JPP Journal of Pharmacy and Pharmacology
ROYAL PHARMACEUTICAL SOCIETY

JPP Journal of Pharmacy and Pharmacology

JPP 2011, 63: 1043–1055

Received December 9, 2010
Accepted April 18, 2011
DOI
10.1111/j.2042-7158.2011.01307.x
ISSN 0022-3573

Research Paper

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

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Abstract

Objectives G protein coupled receptor (GPCR)-Gα fusion proteins are often employed to investigate receptor/G protein interaction. In this study, the impact of Gα fusion proteins on pharmacology of CBRs, both mediating signals through $G\alpha_i$ proteins, were investigated. $G\alpha_{i2}$ was fused to the C-terminus of the CBRs or co-expressed with non-fused $G\alpha_{i2}$ in Sf9 cells, always together with $G\beta_1\gamma_2$. Furthermore, the impact of RGS proteins on CBR signaling in combination with the CBR fusion approach was examined, using RGS4 and RGS19 as paradigms.

Methods CBR ligands were characterized in the steady-state GTPase assay and pharmacological properties of ligands in the different test systems were correlated.

Key findings Fusion of CBRs to $G\alpha_{i2}$ enhanced the maximal stimulatory effects of ligands compared to the co-expression system, especially for 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.

Conclusions 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 conformations. Finally, this paper describes the most sensitive CBR test system currently available.

Keywords cannabinoid receptors; fusion proteins; G protein coupled receptors; RGS proteins; steady-state GTPase assay

Introduction

Many hormones and neurotransmitters exert their physiological effects through G protein coupled receptors (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 guanosine 5′-diphosphate (GDP) to guanosine 5′-triphosphate (GTP) and dissociation of the Gα-GTP-G$\beta\gamma$ complex into the subunits Gα-GTP and G$\beta\gamma$.[1] Both subunits can regulate effector systems, for example adenylyl 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, hydrolysing GTP to GDP and P_i. Subsequently, reassociation of Gα, GDP and G$\beta\gamma$ completes the G protein cycle. According to the two-state model of GPCR activation,[2] 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 the 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.[3]

The efficacy of receptor-G protein coupling is highly influenced by protein expression levels and the stoichiometry of signaling partners.[4] One limitation concerning the use of GPCR co-expression systems in assay development or functional studies is the lack of a 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.[4,5] As the binding of ligands is accompanied by a conformational change in receptors, it is of substantial interest whether

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the pharmacological properties of ligands and receptors are influenced by the fusion to the Gα subunit. It has been reported that pharmacological properties of ligands of the α_{2A}-adrenoceptor are altered as a result of the fusion,[6] and for other systems, such as the 5-HT_{1A}-receptor, similar potencies and efficacies have been observed.[7]

To address this question for the two human cannabinoid receptors (CBRs), which belong to the family A of GPCRs and couple to pertussis-toxin (PTX) sensitive G$\alpha_{i/o}$,[8] receptors were fused C-terminally to the N-terminus of the Gα_{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-1*H*-pyrazole-3-carboxamide) (at CB_1R) and antagonist AM 630 (6-iodo-2-methyl-1-[2–4(morpholinyl)-ethyl]-[1*H*-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.[2] Data obtained in the fusion protein system were compared with those obtained in a system where the CBRs were co-transfected with the Gα_{i2} subunit.

G protein signaling regulators (RGS) interfere with the sensitivity of G protein signaling pathways and shorten the period of time in which the Gα subunit is in its active conformation.[9] 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.[10,11] GAP activity of RGS proteins, as key modulators of the amplitude and duration of G protein mediated signaling, were described for Gα_i and Gα_q subunits.[12]

As it has been reported that RGS4 and RGS19 are GAPs for the Gα_i subfamily[13] and that RGS proteins can participate in the formation of a quaternary complex consisting of agonist, receptor, G protein and RGS protein,[14] 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. *Spodoptera frugiperda* (Sf9) cells were therefore infected with baculoviruses encoding for CBRs-Gα_{i2} or CBRs co-transfected with Gα_{i2}, always together with G$\beta_1\gamma_2$ and in the absence or presence of RGS4 or RGS19. This paper describes the most sensitive CBR test system currently available.

Materials and Methods

cDNAs for hCB1R and hCB2R in pcDNA 3.1 were obtained from the cDNA bank of the University of Missouri (Rolla, MO, USA). For preparation of the SF-hCBR-His_6-Gα_{i2} fusion proteins, the hexahistidine-tagged C-terminus of hCB_1R and hCB_2R was fused to the N-terminus of Gα_{i2} according to a previously described strategy using overlap-extension PCR.[15] Baculoviruses were generated in Sf9 cells using a BaculoGOLD transfection kit according to the instructions of the manufacturer (BD PharMingen, San Diego, CA, USA).

Recombinant baculovirus encoding Gα_{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 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.[16]

Correct transfection of Sf9 membranes expressing different proteins were confirmed with SDS gel electrophoresis and immunoblotting. The antibodies used were M1 anti-FLAG (St Louis, MO, USA), anti-Gα_{i2} (Calbiochem, San Diego, CA, USA), anti RGS4, anti-RGS19 (Santa Cruz, CA, USA) and anti-Gβ (Gβ_{common}; AS398/9), kindly provided by Dr B. Nürnberg (Institute of Pharmacology, University of Tübingen, Germany).

The GTPase assay was performed as described previously.[17] Assay tubes contained membranes (Gα_{i2} co-transfected membranes, 10 µg of protein/tube; Gα_{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) bovine serum albumin in 50 mM Tris/HCl, pH 7.4 to prevent binding of protein or ligand to the polystyrol tubes, CB_1R and CB_2R ligands at various concentrations and 20 µl of [γ-^{32}P]GTP (0.1 µCi/tube). Reactions were conducted for 20 min at 25°C for co-transfected membranes and 10 min for fusion protein membranes.

Data shown in Tables 2 and 3 were analysed statistically by one-way ANOVA, followed by Dunnett's multiple comparison test.[17]

Results

Generation of baculoviruses and immunoblotting

For expression of the CBRs, 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 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

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

CBRs-$G\alpha_{i2}$ fusion proteins, the hexahistidine tag was used as overlap for the $G\alpha_{i2}$ subunit.

We infected 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 1. Correct protein expression was confirmed with immunoblotting. As shown in Figures 1a and 2a, the M1 anti-FLAG recognized CBRs and CBR-$G\alpha_{i2}$ fusion proteins. CB_1R showed the expected band at ~57 kDa.[18] 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.[19] 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 (Figures 1b and 2b).

$G\beta_1$ was detected with a $G\beta_{common}$ antibody in the CB_1R systems (Figure 1c). In the CB_1R-$G\alpha_{i2}$ systems an additional band with relatively high molecular mass was particularly evident, whereas for the CB_2R protein expression systems (Figure 2c) a second weak band was seen near the band for $G\beta_1$ (~36 kDa). 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 (Figures 1d, 1e, 2d and 2e). As expected, the bands for RGS4 were about ~1 kDa lower than those for RGS19. Additional

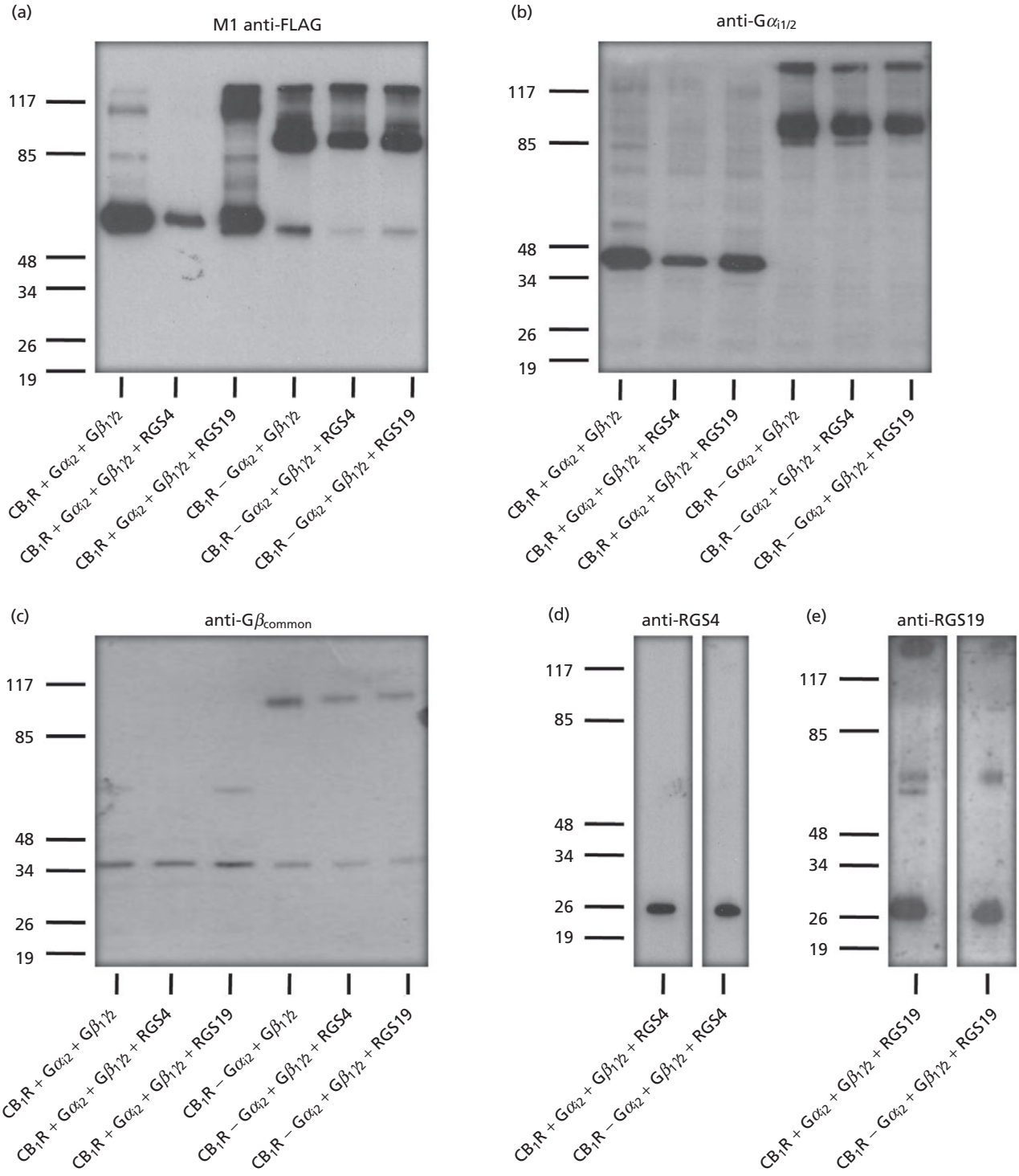

Figure 1 Immunoblot analysis of recombinant proteins in Sf9 cell membranes for CB_1R test systems. Immunological detection of CB_1R, $G\alpha_{i2}$, $G\beta_1\gamma_2$ and RGS proteins expressed in Sf9 cell membranes was performed as described under *Materials and Methods*. Each lane was loaded with 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. (b) Visualization of $G\alpha_{i2}$ with anti-$G\alpha_{i1/2}$. (c) Membranes were reacted with $G\beta_{common}$. (d) Detection of RGS4. (e) Detection of RGS19.

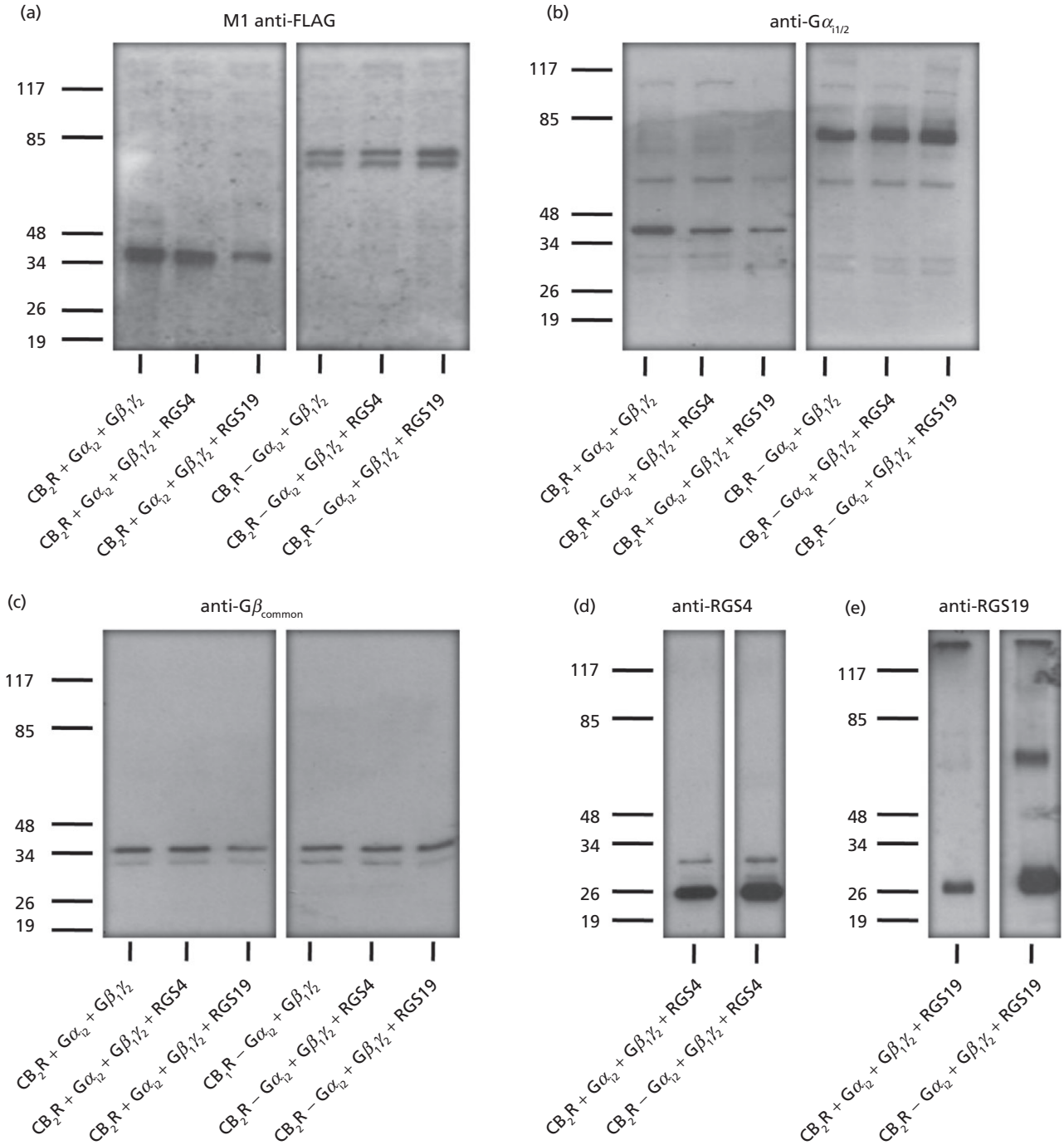

Figure 2 Immunoblot analysis of recombinant proteins in Sf9 cell membranes for CB_2R test systems. Immunological detection of CB_2R, $G\alpha_{i2}$, $G\beta_1\gamma_2$ and RGS proteins expressed in Sf9 cell membranes was performed as described under *Materials and Methods*. Each lane was loaded with 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. (b) Visualization of $G\alpha_{i2}$ with anti-$G\alpha_{i1/2}$. (c) Membranes were reacted with $G\beta_{common}$. (d) Detection of RGS4. (e) Detection of RGS19.

bands were particularly evident for the anti-RGS19, probably representing oligomeric forms.

Basal GTPase activity and stimulation of GTPase by CP 55,940

We performed steady-state GTPase assays with Sf9 membranes co-expressing the proteins shown in Table 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, 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 GAP[20] (Tables 2 and 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, the CB_2R-$G\alpha_{i2}$ fusion protein co-transfected with RGS4 showed the highest GTPase stimulation, amounting to 393 ± 30%.

Figure 3 shows representative concentration-response curves obtained for CP 55 940. 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 $G\alpha_{i2}$.

Potencies and efficacies of CBR ligands in the GTPase assay in the absence and presence of RGS proteins

We evaluated the potential effects of GAPs[20] and used RGS4 and RGS19 as paradigms, $G\beta_1\gamma_2$ always being present. In previous studies, both RGS proteins enhanced agonist-stimulated GTP hydrolysis.[10,11]

The potencies and efficacies of several ligands (Tables 2 and 3), specifically anandamide, 2-AG, CP 55,940, WIN 55,212-2, AM 251, AM 281 and AM 630 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 2), no significant changes in $logEC_{50}$/$logIC_{50}$ values were detected for all analysed 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 resulted in higher stimulation 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 at 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). 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 $CB_2R + G\alpha_{i2} + G\beta_1\gamma_2$ vs 73 ± 13% in the presence of RGS4; the difference was not significant) and WIN 55 212 in the fusion system (192 ± 12% simulation in the standard fusion system vs 255 ± 29% in the presence of RGS4; again a difference that was not significant). RGS4 altered $logEC_{50}$ values for anandamide and WIN 55 212 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 55 211 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.

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

The use of a $G\alpha$ co-expression system is always associated with the problem that the expression levels of the signaling partners are difficult to control.[1] 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.[21] To compare the CBR co-expression system with the fusion system, we used the ubiquitously expressed $G\alpha_{i2}$ subunit.[22] Figure 4 shows correlations of potency and efficacy (calculated as stimulation relative to 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 4).

In contrast, the goodness-of-fit and slope values obtained from the comparisons of the CB_2R systems (Figure 5) indicate that the fusion of the CB_2R to $G\alpha_{i2}$ substantially altered the pharmacological parameters of the ligands. The efficacies (Figure 5a, 5c and 5e) and potencies (Figure 5b, 5d and 5f) of ligands studied in the absence and presence of RGS proteins differed from each other, depending on whether the receptor was fused or co-expressed with $G\alpha_{i2}$. The most impressive differences were obtained when RGS proteins were co-expressed. An r^2 value of 0.687 and slope of 0.551 ± 0.215 for RGS4 (Figure 5d) and an r^2 value 0.815 and slope of 0.594 ± 0.163 for RGS19 (Figure 5f) indicate a poor correlation between the co-expression and fusion protein system.

Discussion

The detailed pharmacological analysis of CBR ligands in primary cells and native systems is very difficult.[17] In this study the steady-state GTPase assay was used to examine the effects of CBR-$G\alpha$ 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

Table 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-$G\alpha_{i2}$

Ligand labeling in figures			$CB_1R + G\alpha_{i2}$	$CB_1R + G\alpha_{i2}$ + RGS4	$CB_1R + G\alpha_{i2}$ + RGS19	CB_1R-$G\alpha_{i2}$	CB_1R-$G\alpha_{i2}$ + RGS4	CB_1R-$G\alpha_{i2}$ + RGS19
1	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
2	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
3	CP 55,940	Stim (%)	57 ± 8	158 ± 8***	70 ± 16	76 ± 8	304 ± 9***	103 ± 15
		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
4	WIN 55,212	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
5	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
6	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 ± 0.16	–7.52 ± 0.14	–7.66 ± 0.26

Stim, stimulation. 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 three independent experiments performed in duplicate or triplicate 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 analysed by non-linear 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 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-$G\alpha_{i2}$

Ligand labeling in figures			$CB_2R + G\alpha_{i2}$	$CB_2R + G\alpha_{i2}$ + RGS4	$CB_2R + G\alpha_{i2}$ + RGS19	CB_2R-$G\alpha_{i2}$	CB_2R-$G\alpha_{i2}$ + RGS4	CB_2R-$G\alpha_{i2}$ + RGS19
1	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
2	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
3	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*
4	WIN 55,212	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
7	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

Stim, stimulation. 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 three independent experiments performed in duplicate or triplicate 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 analysed by non-linear 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: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

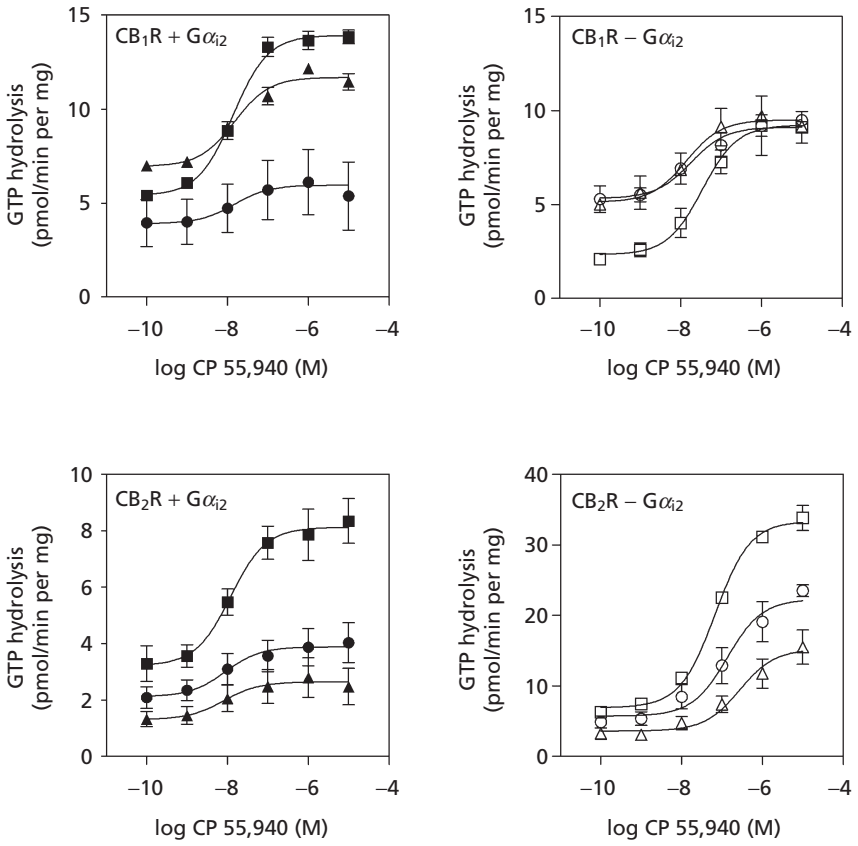

Figure 3 Representative concentration–response curves obtained for CP 55 940 in the $G\alpha_{i2}$ co-expression and $G\alpha_{i2}$ fusion system. Steady-state GTPase activity in Sf9 membranes was determined as described under *Materials and Methods*. Data show representative results performed in triplicates in Sf9 cells expressing CBR + $G\alpha_{i2}$ + $G\beta_1\gamma_2$ without RGS proteins ●, +RGS4 ■, +RGS19 ▲ or CBR-$G\alpha_{i2}$ + $G\beta_1\gamma_2$ without RGS proteins ○, +RGS4 □, +RGS19 Δ. Experiments were replicated three 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 Tables 2 and 3.

ligands. The GTPase assay and the Sf9 cell membrane expression systems were successfully applied to characterize ligands of other $G\alpha_i$-coupled GPCRs.[10,11,23,24] As insect cells do not endogenously express CBRs,[25] infection of Sf9 cells with baculoviruses encoding for CBRs offers advantages in the conduct of functional studies of these GPCRs without interference of endogenous CBRs. Furthermore, mammalian-type $G\alpha_i$ proteins are not expressed in Sf9 cells,[23,26] so that coupling studies of GPCRs to this particular $G\alpha$ 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 agonists.[27] 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 other GPCRs have shown that RGS19 strongly enhances agonist-stimulated GTP hydrolysis.[10,11,28] The data from our study indicate that the capacity of RGS proteins to regulate GTP hydrolysis depends on the GPCR and that the GPCR may govern RGS-G protein interactions.[29] Although the polypeptide size of RGS19 is quite similar to RGS4, its amino-terminal region contains a cysteine string region and a carboxy-terminal PDZ binding motif. The scaffold protein GIPC (GAIP-interacting protein) is required for the binding of RGS19 to the dopamine D_2-receptor.[30] 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 $G\alpha$ subunit is difficult to achieve.[1] The use of GPCR-$G\alpha$ fusion proteins, which ensure close proximity of the signaling partners and anchoring of the $G\alpha$ subunit in the plasma membrane, therefore provides a system for the study of receptor-G protein interaction under defined conditions.[4,5] As CBRs couple to $G_{i/o}$ proteins,[8] a fusion of wild-type $G\alpha_{i2}$ to the CBRs was performed. After successful expression of the desired proteins in Sf9 cells (Figures 1 and 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

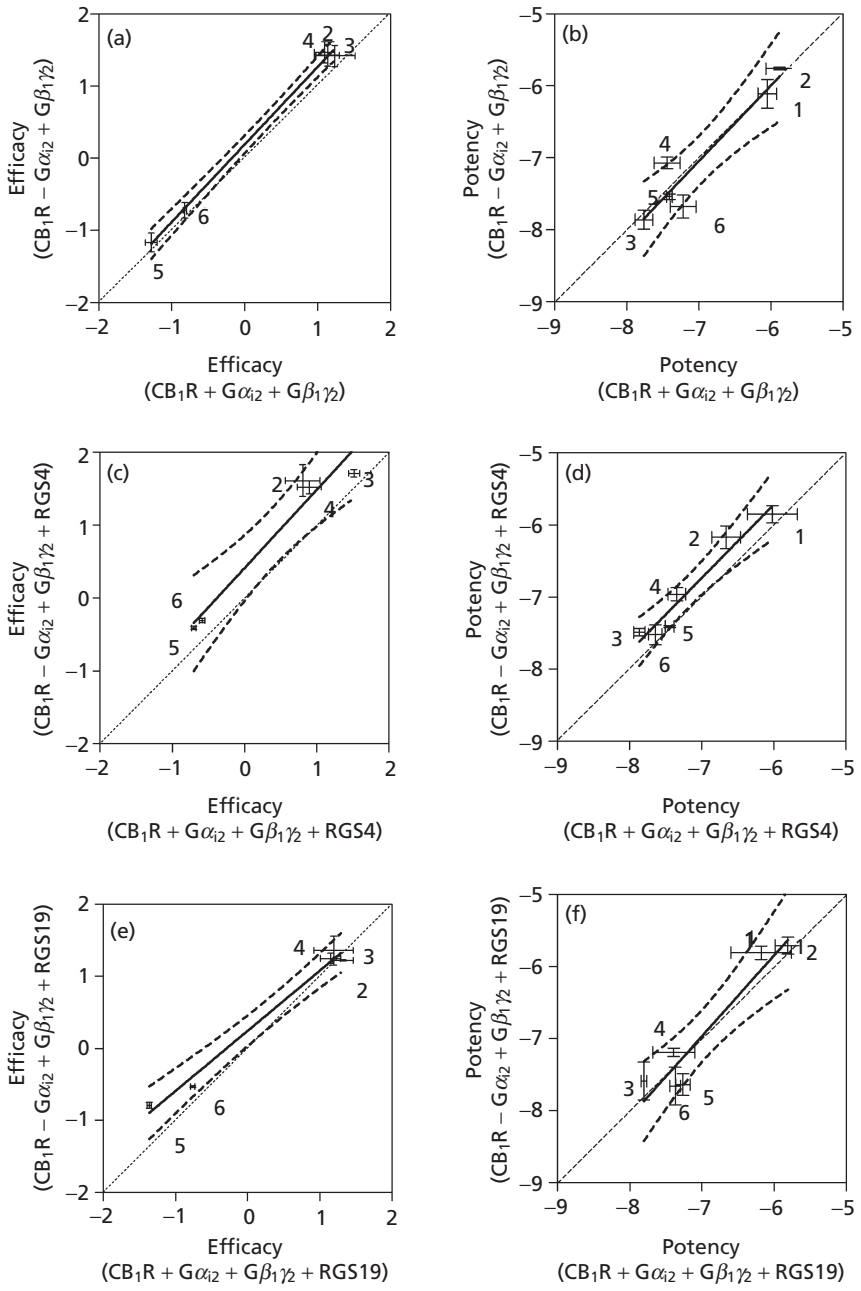

Figure 4 Correlation of potency and efficacy of ligands at the CB_1R between the co-expression and fusion system. Data of Table 2 were analysed by linear regression. (A), (C) and (E), efficacy of ligands at membranes co-expressing CB_1R, $G\alpha_{i2}$ and $G\beta_1\gamma_2$ in the presence or absence 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, $r^2 = 0.997$ and slope = 1.075 ± 0.035; C, $r^2 = 0.946$ and slope = 1.073 ± 0.148; E, $r^2 = 0.986$ and 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, $r^2 = 0.902$ and slope 1.050 ± 0.173; D, $r^2 = 0.940$ and slope = 1.019 ± 0.129. F, $r^2 = 0.905$ and slope = 1.122 ± 0.182. The linear regression lines and the 95% confidence intervals (dotted lines) are shown. 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; 5, AM 251; 6, AM 281.

CB_1R expression systems were higher GTPase activities in the fusion system elicited compared to the co-expression system (Table 2). The forced proximity of the $G\alpha$ 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).

The potencies of some ligands were influenced by the fusion of the CB_2R to the $G\alpha_{i2}$ subunit (Figure 5b, 5d and 5f) and of CB_1R in the presence of RGS4 (Figure 4d). The

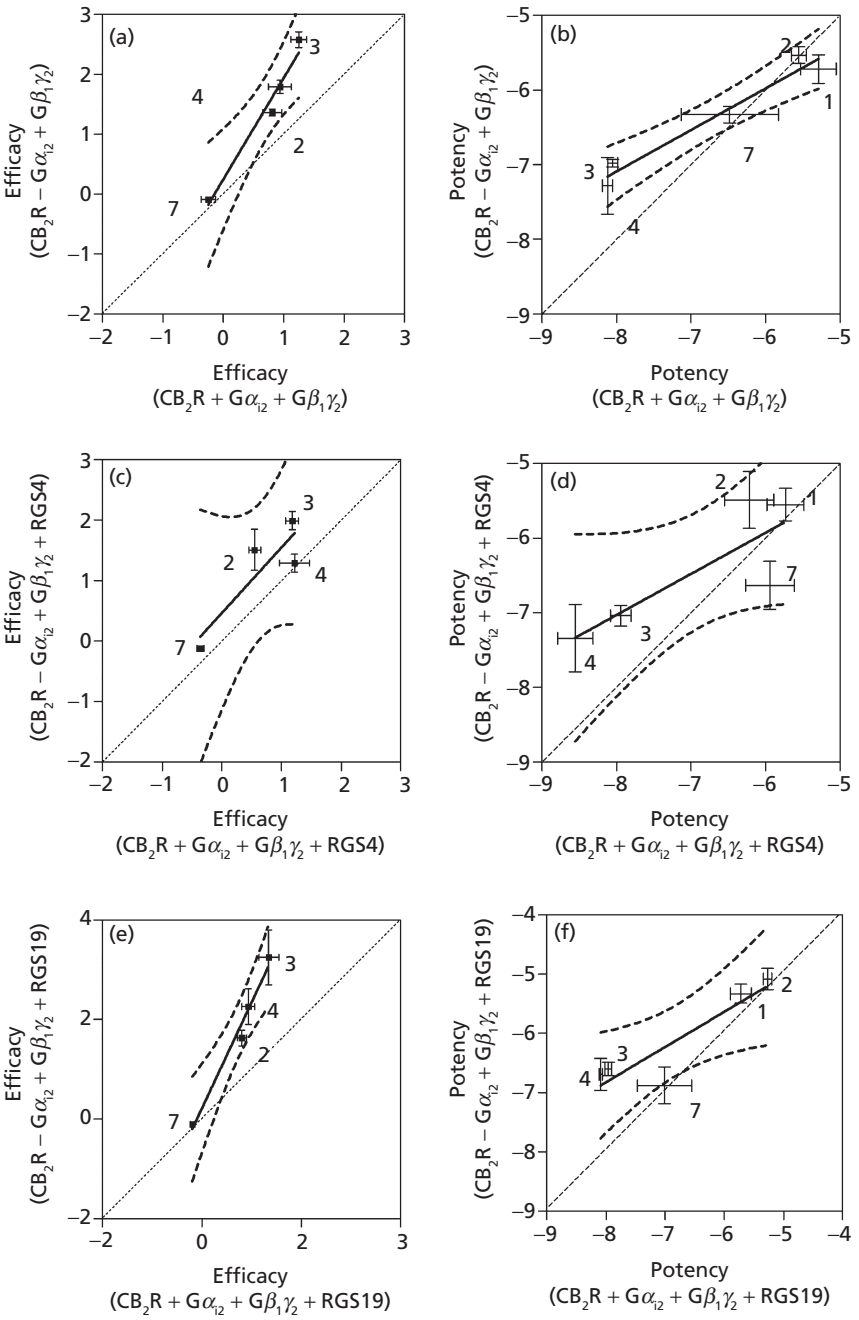

Figure 5 Correlation of potency and efficacy of ligands at the CB_2R between the co-expression and fusion system. Data in Table 3 were analysed by linear regression. A, C, E: efficacy of ligands at membranes co-expressing CB_2R, $G\alpha_{i2}$ and $G\beta_1\gamma_2$ in the presence or absence of RGS 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, $r^2 = 0.967$ and slope = 1.691 ± 0.220; C, $r^2 = 0.778$ and slope = 1.083 ± 0.409; E, $r^2 = 0.978$ and 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, $r^2 = 0.957$ and slope 0.5567 ± 0.068; D, $r^2 = 0.687$ and slope = 0.551 ± 0.215. F, $r^2 = 0.815$ and 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; 7 AM 630.

phenomenon of reduced potencies of ligands in fusion protein expression systems was observed previously[31,32] and it is probably due to physical restrictions inhibiting protein-conformational changes[32] and/or compartmentalization of signaling elements within specific domains of the plasma membrane.[33] For the CB_2R, an altered pattern of phosphorylation can also be considered as a reason for modulated pharmacological parameters. It is known that agonist treatment of Chinese hamster ovary cells stably expressing CB_2R increases basal phosphorylation of Ser^{352}.[34] 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 $G\alpha$ subunit. The relevance of GRKs in Sf9 cells concerning the regulation of GPCR signaling is still unclear,[35] but it is possible that a modulated phosphorylation can in turn affect the efficacy and potency of tested ligands.

The differences in pharmacological properties between fusion and co-expression systems raises the question of whether the $(CB_2)R$-$G\alpha_{i2}$ system is a useful tool for examining pharmacological parameters of new drugs, as the fusion approach is an inherently artificial system. In addition, the co-expression system with its varying GPCR/G-protein stoichiometry may not mirror a physiological environment as it is still unclear to how many G proteins a single receptor has access to. Hence it is difficult to evaluate which of the measured potencies reflects the drug behavior under physiological conditions. A comparable in-vivo assay in mammalian cells, ideally those expressing CBR endogenously, would therefore be required to clarify this issue – a difficult undertaking, as recently reported.[17] However, the use of CBR-$G\alpha_{i2}$ fusion proteins offers a highly sensitive model system and allows the screening of new CBR ligands at a very proximal point of the signaling cascade.

Moreover, the possibility that RGS proteins may alter ligand potencies cannot be excluded, as described previously.[36,37] Interaction between GPCR, RGS and G protein supports the assumption that GPCR function may indeed be modulated by RGS proteins.[14,29] The fact that the potency and efficacies of the ligands are not altered similarly by the fusion and RGS proteins is indicative of ligand-specific receptor conformations. Similarly, at the β_2-adrenoceptor[38,39] and the histamine H_4-receptor,[40] ligands can stabilize unique receptor confirmations[3] differing in their ability to interact with and activate G proteins. It is likely that 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 was shown for CB_2R,[41] offering an opportunity to develop ligands that selectively manipulate physiological functions.[42] Differential modulation of signaling by RGS proteins has also been observed for opioid receptors.[43]

Conclusion

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 systems. 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 well suited to the study of the structure–activity relationships of new CBR ligands. The alterations of pharmacological properties of the CBRs ligands in the different systems examined 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 one, or a few, of the multiple functions regulated by CBRs. As a result, novel therapeutic uses of CBR ligands with fewer side effects may be identified. This paper describes the most sensitive CBR test system currently available. Finally, future studies will have to analyse the effects of RGS protein inhibitors on CBR signaling in intact cells.[44]

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Acknowledgements

We thank Mrs G. Wilberg (Department of Pharmacology and Toxicology, University of Regensburg) for technical assistance concerning cell culture, Dr K. Wenzel-Seifert (Department of Pharmacology and Toxicology, University of Regensburg) for her professional support to construct the fusion proteins and Dr D. Schnell (Department of Pharmacology and Toxicology, University of Regensburg) for his help to synthesize [γ^{32}P]GTP. Thanks are also due to Professor Dr J. Schlossmann (Department of Pharmacology and Toxicology, University of Regensburg) for providing infrastructure. Finally, we thank the reviewers for their helpful critique.

Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

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