase Stimulation [%]**	**log EC_{50}**
anandamide	DMSO	6.01 ± 1.40	82 ± 26	-6.66 ± 0.20	2.64 ± 0.63	73 ± 13	-6.22 ± 0.33
	BSA	4.88 ± 0.80	139 ± 13	-6.49 ± 0.42	2.52 ± 0.98	190 ± 32	-5.42 ± 0.23
CP 55,940	DMSO	5.40 ± 0.02	158 ± 8	-7.86 ± 0.08	3.20 ± 0.46	156 ± 15	-7.94 ± 0.14
	BSA	5.15 ± 0.21	152 ± 4	-7.94 ± 0.10	2.74 ± 0.39	190 ± 8	-7.90 ± 0.12
WIN 55,212-2	DMSO	6.60 ± 1.13	94 ± 18	-7.34 ± 0.12*	2.28 ± 0.40	161 ± 33	-8.54 ± 0.24
	BSA	4.88 ± 0.87	153 ± 26	-7.14 ± 0.06*	2.67 ± 1.13	218 ± 32	-8.10 ± 0.27

Steady-state GTPase assay was performed as described in *Materials and Methods*. Reaction mixtures contained CP 55,940 concentrations from 1 nM to 10 µM to generate sigmoidal concentration-response curves. Data were analyzed by nonlinear regression and best fit to sigmoidal concentration-response curves. Results shown are mean values ± SD and represent 3 independent experiments performed in triplicates with different membrane preparations. Statistical evaluation was performed with the Student´s t-test. No significant difference of the logEC_{50} values could be determined between DMSO and BSA. Only the logEC_{50} value of WIN 55,212-2 showed a significant difference in the tested solvents in the CB_1R system ($p = 0.0482$). (unpaired Student´s t test, *$p \leq 0.05$)

2.4.6 Analysis of potencies and efficacies of CBR ligands in the functional steady-state GTPase assay

The most efficient activation of GTPase by the $G\alpha_{i2}$ + $G\beta_1\gamma_2$ + RGS4 construct directed us to choose this model for further validation of the test system with several ligands (Table 2.4) using the plant-derived agonist Δ^9-THC, the endogenous agonists anandamide and 2-AG, the synthetic agonists CP 55,940 and WIN 55,212-2 as well as the synthetic inverse agonists AM 251 and AM 281 (at CB_1R) and antagonist AM 630 (at CB_2R). According to the two-state model of GPCR activation (Seifert and Wenzel-Seifert, 2002), agonists stabilize the active R* state and increase basal G protein activity, whereas inverse agonists stabilize the inactive R state and decrease basal G protein activity. Antagonists do not change this equilibrium. 2-AG, the most abundant endogenous agonist, acted as a full agonist with similar potency at both CBRs. E_{max} values of all other agonist at a concentration of 10 µM were related to the GTPase activation by 2-AG (E_{max} set 100%). Δ^9-THC acted as partial agonist at CBRs showing a higher efficacy at CB_1R than CB_2R (47 ± 3% versus 29 ± 4%, respectively) which finding is in accordance with literature data (McPartland and Glass, 2003; Pertwee 2008a). Anandamide acted as a partial agonist at CB_1R (79 ± 25%) and at CB_2R (55 ± 10%). WIN 55,212-2 behaved as a nearly full agonist at the CB_1R (90 ± 17%) and as a “superagonist”, with stimulation of GTPase higher than 2-AG, at the CB_2R (122 ± 25%). CP 55,940 showed very similar increase of GTPase activity at both receptors (158 ± 8% versus 156 ± 15%). Compared to 2-AG, CP 55,940 acted as “superagonist” at both receptors (152 ± 8% at the CB_1R and 118 ± 11% at the CB_2R).

Decrease of GTPase activity was observed by the inverse agonists AM 251 and AM 281 at the CB_1R. E_{max} values of these ligands were related to the GTPase inhibition by AM 251 that showed the strongest reduction of GTPase activity (E_{max} set -100%). In relation to AM 251, AM 281 caused a decrease of GTPase activity to -84 ± 5%. An influence of GTPase activity with AM 630 was only observed when tested in the presence of 30 nM CP 55,940. Under these conditions AM 630 reduced GTPase activity to basal GTPase activity assessed with 3% (v/v) DMSO. With this experimental design, the reversibility of agonist CP 55,940 effect by an antagonist can be demonstrated. AM 630 inhibited GTPase stimulation by CP 55,940 with a K_b value of 632 ± 93 nM. The same procedure was performed for the CB_1R, where AM 251 competed with CP 55,940 and consequently reduced the enhanced GTPase

activity. The K_b value for AM 251 at the CB_1R was 52 ± 6 nM. Figure 2.4 shows typical concentration-response curves for selected CBR ligands.

Table 2.4: Analysis of potencies and efficacies of CBR ligands in the functional steady-state [γ-^{32}P]GTPase assay

ligand	$G\alpha_{i2}$ + $G\beta_1\gamma_2$ + RGS4	GTPase stimulation / inhibition [%]	E_{max} [%]	$logEC_{50}$ / $logIC_{50}$	$logIC_{50}$ (literature)
Δ^9-THC	CB_1R	49 ± 3	47 ± 3	-7.13 ± 0.14	-7.27 [1]
	CB_2R	38 ± 5	29 ± 4	-6.77 ± 0.08	-7.12 [1]
anandamide	CB_1R	82 ± 26	79 ± 25	-6.66 ± 0.20	-7.05 [1]
	CB_2R	73 ± 13	55 ± 10	-6.22 ± 0.33	-6.43 [1]
2-AG	CB_1R	104 ± 14	100	-6.02 ± 0.35	-6.33 [2]
	CB_2R	132 ± 13	100	-5.73 ± 0.25	-5.85 [2]
CP 55,940	CB_1R	158 ± 8	152 ± 8	-7.86 ± 0.08	-8.30 [1]
	CB_2R	156 ± 15	118 ± 11	-7.94 ± 0.14	-8.74 [1]
WIN 55,212-2	CB_1R	94 ± 18	90 ± 17	-7.34 ± 0.12	-6.91 [1]
	CB_2R	161 ± 33	122 ± 25	-8.55 ± 0.24	-8.39 [1]
AM 251	CB_1R	-73 ± 3	-100	-7.44 ± 0.06	-8.13 [3]
AM 281	CB_1R	-61 ± 4	-84 ± 5	-7.64 ± 0.09	-7.92 [4]
AM 630*	CB_2R	-48 ± 8	n.d.**	-5.94 ± 0.33	-7.51 [5]

Regulation of GTPase activity by different CBR ligands compared to basal GTPase activity assessed with 3% (v/v) DMSO. Steady-state GTPase assay was performed as described in *Materials and Methods.* E_{max} values represent the stimulation of ligands [10 µM] relative to the endogenous agonist 2-AG (defined as 100% response) respectively the inhibition relative to AM 251 (defined as -100% inhibition). Reaction mixtures contained CBR ligands at various concentrations (1 nM - 10 µM). Data were analyzed by nonlinear regression and best fit to sigmoidal concentration-response curves. Basal activities ranged between 5.11 and 7.71 pmol x mg^{-1} x min^{-1} for CB_1R and between 1.57 and 3.33 pmol x mg^{-1} x min^{-1} for CB_2R. The maximal stimulatory effect of 2-AG constituted 65 to 137% for CB_1R and 116 to 148 % for CB_2R over basal. The results are expressed as mean values ± SD and represent 3 independent experiments performed in triplicates with different membrane preparations.
* Data determined in the presence of 30 nM CP 55,940, ** not determined,
[1] Pertwee, 1999, [2] Mechoulam et al., 1995, [3] Lan et al., 1999a, [4] Lan et al., 1999b, [5] Ross et al., 1999

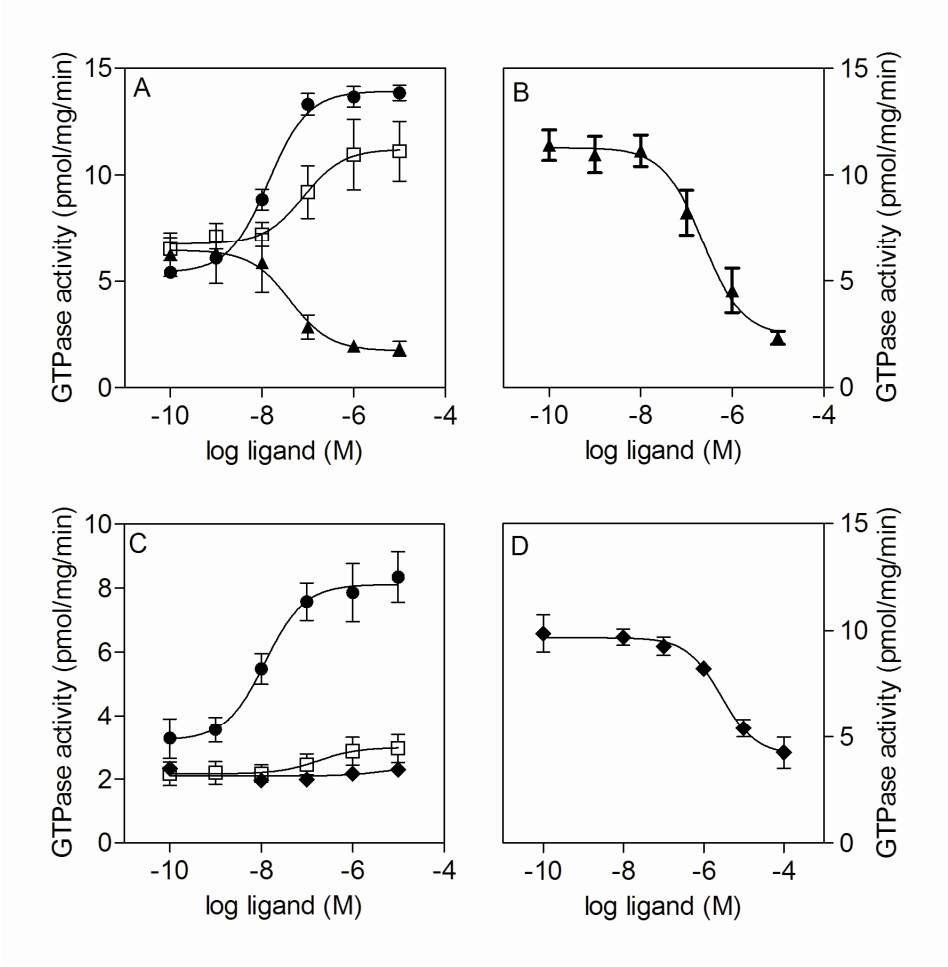


Figure 2.4: Representative concentration-response curves obtained in the steady-state GTPase assay with CBRs co-expressed with $G\alpha_{i2}$ + $G\beta_1\gamma_2$ + RGS4 in Sf9 cells

GTPase activity was determined as described in *Materials and Methods*. Data show merged results of 3 independent GTPase assays obtained from different ligands in concentrations indicated on the abscissa. **A** Effects of CP 55,940 (●), Δ^9-THC (□) and AM 251 (▲) on GTPase activity in Sf9 cells co-expressing CB_1R + $G\alpha_{i2}$ + $G\beta_1\gamma_2$ + RGS4. **B** Effects of AM 251 (▲) on GTPase activity in the antagonist mode in the presence of 30 nM CP 55,940. **C** Effects of CP 55,940 (●), Δ^9-THC (□) and AM 630 (♦) on GTPase activity in Sf9 cells co-expressing CB_2R + $G\alpha_{i2}$ + $G\beta_1\gamma_2$ + RGS4. **D** Effect of AM 630 (♦) on GTPase activity in the antagonist mode in the presence of 30 nM CP 55,940.

2.4.7 Analysis of *C. sativa* extract in the functional steady-state GTPase assay

As natural compounds always occur in a matrix of other secondary metabolites we evaluated whether this system is sensitive enough to detect an agonist at CBRs in a mixture of other compounds. Therefore we added 10% (m/m) Δ^9-THC artificially to a Δ^9-THC free cannabis extract and obtained the expected increase of GTPase activity (Figure 2.5).

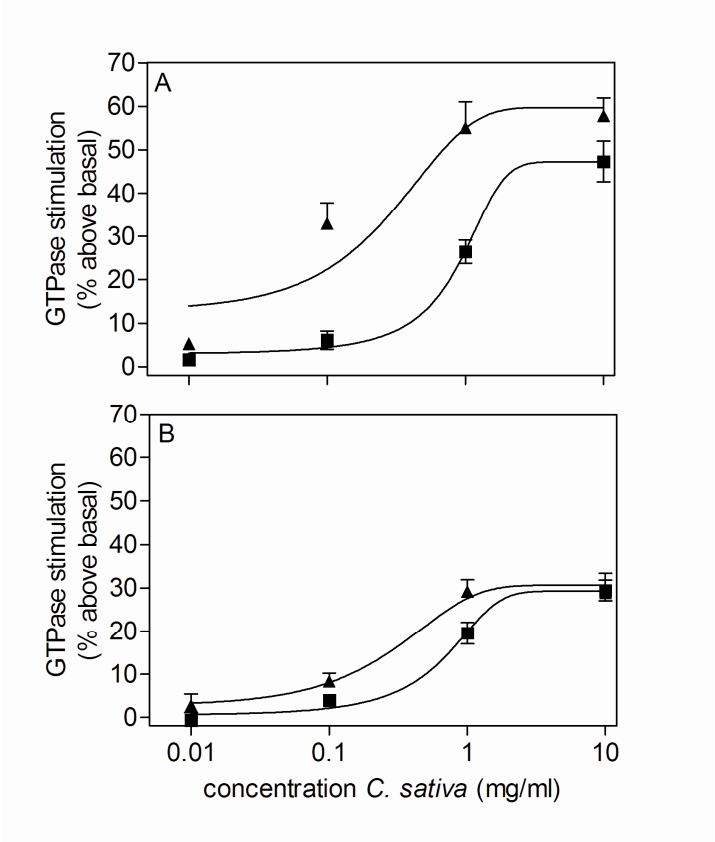


Figure 2.5: Effect of *C. sativa* extract in the steady-state GTPase assay
GTPase activity was determined as described in *Materials and Methods*. Data show the merged results of 3 independent GTPase assays obtained for a Δ^9-THC free *C. sativa* extract (■) and *C. sativa* supplemented with 10% Δ^9-THC (▲) in Sf9 cell membranes co-expressing CB_1R + $G\alpha_{i2}$ + $G\beta_1\gamma_2$ + RGS4 (**A**) and CB_2R + $G\alpha_{i2}$ + $G\beta_1\gamma_2$ + RGS4 (**B**).

2.4.8 Analysis of dodeca-2*E*,4*E*-dienoic acid isobutylamide and *Echinacea purpurea* extract

The analysis of the CB_2R ligand dodeca-2*E*,4*E*-dienoic acid isobutylamide (Raduner et al., 2006) revealed lacking effects in the GTPase assay. Even in the presence of 10 nM CP 55,940, a test mode in which a neutral antagonist can be characterized, no effect was detectable (Figure 2.6A). Furthermore, the lipophilic hexane extract of *Echinacea purpurea* roots containing several alkamides (Perry et al., 1997) showed no activity in the GTPase assay, also tested in both modes (Figure 2.6B).

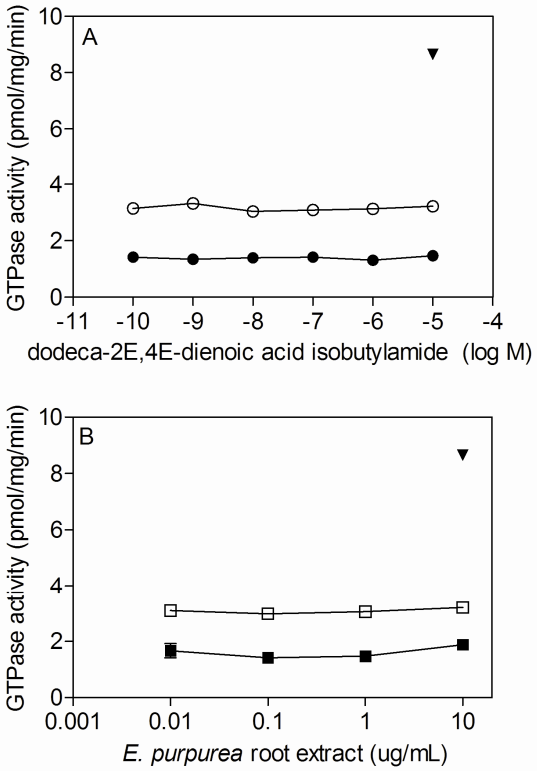


Figure 2.6: Effect of dodeca-2*E*,4*E*-dienoic acid isobutylamide and *Echinacea purpurea* root hexane extract on GTPase activity of the CB_2R

GTPase activity was determined as described in *Materials and Methods.* Data show a representative result performed in triplicates in Sf9 cells expressing CB_2R + $G\alpha_{i2}$ + $G\beta_1\gamma_2$ + RGS4. The experiment was replicated 3 independent times with different membrane preparations. 10 µM CP 55,940 was used as a positive control (▼). **A**, effect of dodeca-2*E*,4*E*-dienoic acid isobutylamide on GTPase activity (●) and in the antagonist mode in the presence of 10 nM CP 55,940 (○). **B**, effect of *Echinacea purpurea* root hexane extract on GTPase activity (■) and in the antagonist mode in the presence of 10 nM CP 55,940 (□).

2.5 Discussion

In this study a functional steady-state GTPase test system for the pharmacological characterization of known CB_1R and CB_2R ligands, as well as for functional studies and the search of new agonists and antagonists in complex natural matrices was established and validated. The assay was developed by using different cell lines or tissues endogenously or heterogeneously expressing CBR. Efforts were made to establish this highly sensitive assay efficient at mammalian test systems, but the superior expression of CBRs in Sf9 cells and the use of the baculovirus expression system offered a unique chance to analyze ligands, even partial agonists and inverse agonists.

Using membranes of CBR transfected HEK 293 cells did not lead to a successful outcome. Imaging of the transfected HEK 293 cells illustrates the difference of CB_1R and CB_2R concerning their basal localization. Like in our present study, the localization of CB_1R in intracellular vesicles was already reported earlier (Leterrier et al., 2004). Possibly, the high constitutive CB_1R activity results in constitutive internalization and constitutive desensitization with subsequent G protein uncoupling and lack of GTPase activation as was previously shown for a constitutively active β_2 adrenergic receptor mutant (Pei et al., 1994). However, in Sf9 cells, desensitization and internalization of GPCRs is much less pronounced. In fact, even in the presence of agonists, usually resulting in desensitization, enhanced GPCR expression is observed (Schneider et al., 2009; Gether et al., 1997).

Establishing the steady-state GTPase assay in rat tissue membranes, only cerebellum membranes led to the expected effect of the tested ligands. Suppression of ligand-independent GTPase activity with NaCl increased the effect of a full agonist such as CP 55,940 in a manner that a measurement of GTPase stimulation was quite possible. However, for qualitative analysis of a partial agonist or a partial inverse agonist the sensitivity of the rat cerebellum membrane system was insufficient and the use of rat spleen membranes did not lead to a successful outcome. Therefore, we established the [^{35}S]GTPγS binding assay with mammalian membranes endogenously expressing CBRs that is described as a highly sensitive assay with a better signal-to-noise ratio than the GTPase assay (Seifert et al., 1998; Gierschik et al., 1989). In agreement with other systems, GDP reduced basal GTPγS binding in rat cerebellum and rat spleen membranes. In contrast to previously published experiments – such as in CHO cell membranes (Ross et al., 1999) - GDP

did not unmask an agonist effect on GTPγS binding. Possibly, the density of CBRs and/or the cognate coupling G proteins in rat cerebellum is too low. The lack of agonist response in these test systems indicates that the use of mammalian tissues expressing CBRs is not suitable for detailed functional and pharmacological studies.

Furthermore, the use of rat tissue for pharmacological screening studies is less satisfactory than transfected cell membranes. Although rat brain is highly populated with CB_1Rs, CB_2Rs are expressed in brain as well (Skaper et al. 1999). Along the same line in spleen, although most of the CBRs present in this system are CB_2Rs, some CB_1Rs are expressed, too (Howlett et al., 2002).

The use of Sf9 cells and the baculovirus expression system offers several advantages. These insect cells do not possess mammalian G proteins or GPCRs, especially the absence of CBRs is described in literature (McPartland et al., 2001), and - in contrast to a mammalian cell line - the production of endogenous ligands can be excluded. The experimental design ensures that data are not influenced by fluctuating conditions of a living cell. Hence, by co-infection of baculoviruses encoding for a certain GPCR and G proteins, functional studies can be performed (Nickl et al., 2008; Wenzel-Seifert and Seifert, 2003) without the background noise of endogenously existing, potentially constitutively active receptors. Furthermore, it is relatively insensitive in terms of solvents, affords easy reconstitution of GPCRs and G proteins and enables high GPCR expression (6.6 ± 0.9 pmol/mg CB_1R and 3.4 ± 1.7 CB_1R) and $G\alpha_{i2}$ protein expression (480 ± 34 pmol/mg in CB_1R membranes and 167 ± 68 pmol/mg in CB_2R membranes) expression. Also, the Gβγ subunit protein expression (5.8 ± 1.2 pmol/mg in CB_1R membranes and 4.3 ± 1.5 pmol/mg in CB_2R membranes) is in a similar expression level as the receptor proteins (Nickl et al., 2008).

However, the use of insect cells renders the assay somewhat artificial due to the fact that in native mammalian cells different Gα subunits as well as several proteins with RGS domains are expressed in parallel (Burchett 2000). A highly specific interaction of a compound via a certain Gα subunit and/or a certain RGS protein (Seifert and Dove 2009) cannot be excluded so that a lacking activity in a defined G protein co-expression system and the respective assay is not a general proof of lacking activity at the receptor. Furthermore, misfolding or proteolysis of expressed proteins had to be considered. Another matter of fact that should be kept in mind is, that the efficiency of coupling between the receptors and the Gα subunit

depends on the density of the subunit in the membrane and its proximity to the receptors. This proximity of the signaling partners is necessary for forming the ternary complex which is a crucial step in the G protein cycle and therewith allowing the measurement of GTPase activity. As already published (Nickl et al., 2008), the Gα subunit in the established assay is expressed at a high level with a GPCR-Gα subunit ratio of 1:73 (CB_1R) and 1: 48 (CB_2R). This ensures statistical room for coupling of the Gα subunit to the receptor. Anyway, a defined 1:1 stoichiometry and a close proximity of the receptor and the Gα subunit would be guaranteed by GPCR-Gα fusion proteins and is an interesting further development for the characterization of ligands to CBR in the steady-state GTPase (Seifert et al., 1999b).

As CBRs are $G_{i/0}$ protein coupled GPCRs, in a first step, $G\alpha_0$ and $G\alpha_{i2}$ subunits as well as two RGS proteins, RGS4 and RGS19, were co-expressed to develop an assay with high GTPase activity and thus a high sensitivity required for a screening procedure usually applied for a bioactivity-guided isolation of natural products. The construct of CBR + $G\alpha_{i2}$ + $G\beta_1\gamma_2$ + RGS4 was the most efficient system. This is in accordance with literature data (Kleemann et al., 2008) where the RGS4 protein enhanced the absolute and relative values of GTPase stimulation with an increase of the basal GTPase activity values. This increase of GTPase stimulation could be observed in our CBR assay system, resulting in a higher sensitivity. These data also imply that under our assay conditions, GTP hydrolysis can become the rate-limiting step of the G protein cycle (Schneider and Seifert 2009; Kleemann et al., 2008). The optimized assays allow the characterization of ligands as partial, full or inverse agonists and antagonists at a very proximal point of the signal cascade of GPCRs. Various agonists, inverse agonists and antagonists like anandamide, 2-AG, Δ^9-THC, CP 55,940, WIN 55,212-2, AM 251, AM 281 and AM 630 were characterized to ensure the validity of the test systems. In agreement with the literature, Δ^9-THC was characterized as partial agonist with higher affinity to CB_1R than to CB_2R (McPartland and Glass, 2003; Pertwee, 2008b). CP 55,940 and WIN 55,212-2 are full agonists at CB_1R with EC_{50} values in the low nM range, also matching the literature data (Felder et al., 1995, Griffin et al 1998). AM 251 and AM 281 were described as selective CB_1R antagonists with strong inverse agonist properties (Cosenza et al., 2000). This finding was also demonstrated in our assay as both reduced CB_1R GTPase activity by 73 ± 3 % and 61 ± 4%, respectively with AM 251 being the more efficacious inverse agonist. In our assay AM 630 behaved as a neutral CB_2R antagonist with

reduction of GTPase activity of -48 ± 8% in the presence of 30 nM CP 55,940. This is in contrast to literature data, where AM 630 is described as a antagonist of CB_2R with the properties of an inverse agonist (Howlett et al., 2002; Ross et al., 1999). For a final conclusion of its pharmacological behavior further experiments are required. The K_b values of 52 ± 6 nM for AM 251 and 632 ± 93 nM for AM 630 determined in our hands differ considerably compared to published data, where the pharmacological parameters of these ligands were evaluated in CBR competition binding experiments (Lan et al., 1999a; Ross et al., 1999). For AM 251 a K_i value of 7.49 nM (Lan et al., 1999) and for AM 630 a K_i value of 31 nM (Ross et al., 1999) can be found in literature. In addition to the fact, that these literature values were not determined in a functional assay but in CBR competition binding assays with different assay conditions, this discrepancy can be explained by the concept of ligand specific receptor conformation. As it is described for example for the β_2 adrenergic receptor (Seifert et al. 2001) and the histamine H_4 receptor (Schneider et al., 2009), ligands can stabilize unique receptor confirmations. It is possible that in our assay the agonist CP 55,940, stabilizes a specific GDP/GTP exchange-promoting conformation of the GPCR for which the affinity of the inverse agonist/antagonist is lower as compared to the CP 55,940 conformation detected in the radioligand binding assay (Lan et al., 1999; Ross et al., 1999). In accordance with these dissociations between functional K_b values and K_i values in ligand binding experiments at CBRs, we observed dissociations between receptor conformations with respect to high-affinity agonist binding and GDP/GTP exchange at a constitutively active mutant of the β_2 adrenergic receptor (Seifert et al., 2001)

In accordance with the literature data, anandamide acted as a partial agonist at both receptors (Howlett et al., 2002). Furthermore, Song et al. (1999) showed that WIN 55,212-2 was more efficacious at CB_2R, explaining this finding by an amino acid residue change from valine in CB_1R to phenylalanine in CB_2R at position 46 in transmembrane helix 5. This finding could also be replicated in our established functional assay, where WIN 55,212-2 showed the characteristics of a full agonist at the CB_1R and a “superagonist” at the CB_2R. In our assay CP 55,940 acted on both CBR with very similar affinity, a finding that was expected since an equal binding affinity on both receptors was published in several papers (Thomas et al., 1998; Pertwee, 2008a). Compared to the endogenous ligand 2-AG, in our test system

CP 55,940 behaved like a “superagonist” at both receptors with an increase of GTPase activity with 51% at the CB_1R and 18% at the CB_2R more than 2-AG.

The validation also included the influence of the solvent enhancer DMSO and BSA due to the fact that the ligands of CBRs are often lipophilic and thus not quantitatively water-soluble. DMSO did not affect the outcome up to a concentration of 3% (v/v) and BSA was usable in a concentration of 1 mg/ml as recommended by Breivogel (Breivogel, 2006). We recommend the use of DMSO due to the fact that lipophilic, unsaturated compounds undergo the risk of binding and autoxidation processes in highly concentrated protein solvents like BSA and FCS containing for example Fe^{2+}/Fe^{3+} ions. Furthermore, the handling of DMSO solutions is easier because BSA causes foamy solutions after mixing which makes it more difficult to pipette accurately and tends to absorb to plastic which might influence the final BSA concentration.

The assay was sensitive enough to detect the partial agonist Δ^9-THC in a matrix of a Δ^9-THC free *Cannabis* extract and thus it is usable for a bioactivity-guided fractionation protocol for natural products (Heilmann, 2007). A more complex problem and a limitation for such a screening procedure could be the possible simultaneous presence of antagonists and agonists in the matrix which can abolish or mask the GTPase activity.

The recently identified CB_2R ligand dodeca-2*E*,4*E*-dienoic acid isobutylamide isolated from *Echinacea purpurea* (Raduner et al., 2006) as well as a *Echinacea purpurea* root extract containing several other alkamides (Perry et al., 1997) was tested for agonism, neutral antagonism and inverse agonism in our GTPase assay and showed no activity in all modes with the used membrane construct. This is in contrast to literature data where not only binding, but also CB_2R-dependent effects have been reported in mammalian cellular systems for dodeca-2*E*,4*E*-dienoic acid isobutylamide. Raduner et al. (2006) measured elevated intracellular Ca^{2+} concentration in CB_2-positive cells, an effect that was inhibited by the CB_2R antagonist SR144528. It can be discussed that a highly cell-specific coupling to a G protein heterotrimer subunit is necessary for the effect of the alkamides.

For further prospective GPCR-Gα fusion proteins for characterization of CBRs are in progress.

In conclusion, we established and validated a steady-state GTPase assay for hCBRs as a highly sensitive test system that allows the characterization of ligands at

a very proximal point of the signal cascade of this type of GPCRs. Efforts were made to establish a functional assay in mammalian tissue membranes, but the resulting signal were far too small. Showing very sensitive and practical properties, the test system with CBR expressing Sf9 cell membranes is also suitable for screening procedures and bioactivity-guided isolation of natural compounds.

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3 Impact of fusion to $G\alpha_{i2}$ and co-expression with RGS proteins on pharmacological properties of human cannabinoid receptors CB_1R and CB_2R*

3.1 Abstract

G protein coupled receptor (GPCR)-Gα fusion proteins are often employed to investigate in detail receptor-G protein interaction. In the current study, the impact of Gα fusion proteins on the pharmacology of CBRs, both mediating signals through $G\alpha_i$ proteins, were investigated. The $G\alpha_{i2}$ protein was fused to the C-terminus of the CBRs or co-expressed with non-fused $G\alpha_{i2}$ in Sf9 cells, always together with $G\beta_1\gamma_2$. As it is known that RGS proteins can regulate the sensitivity of G protein pathways, the impact of these proteins on CBR signaling in combination with the fusion approach was examined as well, using RGS4 and RGS19 as paradigms. Known CBR ligands were characterized in the steady-state GTPase assay and pharmacological properties of ligands in the different test systems were correlated. These studies showed the following: Fusion of CBRs to $G\alpha_{i2}$ enhanced the maximal stimulatory effects of ligands compared to the co-expression system, especially for the CB_2R. RGS4, but not RGS19 behaved as a GTPase activating protein at CBRs in the $G\alpha_{i2}$ co-expression and fusion system. Fusion of GPCR, most prominently CB_2R, to $G\alpha_{i2}$ and co-expression with RGS4 altered the pharmacological properties of ligands. Our data suggest that fusion of CB_2R to $G\alpha_{i2}$ and co-expression with RGS4 impedes with conformational changes. Moreover, our results support the concept of ligand-specific receptor conformation because not all ligands are altered similarly.

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3.2 Introduction

Many hormones and neurotransmitters exert their physiological effects through GPCRs. G proteins play an important role as mediators of signals between GPCRs and intracellular effector molecules. The binding of an agonist to a GPCR induces a conformational change accompanied by the exchange of bound GDP to GTP and dissociation of the Gα-GTP-Gβγ complex into the subunits Gα-GTP and Gβγ (Gille and Seifert, 2003). Both subunits can regulate effector systems, for example adenyly cyclase- and mitogen-activated protein (MAP) kinase activity in the case of $G\alpha_i$. Deactivation of the G protein is accomplished by the intrinsic GTPase activity of the Gα subunit, hydrolyzing GTP to GDP and P_i. Subsequently, reassociation of Gα, GDP and Gβγ completes the G protein cycle. According to the two-state model of GPCR activation (Seifert and Wenzel-Seifert, 2002), agonists stabilize the active R* state and increase basal G protein activity, whereas inverse agonists stabilize the inactive R state and decrease basal G protein activity. Antagonists do not change this equilibrium. Refinements of this two-state model were derived from observations of agonist-specific trafficking of a receptor stimulus. In accordance with this model, each agonist is capable of stabilizing or selecting a unique receptor conformation, which results in an unlimited number of active receptor states (Kenakin, 1995).

The efficacy of receptor-G protein coupling is highly influenced by protein expression levels and stoichiometry of signaling partners (Seifert et al., 1999). One limitation concerning the use of GPCR co-expression systems in assay development or functional studies is the lacking guarantee that every receptor molecule is spatially associated with its signaling partner. The use of receptor-Gα fusion proteins offers the advantage of a defined stoichiometry combined with a close proximity of GPCR and G protein (Seifert et al., 1999; Milligan, 2000). As the binding of ligands is accompanied by a conformational change in receptors, it is of substantial interest whether the pharmacological properties of ligands and receptors are influenced by the fusion to the Gα subunit. Some authors reported on alterations in pharmacological properties of ligands of the α_{2A} adrenoceptor as a result of the fusion (Burt et al., 1998), whereas for others like the 5-HT_{1A} receptor, similar potencies and efficacies were observed (Kellett et al., 1999).

To address this question for the two human cannabinoid receptors (CBRs), which belong to family A GPCRs and couple to pertussis-toxin (PTX) sensitive $G\alpha_{i/o}$ (Howlett et al., 2002), receptors were fused C-terminally to the N-terminus of the $G\alpha_{i2}$

subunit. Afterwards the pharmacological properties of the endogenous agonists anandamide and 2-arachidonoyl glycerol (2-AG), the synthetic agonists CP 55,940 ((-)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl) cyclohexanol) and WIN 55,212-2 ((R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1 naphthalenylmethanone mesylate), as well as the synthetic inverse agonists AM 251 (N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide) and AM 281 (1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-4-morpholinyl-1H-pyrazole-3-carboxamide) (at CB_1R) and antagonist AM 630 (6-iodo-2-methyl-1-[2-4(morpholinyl)-ethyl]-[1H-indol-3-yl]-(4-methoxyphenyl)methanone) (at CB_2R), were determined. To examine the potencies and efficacies of these ligands, the steady-state GTPase assay, a reliable and sensitive assay system, was employed (Geiger et al., 2010; Seifert and Wenzel-Seifert, 2002). Data obtained in the fusion protein system were compared with those obtained in a system where the CBRs were co-transfected with the $G\alpha_{i2}$ subunit.

Regulators of G protein signaling (RGS) proteins are a protein family regulating the sensitivity of G protein signaling pathways. RGS proteins can serve as GTPase activating protein (GAPs) for Gα subunits (Wettschureck and Offermanns, 2005) and shorten the period of time in which the Gα subunit is in its active conformation. Thereby, RGS proteins facilitate GPCR signal termination. Studies with RGS proteins revealed that GTP hydrolysis can become the rate-limiting step of the G protein cycle and that the G protein GTPase kinetics are altered by RGS proteins (Kleemann et al., 2008; Schneider and Seifert, 2009). GAP activity of RGS proteins, as key modulators in amplitude and duration of G protein mediated signaling, were described for $G\alpha_i$ and $G\alpha_q$ subunits (Hollinger and Hepler, 2002).

As it has been reported that RGS4 and RGS19 are GAPs for the $G\alpha_i$ subfamily (Berman et al., 1996) and that RGS proteins can participate in the formation of a quaternary complex consisting of agonist, receptor, G protein and RGS protein (Benians et al., 2005), another aim of this study was to investigate the influences of these RGS proteins on the pharmacological properties of CBR ligands in fusion and co-expression systems. Therefore, *Spodoptera frugiperda* (Sf9) cells were infected with baculoviruses encoding for CBRs-$G\alpha_{i2}$ or CBRs co-transfected with $G\alpha_{i2}$ always together with $G\beta_1\gamma_2$ and in the absence or presence of RGS4 or RGS19.

3.3 Materials and Methods

3.3.1 Materials

The DNA primers for PCR were synthesized by MWG Biotech (Ebersberg, Germany). PfuUltra II fusion HS polymerase was from Stratagene (La Jolla, CA, USA). Restriction enzymes were purchased from New England Biolabs (Ipswich, MA, USA). Recombinant baculovirus encoding $G\alpha_{i2}$ was generously provided by Dr. A. G. Gilman (Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, TX, USA). Recombinant baculovirus encoding $G\beta_1\gamma_2$ was a kind gift from Dr. P. Gierschik (Department of Pharmacology, University of Ulm, Germany). Baculoviruses encoding for mammalian RGS4 and RGS19 were a kind gift from Dr. E. Ross (Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, TX, USA).

The M1 anti-FLAG antibody was obtained from Sigma (St. Louis, MO, USA); the anti-$G\alpha_{i2}$ antibody was purchased from Calbiochem (San Diego, CA, USA). Antibodies selective for RGS4 and RGS19 were from Santa Cruz (Santa Cruz, CA, USA) and antibody for Gβ subunit ($G\beta_{common}$; AS398/9) was kindly provided by Dr. B. Nürnberg (Institute of Pharmacology, University of Tübingen, Germany).

The CBR ligands anandamide, 2-AG, CP 55,940, WIN 55,212-2, AM 251, AM 281 and AM 630 were purchased from Tocris Cookson (Ballwin, MO, USA). The 10 mM stock solutions of these compounds were prepared with 100% (v/v) DMSO and dilutions of all ligands were prepared with 30% (v/v) DMSO.

[γ-^{32}P]GTP was synthesized by enzymatic phosphorylation of GDP and [^{32}P]P_i (150 mCi/ml orthophosphoric acid) (PerkinElmer Life Sciences, Boston, MA, USA) as described previously (Walseth and Johnson, 1979). Radioactive samples were counted in a PerkinElmer Tricarb-TR liquid scintillation analyzer. Unlabeled nucleotides were from Roche Diagnostics (Indianapolis, IN, USA), and all other reagents were of the highest purity available and from standard suppliers.

3.3.2 Methods

3.3.2.1 *Construction of pVL-1392 plasmids encoding SF-hCBR-His$_6$ and SF-hCBR-His$_6$-Gα_{i2}*

The generation of pVL-SF–hCBR-His$_6$ constructs was previously described (Nickl et al., 2008). For preparation of the SF-hCBR-His$_6$-Gα_{i2} fusion proteins, the hexahistidine-tagged C-terminus of hCB$_1$R and hCB$_2$R was fused to the N-terminus of Gα_{i2} according to a previously described strategy using overlap-extension polymerase chain reaction (Wenzel-Seifert and Seifert, 2000).

In PCR 1a, DNA fragments encoding for the cleavable signal peptide from influenza hemagglutinin (S), the FLAG epitope (F), the cDNA of CBRs and the hexahistidine tag were amplified without stop codons. Therefore, the primers SacI-SF (5´-ATC AGA TCA GCT TGA TTC GAG CTC G-3´) and aHis$_6$ (5´-GTG ATG GTG ATG ATG GTG-3´), were synthesized, annealing at pVL-1392 plasmids encoding for SF–hCB$_1$R-His$_6$ and SF–hCB$_2$R-His$_6$, used as templates in PCR 1a. In PCR 1b, the Gα_{i2} sequence was amplified using a pVL-1392-FPR-His$_6$-Gα_{i2} template. The sense primer for this PCR contained 18 bp encoding for hexahistidine tag (underlined) followed by the first 18 bp of the Gα_{i2} sequence (5´-<u>CAC CAT CAT CAC CAT CAC</u> ATG GGC TGC ACC GTG AGC-3´). The antisense primer for SF-hCB$_1$R-His$_6$-Gα_{i2} fusion protein aGα_{i2}-XbaI (5´-GGT CGA CTC TAG AGG TCA GAA GAG GCC ACA GTC) contained a XbaI site 3´ of the stop codon of Gα_{i2}; the antisense primer for SF-hCB$_2$R-His$_6$-Gα_{i2} fusion protein aGα_{i2}-XmaI (5´-ATC CTA CCC GGG TCA GAA GAG GCC ACA GTC-3´) contained an extra XmaI site 3´ of the stop codon. In PCR 2, the product of PCR 1a and 1b were used as templates together with SacI-SF and aGα_{i2}-XbaI (for CB$_1$R) respectively aGα_{i2}-XmaI (for CB$_2$R) as primers. PCR 2 resulted in fragments, consisting of a signal flag and a FLAG tag, the cDNA for hCBRs, followed by a hexahistidine tag and the Gα_{i2} sequence with a XbaI restriction site for CB$_1$R and a XmaI restriction site for CB$_2$R. PCR 2 product containing SF-hCB$_1$R-His$_6$-Gα_{i2} were double-digested with SacI and XbaI and cloned into baculovirus transfer vector pVL-1392-SF-hCB$_1$R-His$_6$ via SacI and XbaI restriction site; PCR products encoding for SF-hCB$_2$R-His$_6$-Gα_{i2} were double-digested with SacI and XmaI and cloned into baculovirus expression vector pVL-1392-SF-hCB$_2$R-His$_6$ via SacI and XmaI restriction site. Correct assembly of the constructs was confirmed by extensive restriction enzyme analysis and sequencing service of Entelechon (Regensburg, Germany).

3.3.2.2 *Generation of recombinant baculoviruses, cell culture and membrane preparation*

Sf9 cells, derived from *Spodoptera frugiperda* pupal ovarian tissue, were used for the baculovirus expression. Sf9 cells were cultured in Erlenmeyer flasks at 28℃ under rotation at 125 rpm in SF 900 II medium (Life Technology, Carlsbad, CA), supplemented with 5% (v/v) fetal calf serum (Pan-Biotech, Aidenbach, Germany) and 0.1 mg/ml gentamicin sulfate (BioWhittaker, Walkersville, MD). Cells were maintained at a density of 0.5-6.0 x 10^6 cells/ml. Recombinant baculoviruses were generated in Sf9 insect cells using the BaculoGOLD transfection kit (BD PharMingen, San Diego, CA) according to the manufacturer´s instructions. After initial transfection, high-titer virus stocks were generated by two sequential virus amplifications. The supernatant fluid of the second amplification was stored under light protection and used as virus stock for membrane preparations.

For membrane preparation, cells were seeded at a density of 3.0 x 10^6 cells/ml and infected with 1:100 dilutions of baculovirus stock solutions of desired proteins. Cells were cultured for 48 h and Sf9 membranes were prepared as described previously (Geiger et al. 2010) using 1 mM EDTA (Merck, Darmstadt, Germany), 0.2 mM phenylmethylsulfonyl fluoride (Sigma), 10 µg/ml benzamidine (Sigma) and 10 µg/ml leupeptin (Calbiochem, San Diego, CA, USA) as protease inhibitors. Membranes were homogenized in binding buffer containing 75 mM Tris/HCl, pH 7.4, 1 mM EDTA and 12.5 mM $MgCl_2$ and stored in aliquots at -80℃. Protein concentrations were determined using the DC protein assay kit (Bio-Rad, Hercules, CA, USA) according to the instructions of the manufacturer.

3.3.2.3 *SDS-PAGE and immunoblot analysis*

Membranes were diluted in Laemmli buffer (8 M urea, 2.5% (w/v) SDS, 200 mM dithiothreitol, 25 mM Tris, 5% glycerol (v/v), 0.01% (w/v) bromophenolblue) and separated on SDS polyacrylamide gels containing 12% (w/v) acrylamide (Sigma). Proteins were transferred onto 0.45 µm nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA) and then incubated with antibody solution: M1 anti-FLAG (1:1000), anti-$G\alpha_{i2}$ (1:1000), anti-RGS4 (1:500), anti-RGS19 (1:500) and anti-$G\beta_{common}$ (1:1200). Protein bands were visualized with Luminol Enhancer Solution (Pierce Chemical, Rockford, IL, USA) using anti-mouse IgG (Sigma), anti-goat IgG (Santa Cruz, CA, USA) and anti-rabbit IgG (GE Healthcare, Little Chalfont, Buckinghamshire, UK), all coupled to horseradish peroxidase. Chemoluminescently

stained blots were exposed to Hyperfilms ECL (GE Healthcare) and developed with a Cawomat 2000 IR (Böhm Medical, Heiligeneich, Germany).

3.3.2.4 *Steady-state GTPase assay*

The GTPase assay was performed as described (Geiger et al., 2010). Briefly, membranes were thawed, sedimented by centrifugation at 18,000 x g for 10 min at 4℃, and carefully resuspended in 10 mM Tris/HCl, p H 7.4. Assay tubes contained membranes ($G\alpha_{i2}$ co-transfected membranes 10 µg of protein/tube; $G\alpha_{i2}$ fusion protein membranes 5 µg of protein/tube), 1.0 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM ATP, 100 nM GTP, 0.1 mM adenylyl imidodiphosphate, 5 mM creatine phosphate, 40 µg of creatine kinase and 0.2% (w/v) BSA in 50 mM Tris/HCl, pH 7.4, to prevent binding of protein or ligand to the polystyrol tubes, and CB_1R and CB_2R ligands at various concentrations. Reaction mixtures (80 µl) were incubated for 2 min at 25℃ before the addition of 20 µl of [γ-^{32}P]GTP (0.1 µCi/tube). Reactions were conducted for 20 min at 25℃ for co-transfected membranes and 10 min for fusion protein membranes. Reactions were terminated by the addition of 900 µl of slurry consisting of 5% (w/v) activated charcoal and 50 mM NaH_2PO_4, pH 2.0. Charcoal absorbs nucleotides but not $^{32}P_i$. Charcoal-quenched reaction mixtures were centrifuged for 7 min at room temperature at 15,000 x g. Six hundred µl supernatant fluid of reaction mixtures was removed and $^{32}P_i$ was determined by Čerenkov radiation in 3 ml water. Enzyme activities were corrected for spontaneous degradation of [γ-^{32}P]GTP. Spontaneous [γ-^{32}P]GTP degradation was determined in tubes containing all of the above described components plus a high concentration of unlabeled GTP (1 mM) that, by competition with [γ-^{32}P]GTP, prevents [γ-^{32}P]GTP hydrolysis by enzymatic activities present in Sf9 membranes. Spontaneous [γ-^{32}P]GTP degradation amounted to <1% of the total amount of radioactivity added. The experimental conditions chosen ensured that not more than 20% of the total amount of [γ-^{32}P]GTP added was converted to $^{32}P_i$. Neutral antagonism property of AM 630 was determined in the presence of 30 nM CP 55,940.

3.3.2.5 *Calculations and statistics*

Data are expressed as means ± SD and represent a minimum of 3 independent experiments, each performed in triplicate. Statistical evaluations and curve fittings were performed using the Prism 4 software (GraphPad Prism, La Jolla, CA, USA) and the Microsoft Excel 2007 software. Statistical significance was determined by the one-way ANOVA, followed by the Dunnett post test.

3.4 Results

3.4.1 Generation of baculoviruses and detection of protein expression by immunoblotting

For expression of the human CBRs in insect cells, recombinant baculovirus transfer vectors bearing different constructs were designed. To direct the receptor protein to the cell membrane and in order to allow immunological detection of the recombinant proteins all constructs contained in-frame fusions to a cleavable signal peptide from influenza hemagglutinin, followed by the FLAG tag. In plasmids encoding for CBRs a hexahistidine tag allowing further purification was fused C-terminally to the receptor-coding region. In plasmids encoding for the CBRs-$G\alpha_{i2}$ fusion proteins the hexahistidine tag was used as overlap for the $G\alpha_{i2}$ subunit.

We transfected Sf9 cells with baculovirus stock solutions encoding for CBRs and $G\alpha_{i2}$ or the CBR-$G\alpha_{i2}$ fusion protein, together with $G\beta_1\gamma_2$ and RGS4 or RGS19 to design test systems as described in Table 3.1. Expression of proteins was confirmed with immunoblotting. As shown in Figure 3.1A and Figure 3.2A, the M1 anti-FLAG antibody recognized the CBRs as well as the CBR-$G\alpha_{i2}$ fusion proteins. CB_1R showed the expected band at ~57 kDa (Xu et al., 2005). Additional bands were detected by the M1 antibody, which may reflect oligomeric forms of the CB_1R. The ~41 kDa bands corresponded to CB_2R, which is in accordance with literature data on the molecular mass of CB_2R (Filppula et al., 2004). Regarding fusion proteins, bands for CB_1R-$G\alpha_{i2}$ (~97 kDa) and CB_2R-$G\alpha_{i2}$ (~80 kDa) appeared as expected. Beneath the intense additional bands in CB_1R-$G\alpha_{i2}$ membranes reflecting oligomeric forms of the receptor, a weak band at the level of non-fused CB_1R was detected by the M1 antibody, probably representing a degradation product. Also noticeable is a second band for CB_2R-$G\alpha_{i2}$ which may be due to different glycosylation states of the receptor.

To visualize the $G\alpha_{i2}$ subunit, we used an antibody for $G\alpha_{i1/2}$ proteins and detected intense bands at ~40 kDa in the co-transfection systems and bands matching the molecular mass estimation for CB_1R-$G\alpha_{i2}$ and CB_2R-$G\alpha_{i2}$ in the fusion systems (Figure 3.1B and Figure 3.2B).

Figure 3.1C shows the detection of $G\beta_1$ with a $G\beta_{common}$ antibody in the CB_1R systems. In the CB_1R-$G\alpha_{i2}$ systems an additional band with relatively high molecular mass is particularly evident, whereas for the CB_2R protein expression systems

(Figure 3.2C) a second weak band near by the band for $G\beta_1$ (~36 kDa) was visualized. The identity of these bands is unknown.

The detection of co-expressed RGS4 and RGS19 proteins was performed with specific anti-RGS4 and anti-RGS19 antibodies (Figures 3.1D and E and Figures 3.2D and E). As expected, the bands for RGS4 were about ~1 kDa lower compared to RGS19. Additional bands were particularly evident for the anti-RGS19 antibody, probably representing oligomeric forms.

In conclusion, these data indicate that the desired CBR constructs were correctly produced in Sf9 cells after transfection with recombinant baculoviruses and that the desired proteins are expressed in the Sf9 membranes.

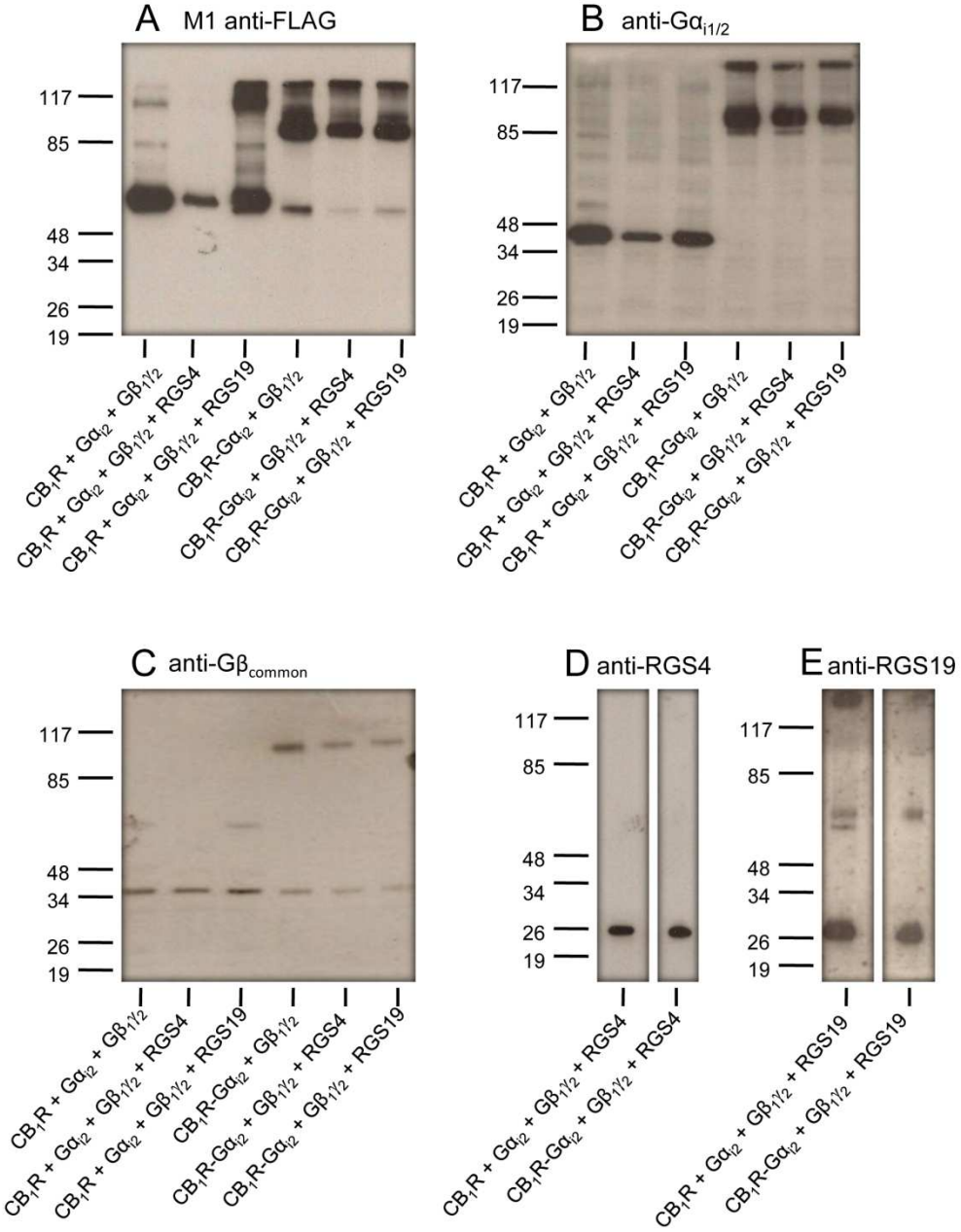

Figure 3.1: Immunoblot analysis of recombinant proteins in Sf9 cell membranes for CB_1R test systems

Immunological detection of CB_1R, $G\alpha_{i2}$, $G\beta_1\gamma_2$ and RGS proteins expressed in Sf9 cell membranes was performed as described under *Materials and Methods.* Each lane was loaded with 10 µg of protein. Numbers on the left indicate masses of marker protein in kilodaltons. **A** Detection of CB_1R and CB_1R-$G\alpha_{i2}$ with the M1 anti-FLAG antibody. **B** Visualization of $G\alpha_{i2}$ with anti-$G\alpha_{i1/2}$. **C** Membranes were reacted with $G\beta_{common}$ antibody. **D** Detection of RGS4. **E** Detection of RGS19.

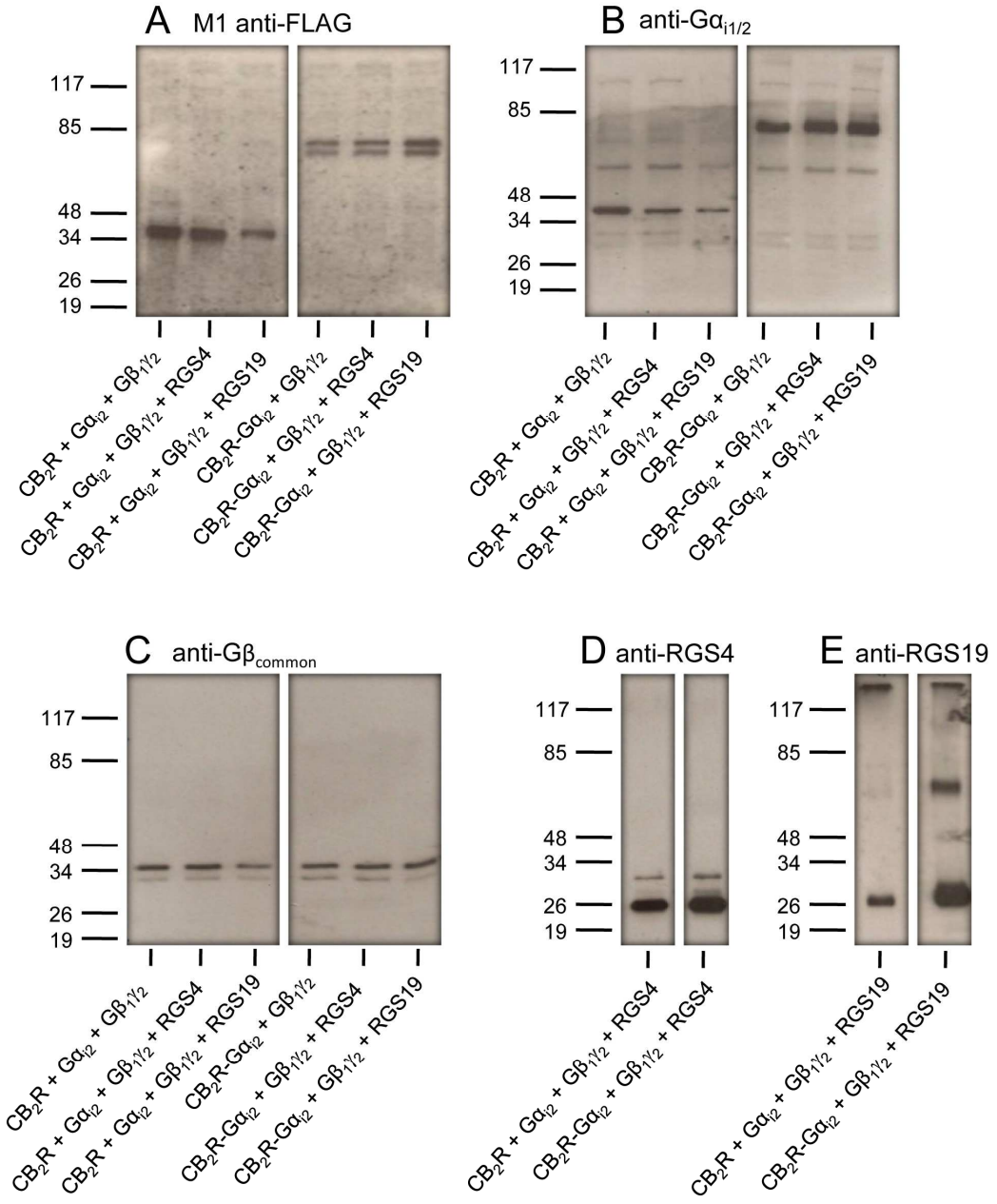

Figure 3.2: Immunoblot analysis of recombinant proteins in Sf9 cell membranes for CB_2R test systems

Immunological detection of CB_2R, $G\alpha_{i2}$, $G\beta_1\gamma_2$ and RGS proteins expressed in Sf9 cell membranes was performed as described under *Materials and Methods.* Each lane was loaded with 10 µg of protein. Numbers on the left indicate masses of marker protein in kilodaltons. **A** Detection of CB_2R and CB_2R-$G\alpha_{i2}$ with the M1 anti-FLAG antibody. **B** Visualization of $G\alpha_{i2}$ with anti-$G\alpha_{i1/2}$. **C** Membranes were reacted with $G\beta_{common}$ antibody. **D** Detection of RGS4. **E** Detection of RGS19.

3.4.2 Basal GTPase activity and stimulation of GTPase by CP 55,940 in the GTPase assay

We performed steady-state GTPase assays with Sf9 membranes co-expressing the proteins shown in Table 3.1 and determined the maximum stimulatory effects of the full agonist CP 55,940. The absolute values of basal GTPase activity differed substantially within the different protein combinations and among various membranes, reflecting different protein expression levels and/or protein integrities. However, in all cases CP 55,940 increased GTPase activity, and addition of RGS4 markedly enhanced the stimulatory effect of CP 55,940. RGS4, but not RGS19, behaved like a GTPase activation protein (Ross and Wilkie, 2000), which is statistically verified in Table 3.2 and Table 3.3. Regarding the CB_1R system, the largest GTPase stimulation was obtained with the fusion system in the presence of RGS4. Here, a mean stimulation of 304 ± 9% above basal was calculated. Also for the CB_2R, the CB_2R-$G\alpha_{i2}$ fusion protein co-transfected with RGS4 showed the highest GTPase stimulation, amounting to 393 ± 30%.

Figure 3.3 shows representative concentration-response curves obtained for CP 55,940 in systems co-expressing CBR and $G\alpha_{i2}$ or expressing CBR-$G\alpha_{i2}$ in the absence or presence of RGS proteins. Particularly remarkable is the enhanced stimulatory effect of the ligand in the CB_2R-$G\alpha_{i2}$ fusion system. The fusion of CB_2R to $G\alpha_{i2}$ revealed 2.5 – 3-fold higher GTPase activities than in systems where CB_2R is co-expressed with $G\alpha_{i2}$.

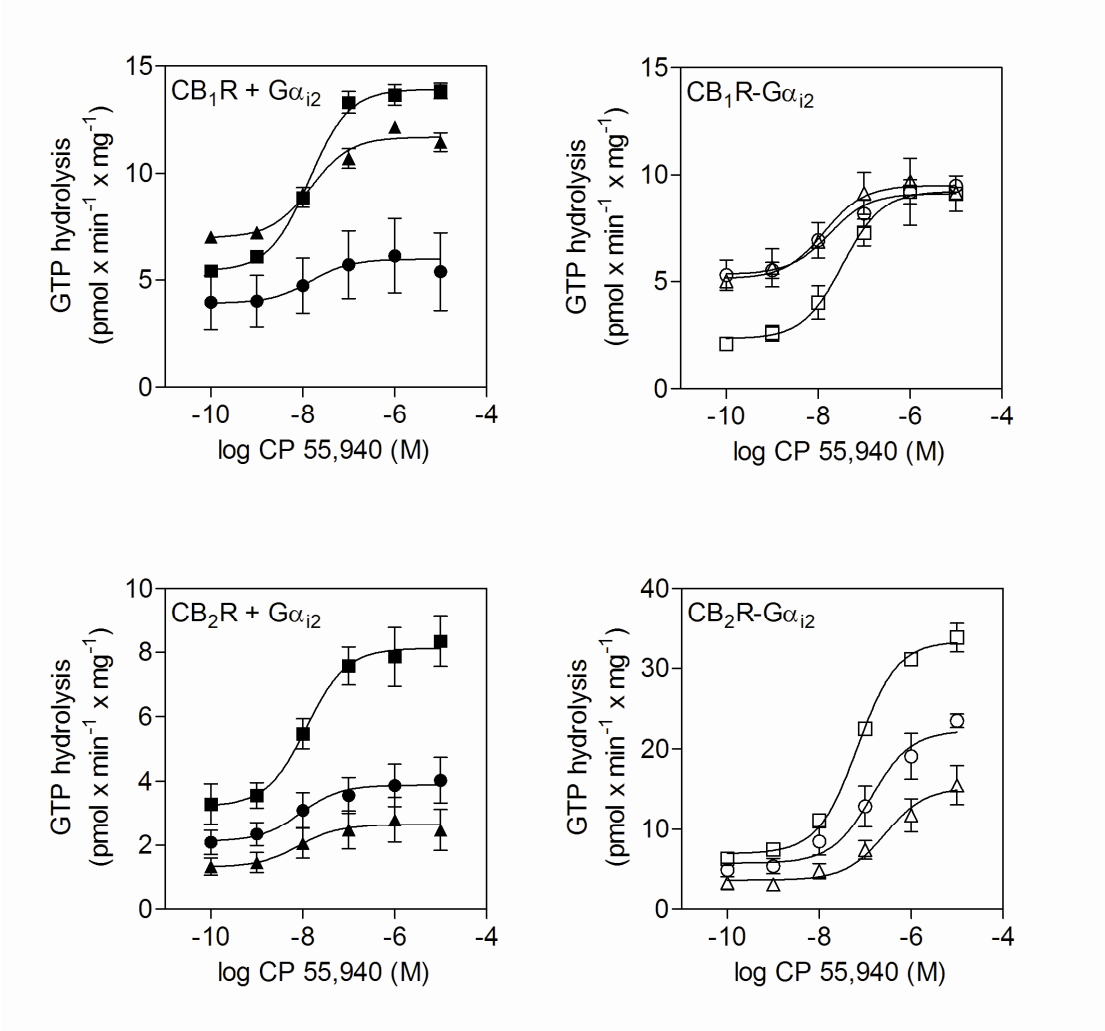

Figure 3.3: Representative concentration-response curves obtained for CP 55,940 in the $G\alpha_{i2}$ co-expression and $G\alpha_{i2}$ fusion system

Steady-state GTPase activity in Sf9 membranes was determined as described under *Materials and Methods.* Data show representative results performed in triplicates in Sf9 cells expressing CBR + $G\alpha_{i2}$ + $G\beta_1\gamma_2$ without ●, + RGS4 ■, + RGS19 ▲ or CBR-$G\alpha_{i2}$ + $G\beta_1\gamma_2$ without ○, + RGS4 □, + RGS19 Δ. Experiments were replicated 3 independent times with different membrane preparations. Reaction mixtures contained CP 55,940 at concentrations from 1 nM to 10 µM. Data were analysed by nonlinear regression and best fit to sigmoidal concentration-response curves. Pharmacological parameters extracted from resulting graphs are shown in Table 3.2 and Table 3.3.

Table 3.1: Impact of RGS proteins and of fusion to $G\alpha_{i2}$ on the basal GTPase activity and effects of full agonist CP 55,940 in the GTPase assay

	Membrane preparation number	Basal [pmol x min^{-1} x mg^{-1}]	Mean value basal	10 µM CP 55,940 [pmol x min^{-1} x mg^{-1}]	% stim over basal
	1578	5.17 ± 0.12		7.56 ± 0.15	46
CB_1R + $G\alpha_{i2}$	988	2.47 ± 0.07	3.92 ± 1.11	3.95 ± 0.05	60
	1797	4.14 ± 0.08		6.83 ± 0.08	65
	1794	5.42 ±0.15		13.97 ± 0.12	158
CB_1R + $G\alpha_{i2}$ + RGS4	1579	5.42 ± 0.13	5.40 ± 0.02	13.48 ± 0.11	149
	1798	5.37 ± 0.14		14.40 ± 0.13	168
	1078	4.13 ± 0.12		6.22 ± 0.13	51
CB_1R + $G\alpha_{i2}$ + RGS19	1244	9.68 ± 0.43	6.94 ± 2.29	18.46 ± 0.36	90
	1233	6.95 ± 0.23		11.70 ± 0.20	68
	1722	5.04 ± 0.29		8.39 ± 0.31	67
CB_1R-$G\alpha_{i2}$	1799	5.11 ± 0.33	5.41 ± 0.10	9.56 ± 0.48	87
	1853	5.40 ± 0.19		9.43 ± 0.19	75
	1800	2.42 ± 0.46		10.07 ± 0.38	317
CB_1R-$G\alpha_{i2}$ + RGS4	1841	5.96 ± 0.43	3.49 ± 1.75	23.60 ± 0.45	296
	1848	2.10 ± 0.23		8.41 ± 0.20	299
	1873	7.32 ± 0.32		14.64 ± 0.29	100
CB_1R-$G\alpha_{i2}$ + RGS19	1875	5.12 ± 0.30	5.32 ± 1.56	9.51 ± 0.25	86
	1879	3.52 ± 0.16		7.85 ± 0.14	123
	1580	2.39 ± 0.13		4.61 ± 0.10	93
CB_2R + $G\alpha_{i2}$	1080	1.71 ± 0.11	2.21 ± 0.30	3.16 ± 0.09	86
	1360	2.27 ± 0.07		3.90 ± 0.05	72
	1581	2.92 ± 0.09		7.42 ± 0.06	154
CB_2R + $G\alpha_{i2}$ + RGS4	1624	2.83 ± 0.13	3.20 ± 0.46	7.79 ± 0.11	175
	1857	3.86 ± 0.14		9.20 ± 0.12	139
	1354	1.30 ± 0.06		2.92 ± 0.05	125
CB_2R + $G\alpha_{i2}$ + RGS19	1058	1.63 ± 0.08	1.33 ± 0.24	3.41 ± 0.08	109
	1003	1.04 ± 0.07		1.94 ± 0.05	86
	1849	4.40 ± 0.40		17.14 ± 0.72	290
CB_2R-$G\alpha_{i2}$	1854	6.18 ± 0.64	5.61 ± 0.86	22.04 ± 0.83	257
	1856	6.25 ± 0.49		23.89 ± 0.62	283
	1842	6.93 ± 0.45		33.38 ± 0.53	382
CB_2R-$G\alpha_{i2}$ + RGS4	1817	8.42 ± 0.76	5.83 ± 2.68	39.04 ± 0.94	364
	1850	2.14 ±0.27		11.42 ± 0.44	435
	1874	3.65 ± 0.27		12.97 ± 0.41	256
CB_2R-$G\alpha_{i2}$ + RGS19	1876	4.26 ± 0.47	3.58 ± 0.58	18.28 ± 0.72	330
	1880	2.83 ± 0.38		13.88 ± 0.91	390

Basal and CP 55,940-stimulated GTPase activities in various CBR expressing Sf9 membranes were determined as described in *Materials and Methods.* All membranes expressed proteins given in the Table and were additionally co-transfected with $G\beta_1\gamma_2$. Numbers designate the specific membrane studied in the GTPase assay. Data shown are the mean ± SD of one assay in triplicates.

3.4.3 Potencies and efficacies of standard ligands of the CBRs in the GTPase assay in the absence and presence of RGS proteins

We evaluated the potential effects of GAPs (Ross and Wilkie, 2000) on GTPase activity and used RGS4 and RGS19 as paradigms, $G\beta_1\gamma_2$ always being present. In previous studies on the chemokine receptor $CXCR_4$ and human histamine H_4 receptor, both RGS proteins enhanced agonist-stimulated GTP hydrolysis (Kleemann et al., 2008; Schneider and Seifert, 2009).

The potencies and efficacies of several ligands (Table 3.2 and Table 3.3), specifically the endogenous agonists anandamide and 2-AG, the synthetic agonists CP 55,940 and WIN 55,212-2 as well as the synthetic inverse agonists AM 251 and AM 281 (at CB_1R) and antagonist AM 630 (at CB_2R) were examined. The results obtained in the presence of RGS proteins were compared to data evaluated in systems where the RGS proteins were absent (CBR + $G\alpha_{i2}$ + $G\beta_1\gamma_2$ and CBR-$G\alpha_{i2}$ + $G\beta_1\gamma_2$, respectively). Regarding CB_1R (Table 3.2), no significant changes in $logEC_{50}$/$logIC_{50}$ values were detected for all analyzed systems. Exceptions are the $logEC_{50}$ of anandamide in the CB_1R + $G\alpha_{i2}$ + $G\beta_1\gamma_2$ + RGS4- and in the CB_1R-$G\alpha_{i2}$ + $G\beta_1\gamma_2$ + RGS4 system, and the $logIC_{50}$ of AM 281 in CB_1R + $G\alpha_{i2}$ + $G\beta_1\gamma_2$ + RGS4 system.

All agonists induced relatively small GTPase activations in the standard co-expression (CB_1R + $G\alpha_{i2}$ + $G\beta_1\gamma_2$) and standard fusion system (CB_1R-$G\alpha_{i2}$ + $G\beta_1\gamma_2$) and the inverse agonists AM 251 and AM 281 reduced GTPase signals to a similar extent. In both systems, addition of RGS4 significantly influenced ligand-regulated GTPase activity, resulting in higher stimulation over basal levels for agonists and a more effective inhibition of GTPase activity for inverse agonists. A divergent result was obtained for anandamide in the co-expression system, where RGS4 did not significantly influence GTPase activity (stimulations of 84 ± 25% with RGS4 and 62 ± 14% in the standard co-expression system), and for the inverse agonist AM 251 in the fusion systems (-73 ± 3% with RGS4 and -62 ± 7% in the standard fusion system). Interestingly, AM 251, tested in the co-expression system, was the only ligand sensitive to RGS19, resulting in a significantly stronger inhibition of GTPase activity with a value -80 ± 2% compared to the standard co-expression system with a value of -64 ± 4%. For calculation of efficacies, the maximal stimulatory effects of the ligands were related to the GTPase activation of 2-AG (E_{max} set 1.00). Interestingly,

only the efficacies of CB_1R inverse agonists AM 251 and AM 281 were significantly altered by RGS protein addition.

As was the case for CB_1R, RGS4, but not RGS19 enhanced GTPase responses of CB_2R (Table 3.3). The co-expression of RGS4 enhanced the stimulatory effects of all ligands except for anandamide in the co-expression system (54 ± 11% stimulation in the standard co-expression system $CB_2R + G\alpha_{i2} + G\beta_1\gamma_2$ *versus* 73 ± 13% in the presence of RGS4) and WIN 55,212-2 in the fusion system (192 ± 12% simulation in the standard fusion system *versus* 255 ± 29% in the presence of RGS4). Although for those two compounds the results were not statistically significant, a tendency of increasing GTPase activity in the presence of RGS4 became apparent. RGS4 altered $logEC_{50}$ values for anandamide and WIN 55,212-2 in the co-expression system and RGS19 influenced the $logEC_{50}$ value for CP 55,940 in the fusion system. For anandamide, a $logEC_{50}$ value of -5.55 ± 0.10 in the $CB_2R + G\alpha_{i2} + G\beta_1\gamma_2$ system was shifted to a $logEC_{50}$ value of -6.22 ± 0.33 obtained in the $CB_2R + G\alpha_{i2} + G\beta_1\gamma_2$ + RGS4 system. Moreover, the potency of WIN 55,212-2 changed from -8.12 ± 0.07 in the $CB_2R + G\alpha_{i2} + G\beta_1\gamma_2$ system to -8.55 ± 0.24 in the system where RGS4 was co-transfected. For CP 55,940 the $logEC_{50}$ value of -6.98 ± 0.05 evaluated in the CB_2R-$G\alpha_{i2} + G\beta_1\gamma_2$ system differs significantly from the $logEC_{50}$ value of -6.60 ± 0.11 obtained in the presence of RGS19.

Table 3.2: GTPase activities of standard ligands and impact of RGS proteins in Sf9 cell membranes expressing CB_1R co-transfected with $G\alpha_{i2}$ or CB_1R-$G\alpha_{i2}$

		$CB_1R + G\alpha_{i2}$	$CB_1R + G\alpha_{i2}$ + RGS4	$CB_1R + G\alpha_{i2}$ + RGS19	CB_1R-$G\alpha_{i2}$	CB_1R-$G\alpha_{i2}$ + RGS4	CB_1R-$G\alpha_{i2}$ + RGS19
2-AG	**Stim [%]**	50 ± 16	104 ± 14**	59 ± 15	53 ± 8	178 ± 52**	76 ± 8
	E_{max}	1.00	1.00	1.00	1.00	1.00	1.00
	logEC_{50}	-6.05 ± 0.13	-6.02 ± 0.35	-6.18 ± 0.42	-6.11 ± 0.20	-5.85 ± 0.12	-5.81 ± 0.09
anandamide	**Stim [%]**	62 ± 14	84 ± 25	78 ± 8	75 ± 8	286 ± 39***	93 ± 1
	E_{max}	1.24 ± 0.28	0.81 ± 0.24	1.32 ± 0.14	1.42 ± 0.15	1.61 ± 0.22	1.22 ± 0.01
	logEC_{50}	-5.88 ± 0.19	-6.66 ± 0.20**	-5.81 ± 0.18	-5.76 ± 0. 02	-6.17 ± 0.16*	-5.71 ± 0.12
CP 55,940	**Stim [%]**	57 ± 8	158 ± 8***	70 ± 16	76 ± 8	304 ± 9***	103 ± 1 5
	E_{max}	1.14 ± 0.16	1.52 ± 0.08	1.19 ± 0.27	1.43 ± 0.15	1.71 ± 0.05	1.36 ± 0.20
	logEC_{50}	-7.76 ± 0.12	-7.86 ± 0.08	-7.80 ± 0.04	-7.86 ± 0.13	-7.49 ± 0.05	-7.59 ± 0.26
WIN 55,212-2	**Stim [%]**	55 ± 7	94 ± 18*	68 ± 8	78 ± 8	271 ± 16***	95 ± 5
	E_{max}	1.10 ± 0.14	0.90 ± 0.17	1.15 ± 0.14	1.47 ± 0.15	1.52 ± 0.09	1.25 ± 0.07
	logEC_{50}	-7.44 ± 0.18	-7.34 ± 0.12	-7.39 ± 0.29	-7.07 ± 0.08	-6.96 ± 0.09	-7.19 ± 0.06
AM 251	**Stim [%]**	-64 ± 4	-73 ± 3*	-80 ± 2**	-62 ± 7	-73 ± 3	-60 ± 3
	E_{max}	-1.28 ± 0.08	-0.70 ± 0.03***	-1.36 ± 0.03	-1.17 ± 0 .13	-0.41 ± 0.02***	-0.79 ± 0.04**
	logIC_{50}	-7.41 ± 0.04	-7.44 ± 0.06	-7.26 ± 0.10	-7.54 ± 0.04	-7.41 ± 0.01	-7.64 ± 0.15
AM 281	**Stim [%]**	-41 ± 1	-61 ± 4***	-45 ± 2	-38 ± 6	-56 ± 6*	-40 ± 1
	E_{max}	-0.82 ± 0.02	-0.59 ± 0.04***	-0.76 ± 0.03	-0.72 ± 0 .11	-0.31 ± 0.03***	-0.53 ± 0.01*
	logIC_{50}	-7.22 ± 0.18	-7.64 ± 0.09*	-7.37 ± 0.07	-7.68 ± 0.1 6	-7.52 ± 0.14	-7.66 ± 0.26

Steady-state GTPase experiments were performed as described in *Materials and Methods.* All membranes were additionally transfected with $G\beta_1\gamma_2$. Reaction mixtures contained 0.1 µCi [γ-^{32}P]GTP and 100 nM unlabeled GTP in the presence of solvent (basal) and CBR ligands at various concentrations (1 nM – 10 µM). Data shown are the mean values ± SD and represent 3 independent experiments performed in duplicates or triplicates with different membrane preparations. The relative agonist-stimulation and inverse agonist-inhibition of GTP hydrolysis (% of basal), were calculated. E_{max} values represent the stimulation of ligands [10 µM] relative to the endogenous agonist 2-AG (defined as 1.00 responses) for each test system. Data were analyzed by nonlinear regression and best fit to sigmoidal concentration-response curves. Statistical evaluations were performed to calculate the impact of RGS proteins on GTPase activity in the co-expression and fusion system. Results in the presence of RGS proteins were compared to data obtained for CBR + $G\alpha_{i2}$ and CBR-$G\alpha_{i2}$, respectively, in one-way ANOVA, followed by the Dunnett´s multiple comparison test. (significant difference: *p < 0.05, **p < 0.01, ***p < 0.001).

Table 3.3: GTPase activities of standard ligands and impact of RGS proteins in Sf9 cell membranes expressing CB_2R co-transfected with $G\alpha_{i2}$ or CB_2R-$G\alpha_{i2}$

		$CB_2R + G\alpha_{i2}$	$CB_2R + G\alpha_{i2}$ + RGS4	$CB_2R + G\alpha_{i2}$ + RGS19	CB_2R-$G\alpha_{i2}$	CB_2R-$G\alpha_{i2}$ + RGS4	CB_2R-$G\alpha_{i2}$ + RGS19
2-AG	**Stim [%]**	67 ± 15	132 ± 13**	79 ± 16	107 ± 20	197 ± 48*	100 ± 7
	E_{max}	1.00	1.00	1.00	1.00	1.00	1.00
	logEC_{50}	-5.29 ± 0.24	-5.73 ± 0.25	-5.72 ± 0.18	-5.72 ± 0.19	-5.55 ± 0.22	-5.33 ± 0.16
anandamide	**Stim [%]**	54 ± 11	73 ± 13	64 ± 7	146 ± 6	297 ± 67*	163 ± 16
	E_{max}	0.81 ± 0.16	0.55 ± 0.10	0.81 ± 0.09	1.36 ± 0.06	1.51 ± 0.34	1.63 ± 0.16
	logEC_{50}	-5.55 ± 0.10	-6.22 ± 0.33*	-5.27 ± 0.07	-5.53 ± 0.11	-5.49 ± 0.38	-5.08 ± 0.18
CP 55,940	**Stim [%]**	84 ± 9	156 ± 15**	107 ± 16	276 ± 14	393 ± 30*	325 ± 55
	E_{max}	1.25 ± 0.13	1.18 ± 0.11	1.35 ± 0.20	2.58 ± 0.13	1.99 ± 0.15	3.25 ± 0.55
	logEC_{50}	-8.05 ± 0.07	-7.94 ± 0.14	-7.96 ± 0.06	-6.98 ± 0.05	-7.04 ± 0.14	-6.60 ± 0.11*
WIN 55,212-2	**Stim [%]**	63 ± 13	161 ± 33**	74 ± 10	192 ± 12	255 ± 29	226 ± 36
	E_{max}	0.94 ± 0.19	1.22 ± 0.25	0.94 ± 0.13	1.79 ± 0.11	1.29 ± 0.15	2.26 ± 0.36
	logEC_{50}	-8.12 ± 0.07	-8.55 ± 0.24*	- 8.09 ± 0.03	-7.28 ± 0. 38	-7.34 ± 0.45	-6.69 ± 0.27
AM 630	**Stim [%]**	-17 ± 8	-48 ± 8**	-15 ± 3	-10 ± 2	-24 ± 1***	-10 ± 2
	E_{max}	-0.25 ± 0.12	-0.36 ± 0.06	-0.19 ± 0.04	-0.09 ± 0.02	-0.12 ± 0.01	-0.10 ± 0.02
	logIC_{50}	-6.48 ± 0.65	-5.94 ± 0.33	-7.01 ± 0.46	-6.33 ± 0.11	-6.63 ± 0.32	-6.88 ± 0.31

Steady-state GTPase experiments were performed as described in *Materials and Methods.* All membranes were additionally transfected with $G\beta_1\gamma_2$. Reaction mixtures contained 0.1 µCi [γ-^{32}P]GTP and 100 nM unlabeled GTP in the presence of solvent (basal) and CBR ligands at various concentrations (1 nM – 10 µM). Data shown are the mean values ± SD and represent 3 independent experiments performed in duplicates or triplicates with different membrane preparations. The relative agonist-stimulation and inverse agonist-inhibition of GTP hydrolysis (% of basal), were calculated. E_{max} values represent the stimulation of ligands [10 µM] relative to the endogenous agonist 2-AG (defined as 1.00 responses) for each test system. Data were analyzed by nonlinear regression and best fit to sigmoidal concentration-response curves. Statistical evaluations were performed to calculate the impact of RGS proteins on GTPase activity in the co-expression and fusion system. Results in the presence of RGS proteins were compared to data obtained for CBR + $G\alpha_{i2}$ and CBR-$G\alpha_{i2}$, respectively, in one-way ANOVA, followed by the Dunnett´s multiple comparison test (significant difference: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

3.4.4 Influence of fusion on ligands potency and efficacy in the absence and presence of RGS proteins

The use of Gα co-expression systems is always associated with the problem that the expression levels and thus a defined stoichiometry of the signaling partners are difficult to control (Gille and Seifert, 2003). This is important since the efficiency of GPCR–G protein interaction is dependent on the relative and absolute density of these proteins in the plasma membrane (Kenakin, 1997). To compare the CBR co-expression systems with systems where the Gα subunit is fused to the CBR, we used the ubiquitously expressed $G\alpha_{i2}$ subunit (Offermanns, 2003). Figure 3.4 shows correlations of potency and efficacy (calculated as stimulation relative to the endogenous agonist 2-AG) of ligands at CB_1R between the co-expression and fusion system in the absence or presence of RGS proteins. As is evident from the slope of the linear regression line and the 95% confidence intervals, linear correlations between the co-expression and the fusion systems concerning potency and efficacy were obtained for CB_1R (Figure 3.4).

In contrast, the goodness of fit and slope values obtained from the comparisons of the CB_2R systems (Figure 3.5) indicate that the fusion of the CB_2R to $G\alpha_{i2}$ substantially altered the pharmacological parameters of the ligands. The efficacies (Figures 3.5A, C and E) and potencies (Figures. 3.5B, D and F) of ligands studied in the absence and presence of RGS proteins clearly differed from each other, depending on whether the receptor was fused or co-expressed with $G\alpha_{i2}$. The most impressive differences were obtained for potency correlations when RGS proteins were co-expressed. A r^2 value of 0.687 and slope of 0.551 ± 0.215 for RGS4 (Figure 3.5D) and a r^2 value 0.815 and slope of 0.594 ± 0.163 for RGS19 (Figure 3.5F) indicate a poor correlation between the co-expression and fusion protein test system.

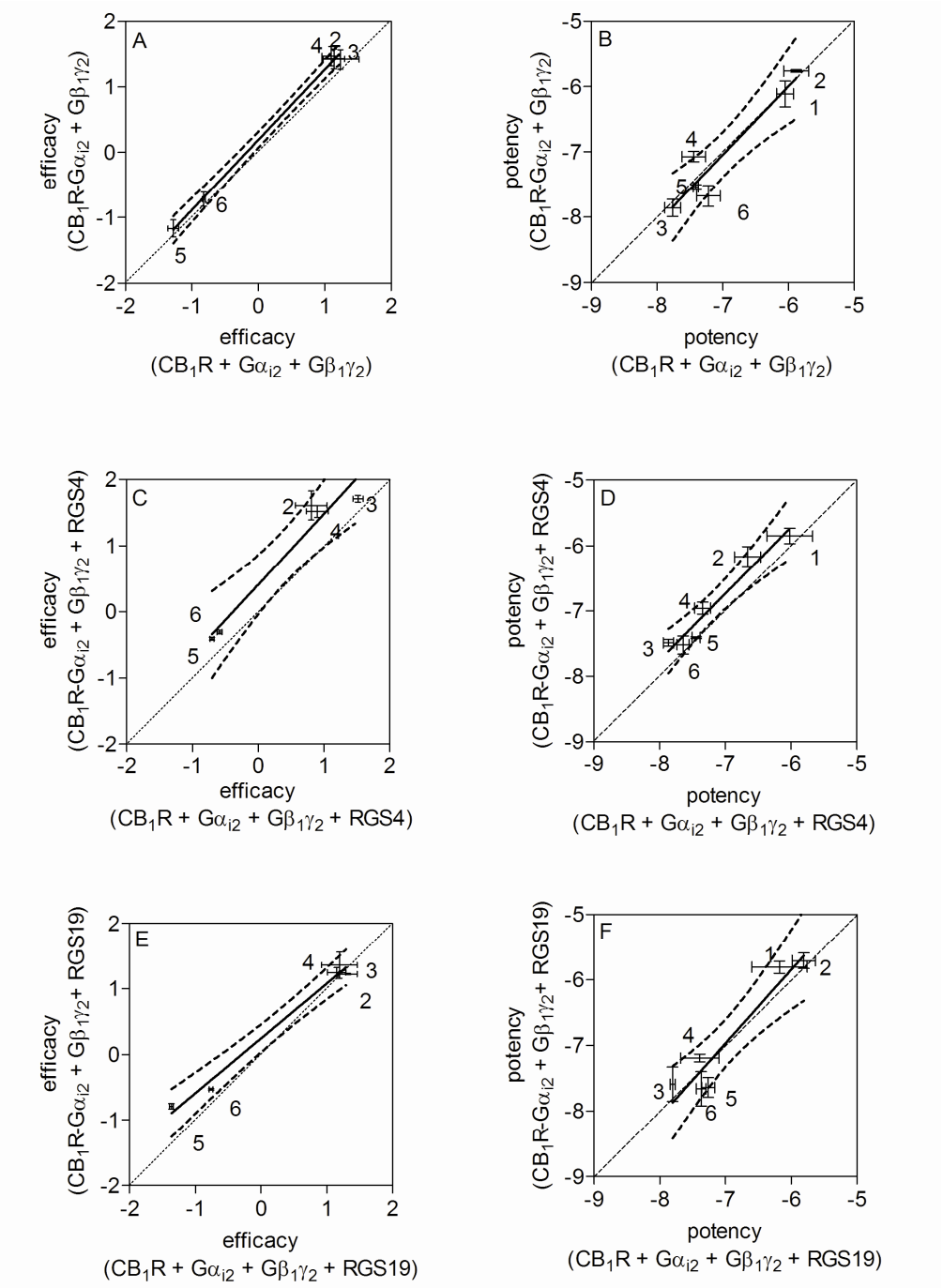

Figure 3.4: Correlation of potency and efficacy of ligands at the CB_1R between the co-expression and fusion system

Data of Table 3.2 were analyzed by linear regression. **A**, **C** and **E**, efficacy of ligands at membranes co-expressing CB_1R, $G\alpha_{i2}$ and $G\beta_1\gamma_2$ in the presence or absence of RGS proteins were correlated with values obtained from membranes expressing CB_1R-$G\alpha_{i2}$ and $G\beta_1\gamma_2$ and the respective RGS proteins. **A**, $r^2 = 0.997$; slope = 1.075 ± 0.035. **C**, $r^2 = 0.946$; slope = 1.073 ± 0.148. **E**, $r^2 = 0.986$; slope = 0.835 ± 0.057. **B**, **D** and **F**, potency of ligands at membranes co-expressing CB_1R, $G\alpha_{i2}$ and $G\beta_1\gamma_2$ in the presence or absence of RGS proteins were correlated with values evaluated at membranes expressing CB_1R-$G\alpha_{i2}$ and $G\beta_1\gamma_2$ and the respective RGS proteins. **B**, $r^2 = 0.902$; slope 1.050 ± 0.173. **D**, $r^2 = 0.940$; slope = 1.019 ± 0.129. **F**, $r^2 = 0.905$; slope = 1.122 ± 0.182. Depicted are the linear regression lines and the 95% confidence intervals (dotted lines). The diagonal line has a slope of 1 and represents a theoretical line for identical values in both systems. **1** 2-AG, **2** anandamide, **3** CP 55,940, **4** WIN 55,212-2, **5** AM 251, **6** AM 281

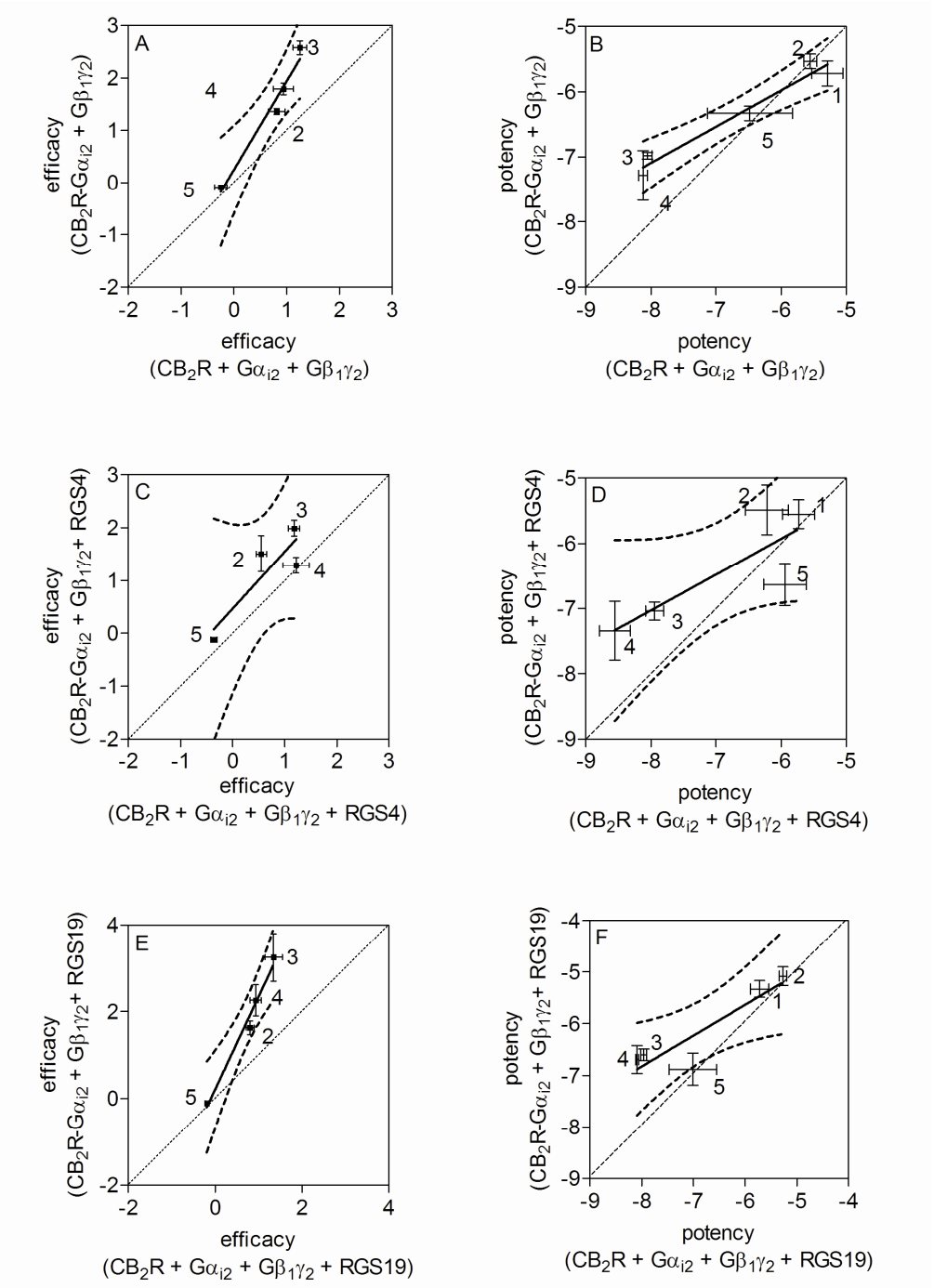

Figure 3.5: Correlation of potency and efficacy of ligands at the CB_2R between the co-expression and fusion system

Data of Table 3.3 were analyzed by linear regression. **A**, **C** and **E**, efficacy of ligands at membranes co-expressing CB_2R, $G\alpha_{i2}$ and $G\beta_1\gamma_2$ in the presence or absence of RGS proteins were correlated with values obtained from membranes expressing CB_2R-$G\alpha_{i2}$ and $G\beta_1\gamma_2$ and the respective RGS proteins. **A**, $r^2 = 0.967$; slope = 1.691 ± 0.220. **C**, $r^2 = 0.778$; slope = 1.083 ± 0.409. **E**, $r^2 = 0.978$; slope = 2.130 ± 0.227. **B**, **D** and **F**, potency of ligands at membranes co-expressing CB_2R, $G\alpha_{i2}$ and $G\beta_1\gamma_2$ in the presence or absence of RGS proteins were correlated with values evaluated at membranes expressing CB_2R-$G\alpha_{i2}$ and $G\beta_1\gamma_2$ and the respective RGS proteins. **B**, $r^2 = 0.957$; slope 0.5567 ± 0.068. **D**, $r^2 = 0.687$; slope = 0.551 ± 0.215. **F**, $r^2 = 0.815$; slope = 0.594 ± 0.163. Depicted are the linear regression lines and the 95% confidence intervals (dotted lines). The diagonal line has a slope of 1 and represents a theoretical line for identical values in both systems. **1** 2-AG, **2** anandamide, **3** CP 55,940, **4** WIN 55,212-2, **5** AM 630

3.4.5 Influence of RGS4 on ligands efficacy and potency in the CB_2R co-expression and CB_2R fusion system

The poor correlations between co-expression and fusion system of CB_2R in the presence of RGS4 directed us to specifically examine the influence of RGS4 on ligand potency and efficacy in the respective systems. As depicted in Figure 3.6, r^2 values of 0.909 (Figure 3.6A), 0.865 (Figure 3.6B), 0.920 (Figure 3.6C) and 0.966 (Figure 3.6D) indicated a good correlation of the examined parameters obtained in the test systems in the absence and presence of RGS4. However, the 95% confidence intervals and standard error values were widely scattered in all cases. This pointed to a substantial deviation of the individual ligands from the ideal linear regression.

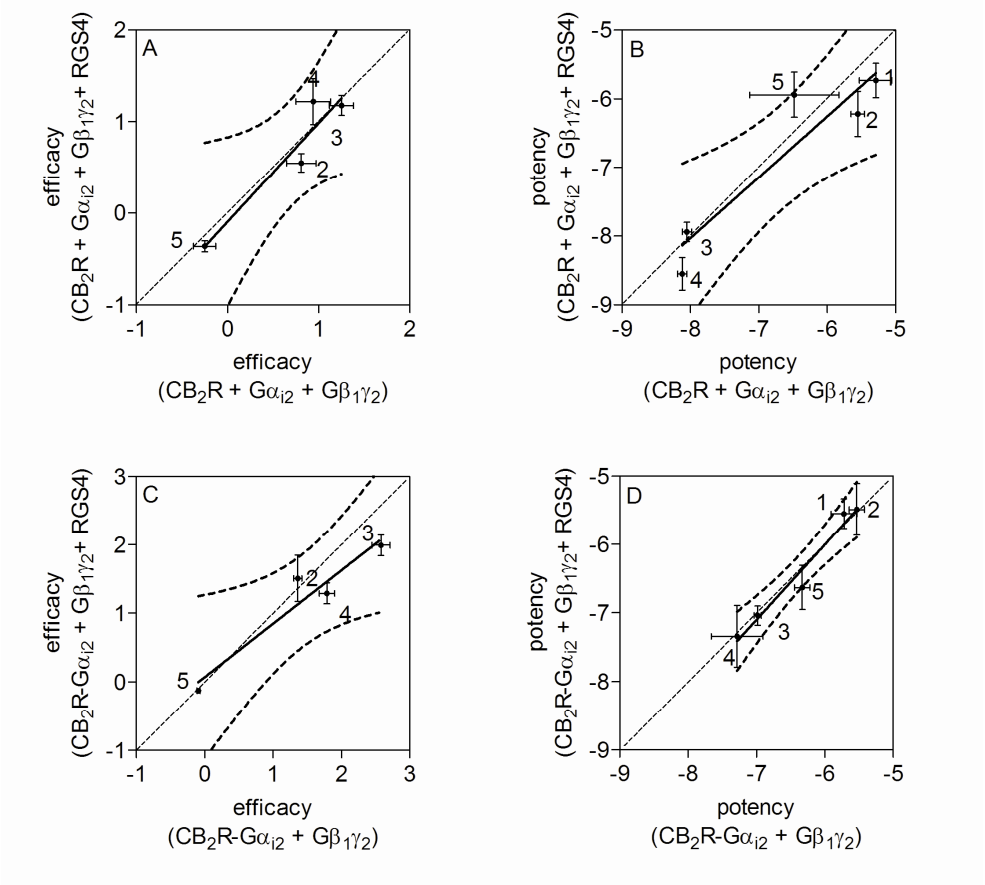

Figure 3.6: Influence of RGS4 protein on potency and efficacy of ligands in the co-expression and fusion system

Correlation of efficacy (**A** and **C**) and potency (**B** and **D**) of ligands evaluated in the absence or presence of RGS4 in membranes expression $CB_2R + G\alpha_{i2}$ (**A** and **B**) or $CB_2R\text{-}G\alpha_{i2}$ (**C** and **D**). **A**, r^2 = 0.909; slope = 1.081 ± 0.241. **B**, r^2 = 0.865; slope = 0.888 ± 0.202. **C**, r^2 = 0.920; slope = 0.776 ± 0.162. **D**, r^2 = 0.966; slope = 1.096 ± 0.118. Depicted are the linear regression lines and the 95% confidence intervals (dotted lines). The diagonal line has a slope of 1 and represents a theoretical curve for identical values in both systems. **1** 2-AG, **2** anandamide, **3** CP 55,940, **4** WIN 55,212-2, **5** AM 630

3.5 Discussion

In this study the steady-state GTPase assay was used to examine the effects of CBR-Gα fusion proteins in comparison to the co-expression system as well as the impact of different RGS proteins on the pharmacological properties of standard CBR ligands. The GTPase assay and the Sf9 cell membrane expression systems were successfully applied to characterize ligands of other $G\alpha_i$ coupled GPCRs such as the formyl peptide receptor FPR-26 (Wenzel-Seifert et al., 1998), chemokine receptor $CXCR_4$ (Kleemann et al., 2008), histamine H_3 receptor (Schnell et al., 2010) and histamine H_4 receptor (Schneider and Seifert, 2009). As insect cells do not endogenously express CBRs (McPartland et al., 2001), infection of Sf9 cells with baculoviruses encoding for CBRs offers the advantages to conduct functional studies of these GPCRs without interference of endogenous CBRs. Furthermore, mammalian-type $G\alpha_i$ proteins are not expressed in Sf9 cells (Quehenberger et al., 1992; Wenzel-Seifert et al., 1998), so that coupling studies of GPCRs to this particular Gα protein can easily be conducted by simultaneous co-transfection with the desired $G\alpha_i$ subunit.

Previous studies showed that RGS proteins can enhance GPCR-stimulated steady-state GTP hydrolysis, facilitating the analysis of partial agonists and inverse agonist (Ward and Milligan, 2004). The fact that in our systems only RGS4 but not RGS19 exhibits an influence on the pharmacological properties of CBR ligands is surprising, since for many GPCRs a similar influence of these GAPs has been described. Studies with GPCRs for instance the histamine H_1 receptor (Houston et al., 2002), histamine H_4 receptor (Schneider and Seifert, 2009) and chemokine receptor $CXCR_4$ (Kleemann et al., 2008), also expressed in Sf9 insect cells, showed that RGS19 strongly enhances agonist-stimulated GTP hydrolysis. The data of our present study indicate that the capacity of RGS proteins to regulate GTP hydrolysis depends on the specific GPCR and that the GPCR may govern RGS-G protein interactions (Abramow-Newerly et al., 2006). It can be argued that the lack of RGS19 effects is attributable to the fact that RGS19 is a member of the RZ family of RGS proteins and does not belong to the R4 family as RGS4 (Ross and Wilkie, 2000). Although the polypeptide size of RGS19 is quite similar to RGS4, its N-terminal region contains a cysteine string region and a C-terminal PDZ binding motif. The scaffold protein GIPC (GAIP-interacting protein) is required for the binding of RGS19 to the dopamine D_2 receptor (Jeanneteau et al., 2004b). As a result, the deficiency of

this specific PDZ domain may be responsible for the lack of effect of RGS19 on the CBRs.

In co-expression systems, expression levels and subcellular distribution of the signaling partners cannot be exactly controlled, and a fixed stoichiometric ratio of GPCR and Gα subunit is difficult to achieve (Gille and Seifert, 2003). Therefore, the use of GPCR-Gα fusion proteins, which ensure close proximity of the signaling partners and anchor of the Gα subunit in the plasma membrane, offers an elegant system to study receptor-G protein interaction under defined conditions (Seifert et al., 1999; Milligan, 2000). One aim of this study was to examine whether the efficacies and potencies of CBR ligands may be altered by the fusion of CBRs to Gα compared to their pharmacological behavior in the co-expression system. Three outcomes are documented in literature; i. e. decreased, enhanced and equal response of $G\alpha_i$ fusion proteins compared to the corresponding co-expression systems. The GTPase assay revealed similar concentration-response curves for the 5-HT_{1A} receptor in HEK 293 cells expressing either the receptor or its fusion protein. In this study, the 5-HT_{1A} receptor was fused to the PTX-resistant mutant $G\alpha_{i1}Cys^{351}Gly$ protein (Kellett et al., 1999). Data obtained in this system were compared to results obtained for the 5-HT_{1A} receptor interacting with endogenous $G\alpha_i$ proteins. Interestingly, fusion of the 5-HT_{1A} receptor to the rat $G\alpha_o$ subunit – also resistant against PTX because of mutation of Cys^{351} to Gly – and expression in COS 7 cells resulted in an 73 ± 2% stimulation by the agonist 8-hydroxy-2-(di-*n*-propylamino)tetralin in the $rG\alpha_oCys^{351}Gly$ co-expression system *versus* 55 ± 7% in the fusion system (Dupuis et al., 1999). The Edg_2 receptor shows an enhanced response for the fusion with $rG\alpha_{i1}Cys^{351}Gly$ compared to signals of co-expressed receptor with wild-type $rG\alpha_{i1}$ and mutant $rG\alpha_{i1}$, respectively (McAllister et al., 2000). In this study, co-expression of receptor with wild-type $rG\alpha_{i1}$ subunit showed the smallest response, indicating that the altered responses are, at least in part, due to the mutation of the Cys^{351} to Gly (McAllister et al., 2000).

CBRs couple to PTX-sensitive $G_{i/o}$ proteins (Howlett et al., 2002). Thus, a fusion of wild-type $G\alpha_{i2}$ to the CBRs was performed. The use of Sf9 cells allowed us to avoid cloning of PTX-resistant $G\alpha_{i2}$ mutants because of the absence of endogenous $G\alpha_i$ (Quehenberger et al., 1992; Wenzel-Seifert et al., 1998). After successful expression of the desired proteins in Sf9 cells (Figure 3.1 and Figure 3.2), we examined several known ligands of the CBRs in the co-expression and fusion

systems and assessed their potency and efficacy to stimulate or inhibit GTPase activity. Fusion of $G\alpha_{i2}$ to CB_1R in the absence of RGS proteins did not significantly alter the stimulatory effects of ligands. Only by adding RGS4 to the CB_1R expression systems higher GTPase activities in the fusion system were elicited compared to the co-expression system (Table 3.2). The forced proximity of the Gα subunit to the CB_2R enhanced the GTPase activation by all ligands. The stimulatory effects of ligands in all CB_2R-$G\alpha_{i2}$ systems were significantly higher than those observed in the corresponding receptor-G protein co-expression systems (Table 3.3).

The higher GTPase stimulations by agonists in the fusion systems, especially for CB_2R, also affected the efficacies of ligands. A prominent example is anandamide in the CB_2R systems that behaves like a partial agonist in the co-expression systems and switches to the status of a “superagonist” with higher efficacy compared to the most abundant endogenous agonist 2-AG in the fusion systems. This may be due to a more efficient coupling between receptor and G protein than in a system, where the signaling partners were expressed as separate entities.

The potencies of some ligands were influenced by the fusion of the CB_2R to the $G\alpha_{i2}$ subunit (Figures 3.5B, D and F) and of CB_1R in the presence of RGS4 (Figure 3.4D). The phenomenon of reduced potencies of ligands in fusion protein expression systems was observed for the α_{2A} adrenoceptor (Sautel and Milligan, 1998; McAllister et al., 2000) and the Edg_2 receptor (McAllister et al., 2000) and it is probably due to physical restrictions inhibiting protein-conformational changes (Sautel and Milligan, 1998) and/or compartmentalization of signaling elements within specific domains of the plasma membrane (Huang et al., 1997). Also, for the CB_2R an altered schema of phosphorylation can be discussed as a reason for modulated pharmacological parameters of ligands in the comparison fusion – co-expression approach. As it is known that agonist treatment of Chinese hamster ovary (CHO) cells stably expressing CB_2R increases basal phosphorylation of Ser^{352} (Bouaboula et al., 1999), it is conceivable that the ability of a G protein coupled receptor kinase (GRK) to phosphorylate the CB_2R is altered due to the tethered Gα subunit. The relevance of GRKs in Sf9 cells concerning the regulation of GPCR signaling it is still unclear (Schneider and Seifert, 2010), but it is likely that a modulated phosphorylation can in turn affect efficacy and potency of tested ligands.

The potential discrepancy of pharmacological parameters between fusion and co-expression systems raises the question whether the $(CB_2)R$-$G\alpha_{i2}$ system is a

usable tool to examine pharmacological parameters of new drugs. The tight tethering of the Gα subunits to the membrane and to the receptor itself dictated by the fusion is an inherently artificial system. But also, the co-expression system with its accidental stoichiometry of receptor-G protein may not mirror a physiological surrounding as it is still unclear to how many G proteins a single receptor has access to. Hence, it is difficult to evaluate which system is closer to reality and which of the measured potencies reflects the drug behavior under physiological condition. Therefore, a comparable *in-vivo* assay in mammalian cells ideally expressing CBR endogenously would be required to clarify this issue – a difficult undertaking as recently reported (Geiger et al., 2010). However, the use of CBR-$G\alpha_{i2}$ fusion proteins offer a highly sensitive model system and allow the screening for new CBR ligands with characterization of pharmacological parameters at a very proximal point of the signaling cascade. With these conditions excellent clues can be provided how the compound behaves *in-vivo.*

Moreover, it cannot be excluded that RGS proteins may alter ligand potencies as well, as it has been described for μ-opioid signaling (Cavalli et al., 2000; Clark et al., 2003) and the α_{2A} adrenoceptor (Cavalli et al., 2000) where under the influence of RGS proteins the potency of agonists were changed profoundly. Interaction between GPCR, RGS and G protein supports the assumption that GPCR function may, indeed, be modulated by RGS proteins (Benians et al., 2005; Abramow-Newerly et al., 2006). In fact, influences of RGS4 on ligand potency and efficacy were observed in the co-expression and fusion system (Figure 3.6) with alterations of slope values and widely scattered standard errors and confidence intervals, reflecting a high variability in ligand properties. Combination of RGS4 protein with the $G\alpha_{i2}$ fusion approach augmented the modifications in pharmacological properties of ligands, supporting the assumption that the conformational flexibility of the receptors is restricted by the spatial proximity of the Gα subunit and the impact of RGS proteins. Our data demonstrate that CB_2R is affected by these restrictions to a greater extent than CB_1R.

The fact that the potency and efficacies of the ligands are not altered similarly by the fusion and RGS proteins is indicative for ligand-specific receptor conformations. Similarly, at the β_2 adrenoceptor (Gether et al., 1995; Seifert et al., 2001) and the histamine H_4 receptor (Schneider et al., 2009), ligands can stabilize unique receptor confirmations (Kenakin, 1995) differing in their ability to interact with

and activate G proteins. Likely, under the chosen assay conditions some ligands stabilize specific GDP/GTP exchange-promoting CBR conformations that are influenced by the forced contact to the $G\alpha_{i2}$ subunit and the addition of RGS protein. Continuing this concept, functional selectivity of CBRs can in turn activate specific signaling cascades as it was shown for the CB_2R (Shoemaker et al., 2005) which offers a great opportunity to develop ligands that selectively manipulate physiological functions (Bosier et at., 2010).

In conclusion, we have shown that RGS4 but not RGS19 behaves as a GAP at CBRs in the $G\alpha_{i2}$ co-expression and fusion system. We demonstrated that the fusion of CBRs to $G\alpha_{i2}$ increases the sensitivity of the GTPase assay compared to the co-expression system, especially for the CB_2R. The fusion system with its extremely sensitive readout is therewith excellently suited to study for example structure-activity-relation of new CBR ligands. The examined alterations of pharmacological properties of the CBRs ligands in the different systems seem to be the result of complex effects of the fusion approach and RGS protein on ligand-specific receptor conformations. In further studies, the impact of other G_i/G_o protein subtypes, different $G\beta_x\gamma_y$ complexes as well as other RGS proteins on pharmacological properties will have to be studied. Ultimately, these studies may result in the development of ligands that modulate only a single or few of the multiple functions regulated by CBRs. As a result, novel therapeutic uses of CBR ligands with fewer side effects may be identified.

3.6 References

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4 Cannabinoid receptor activity of synthetic 2,3-disubstituted indole derivatives and several polyacetylenes, polyenes and alkamides isolated from *Echinacea* species

4.1 Abstract

Cannabis sativa is among the earliest plants used for medicinal purposes. It reached the western medicine in the beginning of the 19th century but its widespread use was soon replaced by other analgesic and narcotic medications. The identification of the main psychoactive compound of *Cannabis sativa*, the cannabinoid receptors (CBRs) and the endocannabinoids system (ECS) renewed the scientific interest. Since the 1990s investigations on the pharmacological potential and on modulations of the physiological functions of the ECS are ongoing and effects on nausea and emesis caused by cancer chemotherapy, appetite regulation, painful conditions and symptoms of multiple sclerosis are demonstrated. For targeting the CBRs and to modulate the physiological action of the ECS, new CBRs ligands are needed. In this study, a screening of natural compounds isolated from *Echinacea* species and synthetic 2,3-disubstituted indole derivatives was conducted to examine their CBR activity. To evaluate the pharmacological properties the steady-state GTPase assay was employed. As protein expression system Sf9 cell membranes were used and CBR-Gα_{i2} fusion proteins were co-expressed with G$\beta_1\gamma_2$ and RGS4 (regulator of G protein signaling 4). The screening of the natural compounds showed that the alkamides dodeca-2*E*,4*Z*-diene-8,10-diynoic acid isobutylamide, dodeca-2*E*,4*Z*-diene-8,10-diynoic acid 2-methylbutylamide and dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamides acted as partial agonists at the CB_2R. Polyacetylenes and polyenes did not exert functionality at the CBRs. Concerning the synthesized indole compounds, derivatives with an ester function at position 3 showed the strongest GTPase stimulation and moderate potency at the CB_2R. Thereby, 3-(2-butylindole-3-yl)prop-2-en acid ethyl ester provides a promising base for further structure-activity-relation studies and the development of selective CB_2R ligands.

4.2 Introduction

The use of *Cannabis sativa* for medicinal applications has been reported long before the Christian era in China, India, Tibet and Egypt (Touw, 1981; Zuardi, 2006; Russo, 2007). Written documents, for example the Shén nóng běn cǎo jīng, the world's oldest pharmacopoeia that was compiled in the first or second century A.D. but based on oral traditions from the time of Shén Nóng, already described "great hemp" and its psychoactive properties (Aldrich, 1997). Therapeutic indications for the use of cannabis included, among others, rheumatic pain, intestinal constipation, disorders of the female reproduction system and malaria (Touw, 1981). Also, the application to anesthetize patients during surgical operations is reported (Li and Lin, 1974; Aldrich, 1997). In Western medicine, the use of cannabis was introduced and studied in the beginning of the 19th century. In publications of various trials with human, administration of *Cannabis* extract against rheumatism, convulsions and mainly for muscular spasms of tetanus and rabies was described (O´Shaughnessy, 1852). These studies resulted in a widespread use of cannabis throughout the Western medicine. With the appearance of various drugs (such as chloral hydrate, paraldehyde, barbiturates, opioids and acetylsalicylic acid) with known efficacy for the treatment of the main indications of cannabis (Mikuriya, 1969; Zuardi, 2006), the release of the Marihuana tax act law 1937 and probably because of difficulties to obtain reproducable effects with *Cannabis* tinctures or extracts, the medicinal application of cannabis formulations faded into the background. Only the cannabis consumption for hedonistic or recreational purpose increased rapidly since the 1960s.

With the identification of the main psychoactive compound and the publication of the partial synthesis of Δ^9-THC (Gaoni and Mechoulam, 1964) the scientific interest increased and led to the description and cloning of two specific receptors in the early 1990´s (Matsuda et al., 1990; Munro et al., 1993) and the discovery of the ECS (De Petrocellis et al., 2004). The growing scientific interest led consequently to investigations on the therapeutical impact of the ECS and possibilities to influence its physiological and pharmacological role. Beneficial clinical results of Δ^9-THC on nausea/vomiting, appetite, pain as well as on symptoms of multiple sclerosis are studied very well (Carlini, 2004). Furthermore, regulation of the ECS in cardiovascular, gastrointestinal, and respiratory functions as well as modulation of inflammation, cell metabolism and reproduction are main areas of research.

Moreover, involvements of the ECS on disorders of the CNS, eyes and musculoskeletal apparatus are topics of intense investigations (Pacher et al., 2006).

To target the CBRs for therapeutic applications, new CBR ligands are needed and the search of selective compounds with activity at the CB_1R or CB_2R are in the focus of interest. In this study, a screening of natural and synthetic compounds for their CBR activity was conducted using the steady-state GTPase assay. As both CBRs couple to pertussis-toxin sensitive $G\alpha_{i/o}$ proteins (Howlett et al., 2002), receptors were fused C-terminally to the N-terminus of the $G\alpha_{i2}$ subunit for this screening procedure. Sf9 cell membranes were employed to express CBR-$G\alpha_{i2}$ fusion proteins together with $G\beta_1\gamma_2$ and RGS4 proteins. Previous studies revealed that the use of fusion proteins in combination with RGS4 resulted in a highly sensitive assay that enabled the detection of partial agonists and antagonists (Chapter 3).

Indole derivates disubstituted at carbons 2 and 3 were screened on their CBR activity. In order to mimic the structure of the endocannabinoids 2-arachidonoyl glycerol (2-AG) (Sugiura et al., 1995) and anandamide (Devane et al., 1992), the unsaturated part of the arachidonic acid was replaced by an indole scaffold. Known CBRs ligands with an indole partial structure are comprised in the class of aminoalkylindoles, for example the most highly studied, commercially available compound of the series, CB_1R/CB_2R full agonist WIN 55,212-2 (structure see Figure 4.3; Howlett et al., 2002).

Previous studies have shown that preparations of *Echinacea* species modulate immune responses (Zhai et al., 2007). *Echinacea* preparations are among the best selling over-the-counter herbal medicines, mainly used for the treatment and prevention of the common cold and infections of the upper respiratory tract. It was reported that alkamides, the main compounds of the roots of *Echinacea purpurea*, bind to CB_2Rs with high affinity (Raduner et al., 2006). Based on these results a molecular mode via the CBRs signaling system is discussed for the immunomodulatory effects of *Echinacea* extracts. Therefore, we tested various alkamides isolated from *Echinacea purpurea*, as well as polyacetylenes and polyenes isolated from *Echinacea pallida* concerning their CBR activity.

4.3 Materials and methods

4.3.1 Materials

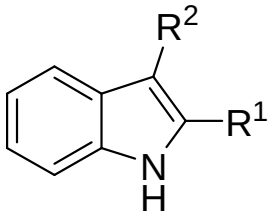
CBR fusion proteins were generated as described in Chapter 3. Baculovirus encoding $G\alpha_{i2}$ was generously provided by Dr. A. G. Gilman (Department of Pharmacology, University of Texas, Southwestern Medical Center, Dallas, TX, USA). Baculovirus encoding $G\beta_1\gamma_2$ was a kind gift from Dr. P. Gierschik (Department of Pharmacology, University of Ulm, Germany). Baculovirus encoding for RGS4 was a kind gift from Dr. E. Ross (University of Texas, Southwestern Medical Center, Dallas, TX, USA). Phenylmethylsulfonylfluoride and leupeptine hemisulfate were purchased from Calbiochem (La Jolla, CA, USA). Benzamidine was from Sigma (99%, St. Louis, MO, USA). Adenylyl imidodiphosphate was obtained from Roche (Mannheim, Germany). [γ-^{32}P]GTP was synthesized through enzymatic phosphorylation of GDP and [^{32}P]orthophosphoric acid (8,000 Ci/mmol, PerkinElmer Life Sciences, Boston, MA, USA) as described previously (Walseth and Johnson, 1979).

All nucleotides, creatine kinase, creatine phosphate and salts (highest purity available) were purchased either from Roche (Mannheim, Germany) or Sigma (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was from Merck (Darmstadt, Germany). Tris base was purchased from USB (Cleveland, OH, USA).

Synthesized indole derivatives (for structures and names see Table 4.1) were kindly provided by Mehrnaz Pirasteh (Department of Pharmaceutical and Medicinal Chemistry, Group of Prof. Bernhard Wünsch, Westfälische Wilhelms University Münster, Germany). Polyacetylenes, polyenes (Table 4.2) and alkamides (Table 4.3) were a kind gift from Elisabeth Feizlmayr (Institute of Pharmaceutical Sciences, Group of Prof. Rudolf Bauer, Karl Franzens-University Graz, Austria).

The 10 mM stock solutions of these compounds were prepared with 100% DMSO and dilutions of all ligands were prepared with 30% DMSO. Final assay concentration of DMSO was always lower than 3%.

Table 4.1: Structures of investigated indole derivatives



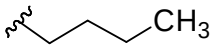
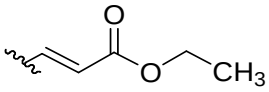
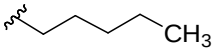
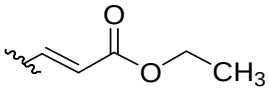
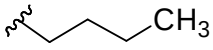
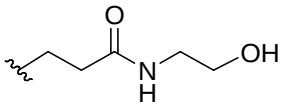
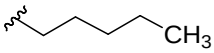
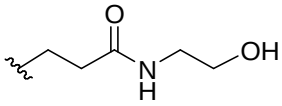
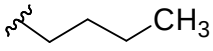
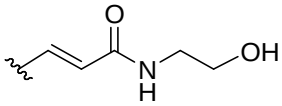
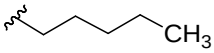
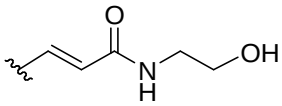
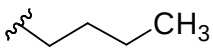
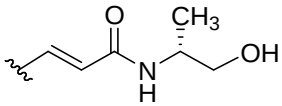
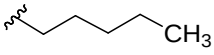
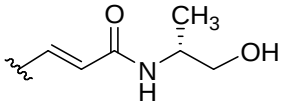
compound	R^1	R^2	name
1			3-(2-butylindole-3-yl) prop-2-en acid ethyl ester
2			3-(2-pentylindole-3-yl) prop-2-en acid ethyl ester
3			3-(2-butylindole-3-yl)-*N*-(2-hydroxy ethyl) propanamide
4			3-(2-pentylindole-3-yl)-*N*-(2-hydroxy ethyl) propanamide
5			3-(2-butylindole-3-yl)-*N*-(2-hydroxy ethyl) prop-2-enamide
6			3-(2-pentylindole-3-yl)-*N*-(2-hydroxy ethyl) prop-2-enamide
7			3-(2-butylindole-3-yl)-*N*-(1*R*-methyl 2-hydroxy ethyl) prop-2-enamide
8			3-(2-pentylindole-3-yl)-*N*-(1*R*-methyl 2-hydroxy ethyl) prop-2-enamide

Table 4.2: Structures of tested ketoalkenes isolated from *Echinacea pallida*

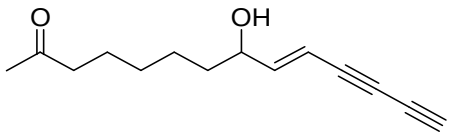
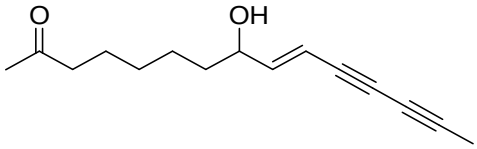
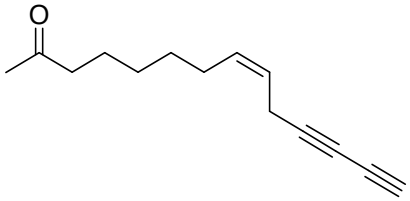
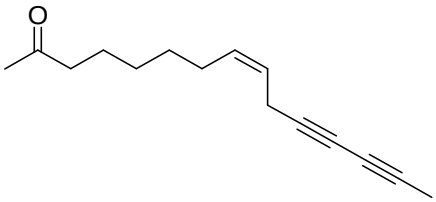
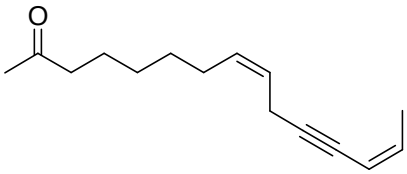
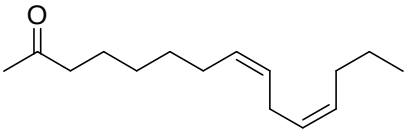
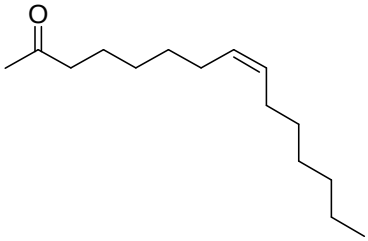
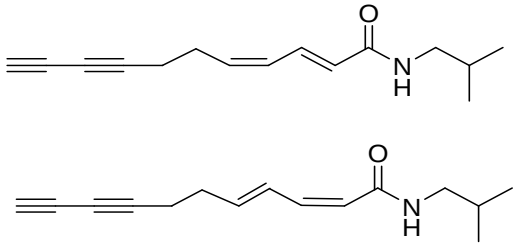
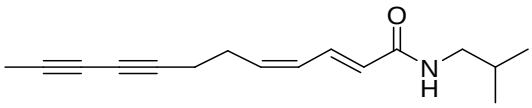
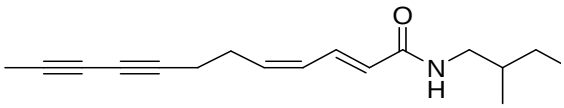
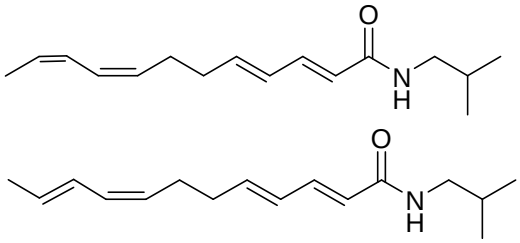
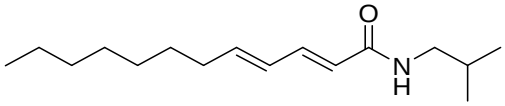
compound	structure	name
9		8-hydroxy-tetradeca-9*E*-ene-11,13-diyn-2-one
10		8-hydroxy-pentadeca-9*E*-ene-11,13-diyn-2-one
11		tetradeca-8*Z*-ene-11,13-diyn-2-one
12		pentadeca-8*Z*-ene-11,13-diyn-2-one
13		pentadeca-8*Z*,13*Z*-diene-11-yn-2-one
14		pentadeca-8*Z*,11*Z*-diene-2-one
15		pentadeca-8*Z*-ene-2-one

Table 4.3: Structures of tested alkamides isolated from *Echinacea purpurea*

compound	structure	name
16		undeca-2*E*/*Z*,4*Z*/*E*-diene-8,10-diynoic acid isobutylamides
17		dodeca-2*E*,4*Z*-diene-8,10-diynoic acid isobutylamide
18		dodeca-2*E*,4*Z*-diene-8,10-diynoic acid 2-methylbutylamide
19		dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamides
20		dodeca-2*E*,4*E*-dienoic acid isobutylamide

4.3.2 Methods

4.3.2.1 *Generation of recombinant baculoviruses and membrane preparation of transfected Sf9 cells*

Sf9 cells, derived from *Spodoptera frugiperda* pupal ovarian tissue, were used for baculovirus expression. Sf9 cells were cultured in Erlenmeyer flasks at 28℃ under rotation at 125 rpm in SF 900 II medium (Invitrogen, Carlsbad, CA, USA), supplemented with fetal calf serum (Pan-Biotech, Aidenbach, Germany) to 5% (v/v) and gentamicin sulfate (BioWhittaker, Walkersville, MD, USA) to 0.1 mg/ml. Recombinant baculoviruses encoding FLAG- and hexahistidine-tagged CB_1R-$G\alpha_{i2}$ and CB_2R-$G\alpha_{i2}$, $G\beta_1\gamma_2$ and RGS4 protein were generated in Sf9 insect cells using the BaculoGOLD transfection kit (BD PharMingen, San Diego, CA) according to the manufacturer´s instructions. After initial transfection, high-titer virus stocks were generated by two sequential virus amplifications.

For transfection, cells were seeded in Erlenmeyer flasks (cell density 3.0 x 10^6 cells/ml) and infected with a 1:100 dilution of high-titer baculovirus stocks. Cells were cultured for 48 h and Sf9 membranes were prepared as described previously (Seifert et al., 1998). Briefly, cells were washed once by centrifugating for 10 min at 170 x g, discarding the supernatant and resuspending the cell pellet in PBS buffer. After repeating the centrifugation step, the supernatant fluid was discarded and the pellet was suspended in lysis buffer (containing 10 mM Tris/HCl pH 7.4, 1 mM EDTA, 0.2 mM phenylmethylsulfonylfluoride, 10 µg/ml benzamidine and 10 µg/ml leupeptin) and homogenized in a Dounce homogenizer with 25 strokes. After centrifugation at 40 x g for 5 min the pellet contained the nuclei and unbroken cells and the supernatant contained the membranes. Therefore, the supernatant fluid was carefully transferred to a plastic tube and spun down by 38,500 x g for 20 min. The pellet containing the membranes was resuspended in lysis buffer and again centrifuged as described above. The resultant membrane pellet was resuspended in buffer containing 75 mM Tris/HCl, pH 7.4, 1 mM EDTA, and 12.5 mM $MgCl_2$, homogenized by a syringe with 20 strokes and stored in aliquots at -80℃. Protein concentrations were determined using the DC protein assay kit (Bio-Rad, Hercules, CA) according to the instructions of the manufacturer. Membranes used in the assays were analyzed by SDS page and immunoblotting with specific antibodies against FLAG-tagged CBRs, G protein subunits and RGS protein to ensure correct transfection (see Chapter 3).

4.3.2.2 *Steady-state GTPase assay*

The GTPase assay was performed as described previously (Preuss et al., 2007). Briefly, membranes were thawed, sedimented by centrifugation at 18,000 x g for 10 min at 4℃, and carefully resuspended in 10 mM Tris/HCl, pH 7.4. Assay tubes contained membranes (5 µg of protein/tube), 1.0 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM ATP, 100 nM GTP, 0.1 mM adenylyl imidodiphosphate, 5 mM creatine phosphate, 40 µg of creatine kinase, 0.2% (w/v) BSA (to prevent binding of protein or ligand to the polystyrol tubes) in 50 mM Tris/HCl, pH 7.4, and test compounds at various concentrations. Reaction mixtures (80 µl) were incubated for 2 min at 25℃ prior to addition of 20 µl of [γ-^{32}P]GTP (0.1 µCi/tube). All stock and work dilutions of [γ-^{32}P]GTP were prepared in 20 mM Tris/HCl, pH 7.4. Reactions were conducted for 10 min at 25℃ and terminated by the addition of 900 µl of slurry consisting of 5% (w/v) activated charcoal and 50 mM NaH_2PO_4, pH 2.0. Charcoal absorbs nucleotides but not $^{32}P_i$. Charcoal-quenched reaction mixtures were centrifuged for 7 min at room temperature at 15,000 x g. 600 µl supernatant fluid of reaction mixtures was removed and $^{32}P_i$ was determined by Čerenkov radiation in 3 ml water. Enzyme activities were corrected for spontaneous degradation of [γ-^{32}P]GTP. Spontaneous [γ-^{32}P]GTP degradation was determined in tubes containing all of the above described components plus a high concentration of unlabeled GTP (1 mM) that, by competition with [γ-^{32}P]GTP, prevents [γ-^{32}P]GTP hydrolysis by enzymatic activities present in Sf9 membranes. Spontaneous [γ-^{32}P]GTP degradation amounted to <1% of the total amount of radioactivity added. The experimental conditions chosen ensured that not more than 20% of the total amount of [γ-^{32}P]GTP added was converted to $^{32}P_i$. Neutral antagonism was determined in the presence of 30 nM CP 55,940.

4.3.2.3 *Calculations and statistics*

Data are expressed as means ± SD and represent a minimum of 2 independent experiments, each performed in triplicates. Compounds that modulated GTPase activity with more than +15% or -15% above basal activity were examined in concentration-response experiments. Statistical evaluations and curve fittings were calculated using the GraphPad Prism 4 software (La Jolla, CA) and the Microsoft Excel 2007 software.

4.4 Results

4.4.1 GTPase activity of synthesized indole derivatives

The potencies and efficacies of indole derivatives (Table 4.1) were examined in the GTPase assay with Sf9 membranes expressing CB_1R-$G\alpha_{i2}$ or CB_2R-$G\alpha_{i2}$, always together with $G\beta_1\gamma_2$ and RGS4. As shown in Figure 4.1, compounds **1**, **2** and **4** altered basal GTPase activity of the CB_2R whereas the basal GTPase activity of the CB_1R is not markedly enhanced or reduced. All compounds without activity in the normal mode were additionally tested in the antagonist mode in the presence of CP 55,940. Again, no effect was detected (data not shown).

Compound **1** showed the highest GTPase stimulation with 126 ± 16% stimulation above basal GTPase activity at the CB_2R. Evaluation of concentration-response curves revealed a $logEC_{50}$ value in a low micromolar range (-5.28 ± 0.15) for this substance showing the strongest pharmacological properties of the tested indole derivatives (Table 4.4). Also compounds **2** and **4** showed effects at the CB_2R with modulation of GTPase activity of 87 ± 18% and 42 ± 6%, respectively. For **2** a $logEC_{50}$ value of -4.79 ± 0.10 and for **4** a $logEC_{50}$ value of -4.67 ± 0.25 was evaluated (Table 4.4).

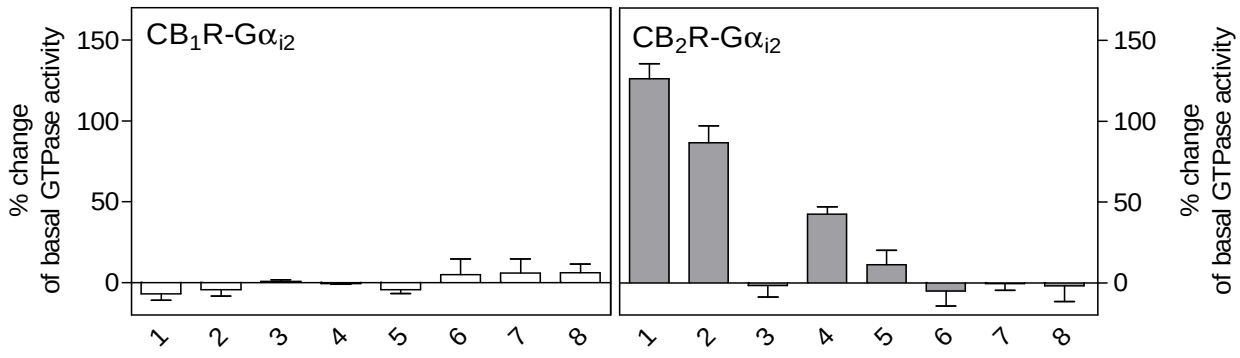


Figure 4.1: Efficacy of indole derivatives analyzed in the GTPase assay with Sf9 membranes
GTPase assays were conducted as described in *Materials and Methods* with Sf9 membranes expressing CB_1R-$G\alpha_{i2}$ or CB_2R-$G\alpha_{i2}$ together with $G\beta_1\gamma_2$ and RGS4. Reaction mixtures contained 0.1 µCi [γ-^{32}P]GTP and 100 nM unlabeled GTP in the presence of solvent (basal) and test compounds (30 µM). Data are given as mean values ± SD and represent at least 2 independent experiments performed in duplicates or triplicates with different membrane preparations.

4.4.2 GTPase activity of compounds isolated from *Echinacea* root extracts

To examine the CBR activity of natural compounds, we tested several polyacetylenes and polyenes isolated from *Echinacea pallida* (Nutt.) Nutt. (Table 4.2) as well as several alkamides isolated from *Echinacea purpurea L.* (Table 4.3) in the GTPase assay. The percental change of basal GTPase activity assessed with 30 µM test substance is depicted in Figure 4.2. While the polyacetylenes and polyenes did not alter the basal activity, compounds **17**, **18** and **19** acted as agonists at the CB_2R. The strongest GTPase stimulations were evaluated for **18** with 136 ± 14% stimulation and **17** with a stimulation of 81 ± 5%. The diastereomers **19**, the main alkamides in *Echinacea purpurea*, altered GTPase activity positively with 64 ± 10%. Also, for these ligands concentration-response curves were mapped and pharmacological parameters calculated (Table 4.4). All compounds without activity in the normal mode were additionally tested in the antagonist mode in the presence of CP 55,940. Again, no effect was detected (data not shown).

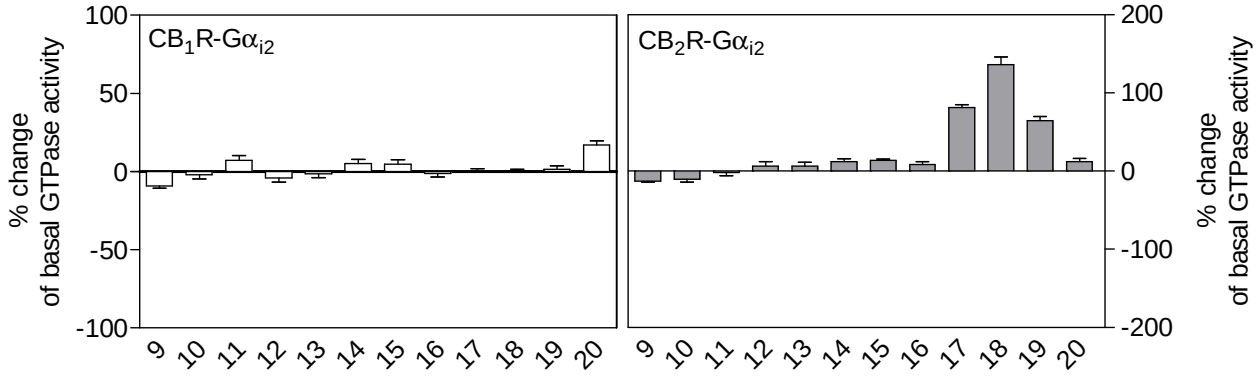


Figure 4.2: Efficacy of polyacetylenes, polyene and alkamides analyzed in the GTPase assay with Sf9 membranes

GTPase assays were conducted as described in *Materials and Methods* with Sf9 membranes expressing CB_1R-$G\alpha_{i2}$ or CB_2R-$G\alpha_{i2}$ together with $G\beta_1\gamma_2$ and RGS4. Reaction mixtures contained 0.1 µCi [γ-^{32}P]GTP and 100 nM unlabeled GTP in the presence of solvent (basal) and test compounds (30 µM). Data are given as mean values ± SD and represent at least 2 independent experiments performed in duplicates or triplicates with different membrane preparations.

Table 4.4: GTPase activities of compounds in Sf9 cell membranes expressing CB_2R-$G\alpha_{i2}$, $G\beta_1\gamma_2$ and RGS4

ligand	GTPase stimulation %	E_{max}	logEC_{50}
2-AG	197 ± 48	1.00	-5.55 ± 0.22
1	126 ± 16	0.64 ± 0.08	-5.28 ± 0.15
2	87 ± 18	0.44 ± 0.04	-4.79 ± 0.10
4	42 ± 6	0.21 ± 0.03	-4.67 ± 0.25
17	81 ± 5	0.41 ± 0.03	-4.86 ± 0.03
18	136 ± 14	0.69 ± 0.07	-5.51 ± 0.10
19	64 ± 10	0.32 ± 0.05	-4.86 ± 0.04

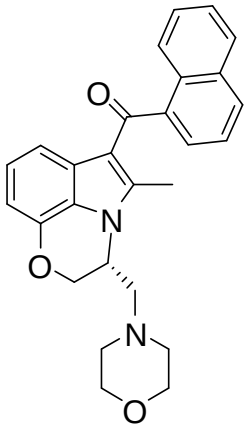
Steady-state GTPase experiments were performed as described in *Materials and Methods.* Reaction mixtures contained 0.1 µCi [γ-^{32}P]GTP and 100 nM unlabeled GTP in the presence of solvent (basal) and CBR ligands at various concentrations (10 nM – 30 µM). The relative agonist-stimulation of GTP hydrolysis was calculated in percent above basal. Data shown are the mean values ± SD and represent at least 3 independent experiments performed in triplicates with different membrane preparations. E_{max} values represent the stimulation of ligand relative to the endogenous agonist 2-AG (defined as 1.00 response). Data were analyzed by nonlinear regression and fit to sigmoidal concentration-response curves.

4.5 Discussion

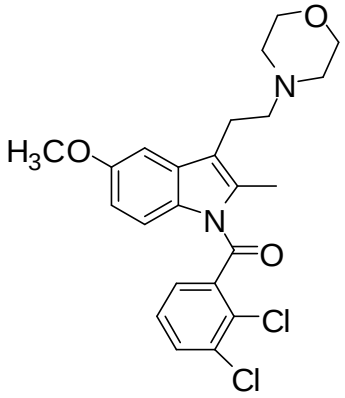
In the current study, the GTPase assay was used to examine the activity of various synthetic and natural compounds at the CBRs. The GTPase assay is a reliable functional test system that is – beneath [^{35}S]GTPγS binding assays, competition binding assays with a radiolabeled ligand, adenylyl cyclase assays and Ca^{2+} determination – well established for the assessment of the pharmacological properties of GPCR ligands (Wieland and Seifert, 2005; Geiger et al., 2010).

Sf9 cell membranes were used as protein expression system and the employment of CBR-Gα_{i2} fusion proteins in combination with RGS4 guaranteed a highly sensitive test system (see Chapter 3). The use of the fusion approach ensures a defined 1:1 stoichiometry and a spatial proximity of the signaling partners (Seifert et al., 1999), which markedly enhanced the sensitivity of the CBR GTPase assay. Furthermore, addition of RGS4 protein was auxiliary for an increased maximal stimulatory effect of standard ligands at the CBRs (Geiger et al., 2010; Chapter 3). These prerequisites provide the base for the detection of even partial agonists and inverse agonists.

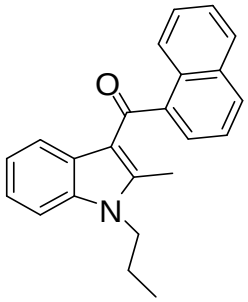
All tested synthetic compounds were indole derivatives. Position 2 was substituted with *n*-butyl or *n*-pentyl chains and various substituents with ester or amide function were placed at position 3 (Table 4.1). These 2,3-disubstituted indole derivatives were synthesized to mimic the structure of the endocannabinoids anandamide and 2-AG (Mackie, 2006). Moreover, there are well known CBRs ligands like WIN 55,212-2, an agonist at both CBRs, and AM 630, a ligand with neutral antagonistic properties at the CB_2R, that comprise this indole scaffold (Pertwee, 1999) showing remarkable potency and efficacy in the GTPase assay (Chapter 3). Structure-activity-relation (SAR) studies of aminoalkylindoles, a large group of synthetic cannabinoids with WIN 55,212-2 (Figure 4.3) as prominent member, were conducted previously. Results from these SAR studies showed that shortening of the side chain of the indole nitrogen may produce agonists with high affinity to the CB_2R – like JWH-018 and JWH-015 (Figure 4.3; Showalter et al., 1996). Other SARs with different indole series propose the use of naphthoyl or 2,3-dihalogenated benzoyl residues at the indole nitrogen. This led to the discovery of L-768,242 (Gallant et al., 1996), an indole derivative that displayed significant selectivity for the CB_2R. As depicted in Figure 4.3 all these compounds contain a methyl group



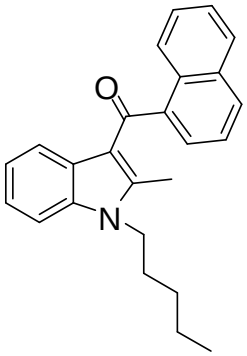
WIN 55,212-2



L-768,242



JWH-015



JWH-018

Figure 4.3: Structures of four indole derivatives with CBR agonistic properties

at position 2 and an alkyl morpholino or a naphthoyl methanon moiety at position 3. Our data showed that displacement of the indole nitrogen substituent in combination with longer alkyl chains in position 2 also results in significant selectivity for the CB_2R.

While all synthesized compounds showed no effects at the CB_1R, compounds **1**, **2** and **4** exhibited properties of partial agonists at the CB_2R compared to the most abundant endogenous agonist 2-AG (E_{max} set as 1.00; Table 4.4). Substituents with an ester function at position 3 showed greater efficacies than substituents with amide function. For the ester compounds **1** and **2**, E_{max} values of 0.64 ± 0.08 and 0.44 ± 0.04, respectively were calculated. Compound **4**, containing an amide function, displays an E_{max} value of 0.21 ± 0.03. It can be assumed that the 2-hydroxy ethyl amides at position 3 are too hydrophilic for the interaction with the CBR binding site.

Furthermore *n*-butyl chains (**1**) were outmatching *n*-pentyl (**2**) substituents at position 2.

To conclude, an ethyl ester function in combination with lipophilic alkyl moieties seems to be a solid base to examine SAR for CB_2R selective compounds.

The investigated natural compounds, all isolated from *Echinacea* species, are in the focus of research because the mechanism of action of *Echinacea* is still unclear. However, a modulation of innate and adaptive immune response is reported (Zhai et al., 2007). Since it was found that the endocannabinoid system is a target of alkamides (Gertsch et al., 2004; Woelkart et al., 2005; Raduner et al., 2006), these lipophilic compounds of the roots of *Echinacea purpurea* and *Echinacea pallida var. angustifolia* are discussed as key substances for the immunomodulatory effects of *Echinacea* preparations. Hence, it is surprising that only three of the tested alkamides showed activity at the CB_2R. Compared to 2-AG, compounds **17**, **18** and **19** belong to the field of partial CB_2R agonists with efficacies of 0.41 ± 0.03 (**17**), 0.69 ± 0.07 (**18**) and 0.32 ± 0.05 for (**19**). For compound **18**, the strongest CB_2R-activator of the tested alkamides, a $logEC_{50}$ of -5.51 ± 0.10 was assessed, showing similar potency compared to 2-AG with a $logEC_{50}$ value of -5.55 ± 0.22. The lacking activity of compound **20** is astonishing since Raduner measured displacement in competition binding with radiolabeled ligand and effects on the Ca^{2+} levels caused by **20** in HL60 cells (Raduner et al., 2006). Tested in the co-expression system (Chapter 2) and also in the higher sensitive fusion protein system no effects at CBRs were measurable. Possible explanations, as interactions with specific G protein subunits, are discussed in Chapter 2.

In contrast to the alkamides, the polyacetylenes and polyenes isolated from *Echinacea pallida* root extracts, did not show any activity at the CBRs. This is in accordance with Egger et al., (2008) who examined ketoalkenes and non-natural conjugated analogues on their activity at CBRs. In this study, only the ketoanalogue of anandamide and the dodeca-8*Z*-10-in-11phenyl-2-one were found to have agonistic properties at CBRs. These results lead to the conclusion that *Echinacea pallida* preparations, which almost lack of alkamides (Binns et al., 2002), do not conduct their immunomodulatory action via the CBRs.

All compounds that revealed lacking effects at the CBRs were tested in the antagonist mode in the presence of 30 nM CP 55,940 to exclude the unlikely occurrence of a neutral antagonist (Kenakin, 2004). Likewise, no alteration of the

basal GTPase activity was measurable indicating that these substances do not interact with the CBRs (data not shown).

In conclusion, among the tested alkamides and polyacetylenes, only the alkamides dodeca-2*E*,4*Z*-diene-8,10-diynoic acid isobutylamide, dodeca-2*E*,4*Z*-diene-8,10-diynoic acid 2-methylbutylamide and dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamides showed measurable activity with selectivity at the CB_2R. Furthermore, new 2,3-disubstituted indole derivatives with activity at the CB_2R were found. Thereby, the 3-(2-butylindole-3-yl)prop-2-en acid ethyl ester showed the strongest effect and provides a promising basis for the development of potent and selective CB_2R ligands.

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5 Conclusion

So far two human cannabinoid receptors (hCBRs) have been identified, both belonging to the family of G protein coupled receptors (GPCRs): the hCB_1R mainly located in the brain and the hCB_2R predominantly located in the periphery on immune cells. Because of their involvement in many physiological functions, such as movement, metabolic regulation, host defense, analgesia and memory, there is still a great interest for targeting CBRs for therapeutic applications.

The aim of this thesis was the establishment of a highly sensitive assay system that is suitable to analyze CBR pharmacology and to screen ligands concerning their CBR activity and their pharmacological profile. Therefore, we established the steady-state [γ-^{32}P]-GTPase assay, a functional, sensitive and reliable approach to study GPCRs with a read out at a very proximal point of the signal cascade. In native systems, this *in-vitro* assay showed a very low sensitivity. In rat cerebellum membrane only a weak stimulation of GTPase activity was measurable. The use of membranes of CBR transfected HEK 293 cells did not lead to a successful outcome as no change of GTPase activity caused by a ligand was detectable. These results directed us to employ *Spodoptera frugiperda* (Sf9) cell membranes as expression system. Here, the co-expression of CBRs, Gα subunit and the Gβγ heterodimer greatly improved the sensitivity of the assay.

As the efficacy of receptor-G protein interaction is highly influenced by the expression levels and density of these proteins in the membrane, we examined the impact of CBR-Gα fusion proteins on pharmacological properties of known CBR ligands. With the defined 1:1 stoichiometry and the forced anchor of the signaling partners in the membrane, the sensitivity of the GTPase assay was dramatically increased. Influences of regulators of G protein signaling (RGS) proteins RGS4 and RGS19 on GTPase activity in the co-expression and fusion system were examined. The results revealed that RGS4 in contrast to RGS19 behaves as a GTPase activating protein (GAP) for CBRs.

Comparing pharmacological properties of known CBR ligands evaluated in the co-expression and fusion systems in the absence or presence of RGS4 and RGS19, altered potencies and efficacies became apparent, especially in the CB_2R test systems. These data suggests that ligands stabilized specific GDP/GTP exchange-promoting receptor conformations that are influenced by the forced contact to the

$G\alpha_{i2}$ subunit and the addition of RGS protein. This phenomenon is indicative for the concept of ligand-specific receptor conformation.

For ligand screening procedures and to evaluate pharmacological parameters of new CBRs ligands, we used Sf9 cell membranes expressing CBR-Gα fusion proteins, the Gβγ-heterodimer and RGS4 as this constellation offers the highest sensitivity among the tested protein combinations. We examined various alkamides, polyacetylenes and polyenes isolated from *Echinacea* species as well as synthesized indole derivatives. From the natural compounds several alkamides showed measurable activity in a CB_2R selective manner. In contrast to the alkamides, polyacetylenes and polyenes did not show any effect, suggesting that they do not exert their immunomodulatory effect via the CBRs. Furthermore, new 2,3-disubstituted indole derivatives with activity at the CB_2R were found. Thereby, the 3-(2-butylindole-3-yl)prop-2-en acid ethyl ester showed the strongest effect and provides a promising basis for the development of potent and selective CB_2R ligands.

6 Appendix

List of abbreviations

2-AG	2-arachidonoyl glycerol
AM 251	*N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide
AM 281	1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-4-morpholinyl-1*H*-pyrazole-3-carboxamide
AM 630	(6-iodo-2-methyl-1-[2-4(morpholinyl)-ethyl]-[1*H*-indol-3-yl]-(4-methoxyphenyl)methanone)
ATP	adenosine 5`-triphosphate
bp	base pair(s)
BSA	bovine serum albumin
cAMP	3´,5´-adenosine monophosphate
cDNA	copy DNA
CNS	central nervous system
CP 55,940	[(-)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol]
CB_1R	human cannabinoid receptor subtype 1
CB_2R	human cannabinoid receptor subtype 2
C-terminus	carboxy-terminus
DAG	1,2-diacylglycerol
DMSO	dimethyl sulfoxide
EC_{50}	agonist concentration which induces 50% of the maximum effect
ECS	endocannabinoid system
EDTA	ethylenediaminetetraacetic acid (Ca^{2+}-chelator)
E_{max}	efficacy (maximal response)
FLAG	octapeptide epitope for the labeling of proteins
$G\alpha_i$	α subunit of G proteins that inhibits adenylyl cyclase
$G\alpha_q$	α subunit of G proteins that activates phospholipase C
$G\alpha_s$	α subunit of G proteins that stimulates adenylyl cyclase
GAP	GTPase activating protein
$G\beta\gamma$	βγ subunit of a heterotrimeric G protein
GDP	guanosine 5´-diphosphate
GIT	gastrointestinal tract
GPCR	G protein coupled receptor
GTP	guanosine 5´-triphosphate
$GTP\gamma S$	guanosine 5´-[γ-thio]triphosphate
h	hour(s)
5-HT	5-hydroxy tryptophan
His_6	hexahistidin tag
IC_{50}	antagonist concentration which suppresses 50% of an agonist induced effect

IP_3	inositoltrisphosphate
kDa	kilodalton
MAPK	mitogen-acitivated protein kinase
N-terminus	amino-terminus
PBS	phosphate buffered saline
PCR	polymerase chain reaction
P_i	inorganic phosphate
PIP_2	phosphatidylinositolbisphosphate
PLC	phospholipase C
r^2	correlation coefficient
rimonabant	*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide
RGS	Regulator of G protein signaling
rpm	revolutions per minute
SAR	structure-activity-relation
SD	standard deviation
SDS-PAGE	sodiumdodecylsulfate polyacrylamide gel electrophoresis
Sf9	insect cell line of *Spodoptera frugiperda*
Δ^9-THC	Δ^9-tetrahydrocannabinol
TM	transmembrane
Tris	tris(hydroxymethyl)aminomethan
WIN 55,212-2	(*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmetha-none mesylate

List of publications

Prior to submission of this thesis, results were published in part or presented as short lectures or posters.

Publications:

Sutor S, Heilmann J, Seifert R (2010) Impact of fusion to $G\alpha_{i2}$ and co-expression with RGS proteins on pharmacological properties of human cannabinoid receptors CB_1R and CB_2R (submitted to Naunyn Schmiedebergs Arch Pharmacol)

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Poster Contribution and Short Lectures:

Feizlmayr E, Sutor S, Stinglmayr I, Kunert O, Heilmann J, Bauer R (2010) Cannabinoid receptor activity of polyacetylenes and polyenes from *Echinacea pallida.* Annual meeting of the Society of Medicinal Plant Research – Poster contribution

Sutor S, Heilmann J, Seifert R (2010) The steady-state [γ-^{32}P]-GTPase assay: a functional and highly sensitive model system for characterization of cannabinoid receptor ligands. Cannabinoid Workshop-The Endocannabinoid system: from Physiology to Pathophysiology – Poster contribution

Geiger S, Heilmann J, Seifert S (2010) Use of the steady-state [γ-^{32}P]-GTPase assay for characterization of cannabinoid receptor ligands: a functional and highly sensitive model system. Deutsche Gesellschaft für Experimentelle und Klinische Pharmakologie und Toxikologie – Short Lecture

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