



Universiteit
Leiden
The Netherlands

Non-ribose ligands for the human adenosine A1 receptor

Klaasse, E.C.

Citation

Klaasse, E. C. (2008, June 10). *Non-ribose ligands for the human adenosine A1 receptor*. Retrieved from <https://hdl.handle.net/1887/12936>

Version: Corrected Publisher's Version

License: [Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden](#)

Downloaded from: <https://hdl.handle.net/1887/12936>

Note: To cite this publication please use the final published version (if applicable).

CHAPTER 3

Allosteric modulation and constitutive activity of
fusion proteins between the adenosine A₁ receptor
and different ³⁵¹Cys-mutated G_iα-subunits

We studied fusion proteins between the human adenosine A₁ receptor and different (³⁵¹Cys) mutated G_{i1}α subunits (A₁-G_iα) with respect to two important concepts in receptor pharmacology, i.e. allosteric modulation and constitutive activity/inverse agonism. The aim of our study was twofold. We first analysed whether such fusion products are still subject to allosteric modulation, and, secondly, we investigated the potential utility of the fusion proteins to study constitutive receptor activity. We determined the pharmacological profile of 9 different A₁-G_iα fusion proteins in radioligand binding studies. In addition, we performed [³⁵S]GTPγS binding experiments to study receptor and G protein activation of selected A₁-G_iα fusion proteins. Compared to unfused adenosine A₁ receptors, the affinity of N⁶-cyclopentyladenosine (CPA) at wild-type A₁-G_iα fusion proteins (³⁵¹Cys) increased more than eight-fold, while the affinity of 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) did not change significantly. Furthermore, we showed that the allosteric enhancer of agonist binding, PD81,723 (2-amino-4,5-dimethyl-3-thienyl-[3-(trifluoromethyl)-phenyl]methanone), elicited similar effects on ligand binding; i.e. CPA binding to the A₁-G_iα fusion proteins was enhanced, whereas the affinity of DPCPX was hardly affected. Moreover, sodium ions were unable to decrease agonist binding to the majority of the A₁-G_iα fusion proteins, presumably because they exhibit their effect through uncoupling of the R-G complex. From [³⁵S]GTPγS binding experiments we learned that all the A₁-G_iα fusion proteins tested had a higher basal receptor activity than the unfused adenosine A₁ receptor, thereby providing improved conditions to observe inverse agonism. Moreover, the maximal CPA-induced stimulation of basal [³⁵S]GTPγS binding was increased for the five A₁-G_iα fusion proteins tested, whereas the inhibition induced by 8-cyclopentyltheophylline (CPT) was more pronounced at ³⁵¹Cys, ³⁵¹Ile, and ³⁵¹Val A₁-G_iα fusion proteins. Thus, the maximal receptor (de)activation depended on the amino acid at position 351 of the G_iα-subunit. In conclusion, A₁-G_iα fusion proteins, especially with ³⁵¹Cys and ³⁵¹Ile, can be used as research tools to investigate inverse agonism, due to their increased readout window in [³⁵S]GTPγS binding experiments.

Based upon Klaasse E, de Ligt RAF, Roerink SF, Lorenzen A, Milligan G, Leurs R, IJzerman AP., Eur J Pharmacol. 2004, 499:91-98.

Introduction

Adenosine A₁ receptors belong to the superfamily of G protein-coupled receptors (GPCRs). They preferentially interact with G_i and G_o proteins¹. Various strategies have been employed to study the interaction between receptor and G protein. One method is the construction of so-called fusion proteins, in which both signalling partners, GPCR and G α-subunit, are physically linked to each other. In 1994, the first fusion protein (between β₂-adrenoceptor and G_s-subunit) was described and found to be functional in both ligand binding experiments and cAMP determinations².

The same strategy has been applied to adenosine A₁ receptors. Waldhoer et al. analysed the kinetic behaviour of the adenosine A₁ receptor linked to both G_i- and G_o-subunits³. The authors also introduced a fusion protein in which the G_i-subunit (of rat G_{i1}) was mutated at position ³⁵¹Cys. This cysteine residue is known to be ADP-ribosylated by pertussis toxin (PTX); changing it for another amino acid (glycine or isoleucine in this case) renders the fusion protein insensitive to the toxin⁴. Endogenous G proteins present in the cell which the fusion protein is expressed in, remain sensitive to PTX and can be 'knocked out'. Similar constructs were used in studies to analyse the action of both receptor ligands such as adenosine analogues and G protein inhibitors such as suramin and derivatives^{5,6}. Curiously, Bevan et al. introduced a "trivalent" fusion protein between adenosine A₁ receptor, green fluorescent protein, and G_i-subunit, which behaved very much as the wild-type receptor in terms of ligand binding and G protein activation⁷.

In the present study we performed a further analysis of such fusion proteins with two aims that have not yet been subject of investigation. Firstly, we wondered whether the constructs can still be influenced by allosteric modulators. In our laboratory, we have been investigating allosteric modulation of adenosine receptors by, e.g., sodium ions and PD81,723⁸⁻¹⁰.

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

Materials and Methods

Chemicals

CPA, DPCPX, CPT, and PD81,723 were purchased from Research Biochemicals Inc. (Natick, USA). Adenosine deaminase (ADA), diethylaminoethyl (DEAE) dextran, chloroquine, and dithiothreitol were purchased from Sigma, while EDTA, MgCl_2 , GDP,

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

Cell culture and transfection

African green monkey kidney (COS-7) cells were maintained at 37 °C in a humidified atmosphere with 7% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% FCS, penicillin (50 IU/ml), and streptomycin (50 $\mu\text{g}/\text{ml}$). COS-7 cells were transiently transfected with either the unfused adenosine A_1 receptor, or a fusion protein (A_1 - $\text{G}_{i1}\alpha$) using a slightly modified protocol of the 'in suspension' DEAE dextran method previously described by Brakenhoff et al.¹³. In short, COS-7 cells were subcultured one day prior to transfection. The next day cells were trypsinised, counted, and resuspended in RPMI 1640 amino acids solution supplemented with 2% FCS and 100 μM chloroquine (RSC), at a density of 2×10^6 cells/ml. DNA (4-10 $\mu\text{g}/10^6$ cells) and DEAE dextran (400 $\mu\text{g}/\text{ml}$) were mixed in a total volume of 4 ml RSC, and incubated at room temperature for two minutes. Then 0.5 ml (10^6 cells) of the cell suspension was added, and the total mixture was incubated for 60 min at 37 °C and 7% CO_2 . Cells were subsequently spun down at

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

Membrane preparation

After 48 hrs, transiently transfected COS-7 cells were harvested with a cell scraper, and recovered by a 5-min centrifugation at 1000 x g. Cells were then homogenised in ice-cold 50 mM Tris HCl buffer (pH 7.4) with a polytron homogeniser (5 sec, speed 8), and used for radioligand binding studies. To measure [³⁵S]GTP_γS binding, the cell homogenates were purified with two additional centrifugation steps at 4 °C: a 10-min centrifugation at 1000 x g and the obtained supernatant for 30 min at 60,000 x g (Beckman L8-50 M/E Ultracentrifuge). The final pellet was resuspended in 3 ml ice-cold 50 mM Tris HCl buffer (pH 7.4), supplemented with ADA (2 U/ml). Protein concentrations were measured with the bicinchoninic acid method with BSA as a standard¹⁴.

Radioligand binding studies

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

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

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

[³⁵S]GTP_γS binding

[³⁵S]GTP_γS binding was measured in 100 μl containing 50 mM Tris HCl (pH 7.4), 1 mM EDTA, 5 mM MgCl₂, 10 μM GDP, 1 mM dithiothreitol, 100 mM NaCl, 5 U/ml ADA, 0.3 nM [³⁵S]GTP_γS (~50,000 cpm), 0.004% CHAPS, and 0.5% BSA.

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

Data and statistical analysis

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

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

Results

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

Table 3.1. K_d and B_{max} values from saturation experiments with [³H]DPCPX on COS-7 cell homogenates transfected with the human adenosine A₁ receptor or various (mutated) A₁-G_iα fusion proteins (³⁵¹Cys (= wild-type G_iα), ³⁵¹Gly, and ³⁵¹Ile). The experiments were carried out in the presence or absence of 1 M NaCl. Data are expressed as means ± S.E.M. from three independent experiments performed in duplicate.

Construct	control		1 M NaCl	
	K _d (nM)	B _{max} (pmol/mg protein)	K _d (nM)	B _{max} (pmol/mg protein)
unfused A ₁	1.8 ± 0.31	2.18 ± 0.13	0.83/0.83 ^a	2.71/2.81 ^a
A ₁ G _i ³⁵¹ Cys	1.5 ± 0.04	1.29 ± 0.38	0.50 ± 0.06 ^b	1.59 ± 0.41
A ₁ G _i ³⁵¹ Gly	1.9 ± 0.38	1.22 ± 0.21	0.41 ± 0.05 ^c	1.39 ± 0.27
A ₁ G _i ³⁵¹ Ile	1.5 ± 0.03	1.53 ± 0.48	0.50 ± 0.02 ^b	1.58 ± 0.38

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

Next, we determined the IC₅₀ values of CPA and DPCPX for the nine A₁-G_iα constructs and compared the values obtained with their IC₅₀ for the unfused adenosine A₁ receptor. We also studied the modulatory effects of 1 M NaCl and 10 μM PD81,723 on ligand binding to these A₁-G_iα fusion proteins. We could not convert the IC₅₀ values into K_i values, since we did not determine radioligand K_d values for all fusion products under all three conditions, i.e. without or in the presence of 1 M NaCl or PD81,723.

The results from radioligand binding experiments with the adenosine A₁ receptor agonist, CPA, are listed in Table 3.2. CPA had significantly higher affinities for five of the A₁-G_iα fusion proteins (³⁵¹Cys, ³⁵¹Gly, ³⁵¹Ile, ³⁵¹Ser, and ³⁵¹Val) than for the unfused adenosine A₁ receptor. In the presence of 1 M NaCl, CPA's affinity for the unfused adenosine A₁ receptor decreased three-fold. Contrarily, the A₁-G_iα fusion proteins did not seem to be affected very much by sodium ions, exhibiting no significant shifts in the presence of 1 M NaCl, except for ³⁵¹Arg and ³⁵¹Pro. In these two cases, the affinity of CPA decreased five- and eight-fold, respectively, and resembled the affinity of CPA for the unfused adenosine A₁ receptor in the presence of sodium ions (Table 3.2). Furthermore, in the presence of 10 μM PD81,723 all A₁-G_iα fusion proteins exhibited an increased affinity for CPA. This PD81,723-induced enhancement of CPA binding was also observed at unfused adenosine A₁ receptors, which had a four-fold higher affinity for CPA in the presence of 10 μM PD81,723. Yet,

under these conditions all nine A₁-G_iα fusion proteins had a higher affinity for CPA (13-76 nM) than the unfused adenosine A₁ receptor (111 nM, Table 3.2).

Table 3.2. IC₅₀ values (nM) for CPA binding to COS-7 cell homogenates transfected with the human adenosine A₁ receptor or different ³⁵¹Cys-mutated A₁-G_iα fusion proteins. The experiments were carried out in the presence or absence of 1 M NaCl or 10 μM PD81,723. Shifts were calculated by dividing the IC₅₀ (in the presence of modulator) by IC₅₀ (control). Data are expressed as means ± S.E.M. from at least three independent experiments performed in duplicate (n = 3-5).

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

Significant shift compared to unity, ^aP<0.005, ^bP<0.05, ^cP<0.001

The results from radioligand displacement studies with the adenosine A₁ receptor antagonist/inverse agonist DPCPX are presented in Table 3.3. The A₁-G_iα fusion proteins ³⁵¹Ile, ³⁵¹Ser, and ³⁵¹Val had slightly, but significantly lower affinities for DPCPX than the unfused adenosine A₁ receptor, while the other constructs showed statistically similar affinities for DPCPX. Moreover, sodium ions increased the affinity of DPCPX for the unfused adenosine A₁ receptor approximately two-fold. The binding of DPCPX to the A₁-G_iα fusion proteins ³⁵¹Phe, ³⁵¹Pro, ³⁵¹Arg, ³⁵¹Ser, and ³⁵¹Val also increased two- to threefold, while DPCPX binding to the other A₁-G_iα fusion proteins (³⁵¹Cys, ³⁵¹Gly, ³⁵¹Ile, and ³⁵¹His) was unaffected. In the presence of 10 μM PD81,723 the IC₅₀ value of DPCPX at A₁-G_iα fusion protein ³⁵¹Arg increased almost two-fold (p <0.05). However, the affinity of DPCPX for the other A₁-G_iα fusion

proteins, as well as for the unfused adenosine A₁ receptor, was not significantly changed in the presence of 10 μM PD81,723. Since we used 8-cyclopentyltheophylline (CPT) in our GTP S binding studies (see below), we also determined its affinity for one of the fusion proteins. CPT displayed an IC₅₀ value of 30 ± 12 nM on the A₁-G_i³⁵¹Ile product in the absence of any modulator, being sevenfold less active than DPCPX.

Table 3.3. IC₅₀ values (nM) for DPCPX binding to COS-7 cell homogenates transfected with the human adenosine A₁ receptor or different ³⁵¹Cys-mutated A₁-G_iα fusion proteins. The experiments were carried out in the presence or absence of 1 M NaCl or 10 μM PD81,723. Shifts were calculated by dividing the IC₅₀ (in the presence of modulator) by IC₅₀ (control). Data are expressed as means ± S.E.M. from at least three independent experiments performed in duplicate (n = 3-5).

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

Significant shift compared to unity, ^aP<0.05, ^bP<0.005

To study the functionality of the A₁-G_iα fusion proteins, we determined [³⁵S]GTPγS binding on membranes prepared from transfected COS-7 cells, expressing the A₁-G_iα fusion proteins of interest. In these studies, CPT, a more water-soluble analogue of DPCPX, was used as reference adenosine A₁ receptor inverse agonist in [³⁵S]GTPγS binding experiments. Figure 3.1 shows the results of [³⁵S]GTPγS binding to COS-7 membranes, expressing the wild-type A₁-G_iα fusion protein (³⁵¹Cys). The agonist CPA increased basal [³⁵S]GTPγS binding more than seven-fold, while CPT decreased [³⁵S]GTPγS binding to 52% of the basal level. To obtain a reasonable window to observe inhibition of basal [³⁵S]GTPγS binding by the adenosine A₁ receptor inverse agonist CPT, we used more protein (3 μg) than in experiments with the agonist CPA (1 μg of membrane protein).

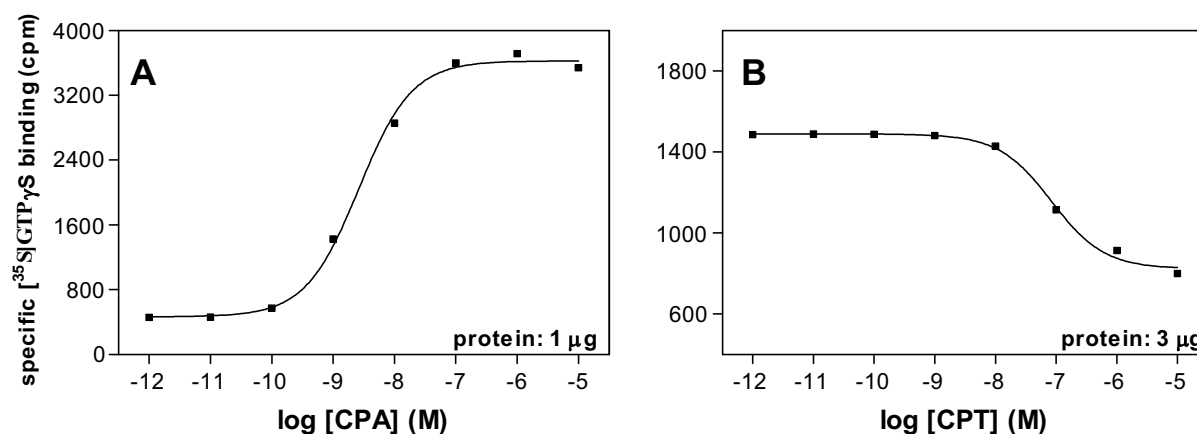


Figure 3.1. Stimulation by CPA (panel A) and inhibition by CPT (panel B) of basal [³⁵S]GTPγS binding in membranes prepared from COS-7 cells transfected with the A₁-G_iα fusion protein ³⁵¹Cys. Data are from a representative experiment performed in duplicate.

We performed similar experiments with COS-7 membranes expressing the unfused adenosine A₁ receptor (10 μg of protein) and the A₁-G_iα fusion proteins ³⁵¹Cys, ³⁵¹Gly, ³⁵¹Ile, ³⁵¹Pro and ³⁵¹Val (1-3 μg of protein). For the latter two A₁-G_iα fusion proteins, we tested only a single concentration of 1 μM CPT. It should be noted that less protein was needed in experiments with the A₁-G_iα fusion proteins to give similar basal [³⁵S]GTPγS binding. The results from these [³⁵S]GTPγS binding experiments are summarised in Table 3.4.

Table 3.4. Modulation by CPA or CPT of basal [³⁵S]GTP_γS binding to the unfused adenosine A₁ receptor and various A₁-G_iα fusion proteins (³⁵¹Cys, ³⁵¹Gly, ³⁵¹Ile, ³⁵¹Pro, and ³⁵¹Val). EC₅₀/IC₅₀ values are expressed with 95% confidence limits (n = 3). Effects of CPA and CPT are expressed as percentage of basal [³⁵S]GTP_γS binding. Basal [³⁵S]GTP_γS binding on membranes with the A₁-G_iα fusion proteins was ~600 cpm and ~1600 cpm in experiments with CPA and CPT, respectively. Basal [³⁵S]GTP_γS binding on membranes expressing the unfused adenosine A₁ receptor was ~800 cpm for both CPA and CPT.

construct	CPA		CPT	
	EC ₅₀ (nM)	effect (% of basal)	IC ₅₀ (nM)	effect (% of basal)
unfused	0.25 (0.15-0.43)	165	14 (7-27)	75
A ₁ -G _i ³⁵¹ Cys	3.74 (2.55-5.50)	744	91 (71-117)	52
A ₁ -G _i ³⁵¹ Gly	49.2 (36.8- 65.9)	580	148 (89-244)	84
A ₁ -G _i ³⁵¹ Ile	5.94 (4.40-8.01)	1014	89 (41-192)	51
A ₁ -G _i ³⁵¹ Ile+PTX	6.03 (5.71-6.36)	996	n.d.	49
A ₁ -G _i ³⁵¹ Pro	1.70 (1.56-1.86)	437	n.d.	76
A ₁ -G _i ³⁵¹ Val	9.03 (8.23-9.90)	967	n.d.	55

n.d. not determined

First, the extent of the CPA-induced stimulation of basal [³⁵S]GTP_γS binding differed for the various membrane preparations. Membranes expressing the unfused adenosine A₁ receptor showed only a modest effect of CPA, i.e. 165% stimulation over basal [³⁵S]GTP_γS binding, whereas the membrane preparations with the selected A₁-G_iα fusion proteins all had larger CPA-induced increases in basal [³⁵S]GTP_γS binding (Table 3.4). Similar observations were made when the inverse agonist, CPT, was present. In all cases, CPT decreased basal [³⁵S]GTP_γS binding, and to a different extent depending on the A₁-G_iα membrane preparation used. The CPT-induced decrease of basal [³⁵S]GTP_γS binding was most prominent in experiments with COS-7 cell membranes expressing A₁-G_iα fusion protein ³⁵¹Cys or ³⁵¹Ile, i.e. a reduction to 52 and 51% (49% in the presence of PTX) of the basal levels, respectively. On membranes with the A₁-G_iα fusion protein ³⁵¹Val, CPT lowered basal [³⁵S]GTP_γS binding to 55% of control values. Finally, the inhibitory effect of CPT on basal [³⁵S]GTP_γS binding measured at the A₁-G_iα fusion proteins, ³⁵¹Gly and ³⁵¹Pro, or at unfused adenosine A₁ receptors, was less pronounced (Table 3.4).

Discussion

In 1994, the first fusion protein between the β_2 -adrenoceptor and its cognate G_s α -subunit was described². The authors showed that this fusion protein (β_2 - $G_s\alpha$), when expressed in S49 cyc⁻ cells, was functional in both ligand binding experiments and cAMP determinations. Since then, fusion proteins have been engineered between various receptors (e.g. α_{2A} -adrenoceptor, adenosine A_1 , serotonin 5-HT_{1A} receptor) and G proteins (e.g. $G_{i1}\alpha$, $G_o\alpha$) (for review see Milligan)¹⁵. Because of the physical connection between receptor and G α -subunit, fusion proteins may resemble the precoupled receptor-G protein complex (RG and/or R*G) (Lefkowitz et al., and references therein)^{16,17}. These R- G_α fusion proteins may also enable the examination of receptor interactions in a system with a fixed R:G (1:1) ratio.

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

In the present and other studies, mutated G α -subunits were used. As demonstrated by Molinari et al. R- G_α fusion proteins might also couple to and activate endogenously expressed G proteins¹⁹. Treatment with pertussis toxin (PTX) assures that receptor activation arises solely through a mutated G α_i -subunit of the fused R-G construct, since the α -subunits of endogenously expressed G proteins are ADP-ribosylated by the toxin, and hence inactivated. Interestingly, we did not find significant differences in modulation of [³⁵S]GTP γ S binding between PTX-treated and untreated membranes of COS-7 cells transfected with A_1 - $G_{i\alpha}$ (³⁵¹Ile) fusion proteins (Table 3.4). In our hands, it thus seemed that the fusion proteins hardly activated endogenously expressed G α -subunits, if at all. Therefore, we did not use PTX-treated membranes in our further experiments.

Saturation experiments with the radiolabelled antagonist/inverse agonist [³H]DPCPX taught us that its affinity was similar for the fusion proteins and the adenosine A_1 receptor itself (see Table 3.1 and text in Results section). Consequently, we found no significant differences in the IC₅₀ values of DPCPX determined in radioligand binding displacement studies with COS-7 membranes expressing the various A_1 - $G_{i\alpha}$ fusion proteins (control column in Table 3.3). On the other hand, the affinity of CPA was higher for most A_1 - $G_{i\alpha}$ fusion proteins compared to the unfused adenosine A_1

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

After this characterization of the fusion proteins under control conditions we next examined allosteric modulation of ligand binding to the mutated A₁-G_iα fusion proteins by sodium ions and PD81,723, as our first objective of this study.

In the presence of 1 M NaCl, CPA's affinity for the unfused adenosine A₁ receptor decreased three-fold (Table 3.2). Sodium ions act through an aspartate residue in transmembrane helix II, which is highly conserved in GPCRs²⁰. Their interaction with this aspartate residue, Asp⁵⁵ in the human adenosine A₁ receptor, presumably induces a change in receptor conformation towards the inactive R state. Moreover, mutation of this residue into an asparagine (Asp⁵⁵Asn) diminished the sodium-induced decrease in agonist affinity²¹. In the latter study it was also shown that concentrations of NaCl lower than 1 M also affect agonist binding, although less outspoken. Remarkably, the affinity of CPA for most of the A₁-G_iα fusion proteins was unaffected by sodium ions. Although the adenosine A₁ receptor was not mutated in the A₁-G_iα fusion proteins, sodium ions appeared unable to elicit their effect. It may be that the physical connection between receptor and G_iα-subunit causes a conformational change, which, in turn, prevents the sodium ions from binding to the adenosine A₁ receptor. Alternatively, sodium ions may promote the formation of an inactive state (R) of the receptor, which cannot or only partly be achieved in receptors already fused with a G protein¹⁷. The latter explanation may make more sense, since the same concentration of NaCl did affect DPCPX binding at the wild-type receptor and at many of the fusion proteins (Table 3.1 and Table 3.3). Apparently, the binding of the antagonist/inverse agonist DPCPX remains sensitive to sodium ions. Surprisingly, sodium ions were able to decrease CPA binding at ³⁵¹Pro and ³⁵¹Arg A₁-G_iα fusion proteins (Table 3.2). Although speculative, the distinct characteristics of proline (ring structure) and arginine (positive charge) residues may have more profound effects on the conformation of the G_iα-subunit, and of the fusion protein as a whole.

The effects of 10 μM PD81,723 on ligand binding were rather similar for both the unfused adenosine A₁ receptor and the different A₁-G_iα fusion proteins. In the presence of PD81,723, CPA's affinity for the unfused receptor increased four-fold. The allosteric modulator increased the affinity of CPA for the various A₁-G_iα fusion proteins to a varying extent, namely two- to six-fold (Table 3.2). It should be noted here that in the presence of 10 μM PD81,723, the affinity of CPA for the unfused

receptor was lower compared to its affinity for the different A₁-G_iα fusion proteins under similar conditions. Thus, PD81,723 is still able to enhance CPA's binding to the adenosine A₁ receptor when the G_iα-subunit is physically linked to it. The molecular mechanism of action for PD81,723 has not been resolved yet, but these findings suggest that PD81,723 has a direct effect on the receptor itself and, subsequently, shifts the receptor equilibrium towards a more activated form (R*) of the receptor¹⁷. The binding of DPCPX (Table 3.3) was not very much affected by PD81,723, suggesting that the antagonist/inverse agonist binding is relatively insensitive to the presence of an allosteric enhancer of agonist binding.

The second objective of this research was to investigate whether these A₁-G_iα fusion proteins may be used to detect inverse agonism at adenosine A₁ receptors more easily. To observe inverse agonistic activity of e.g., DPCPX, a sufficient basal receptor activity is required¹¹. Possibilities to increase basal receptor activation include receptor overexpression¹⁸, or the design of constitutively active mutant receptors²². Assuming that these A₁-G_iα fusion proteins resemble a 'precoupled' and, therefore, an active receptor conformation, we reasoned that basal receptor activity of A₁-G_iα fusion proteins should be higher than the basal activity of unfused adenosine receptors. We indeed found far higher basal [³⁵S]GTPγS binding for all A₁-G_iα fusion proteins tested in [³⁵S]GTPγS binding experiments. Despite lower receptor densities (see Table 3.1 and text in Results section), membranes expressing A₁-G_iα fusion proteins showed more than seven-fold higher basal [³⁵S]GTPγS binding than that found in membranes with the unfused receptor, approx. 600 and 80 cpm/μg of protein, respectively. Moreover, for all A₁-G_iα fusion proteins tested, the maximal stimulation of basal [³⁵S]GTPγS binding was significantly larger than the maximal CPA-induced effect with unfused adenosine A₁ receptors (Table 3.4). For instance CPA caused a 14-fold increase [(1014%-100%)/(165%-100%)] in stimulation on the ³⁵¹Ile fusion protein compared to the unfused receptor. The same was observed for inhibition of basal [³⁵S]GTPγS binding by the adenosine A₁ receptor inverse agonist CPT. This compound decreased basal [³⁵S]GTPγS binding to the ³⁵¹Ile and ³⁵¹Cys fusion proteins very effectively (-49 and -48% vs. -25% for the unfused receptor expressed in COS-7 cells). Differences in absolute numbers are also substantial. We used up to 10-fold less protein when studying the fusion constructs, implicitly showing the enormous increase in "window".

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

proteins and the α_{2A} adrenoceptor, while we used A₁-G_iα fusion proteins. Here, we show that their conclusions regarding the correlation mentioned above were also applicable to our mutated A₁-G_iα fusion proteins.

Conclusions

In the present study we analysed fusion proteins between the adenosine A₁ receptor and (mutated) G protein α-subunits with respect to two novel concepts in receptor pharmacology, i.e. allosteric modulation and constitutive activity.

Allosteric modulation of ligand binding was not always similar for A₁-G_iα fusion proteins compared to the unfused adenosine A₁ receptor. In most cases, sodium ions were unable to decrease CPA binding to membranes expressing A₁-G_iα fusion proteins. Their lack of effect may result from a change in receptor conformation induced by the coupling of the G_iα-subunit. In contrast, allosteric modulation by PD81,723 was comparable for the unfused adenosine A₁ receptor and the A₁-G_iα fusion proteins. Thus, PD81,723 is able to shift the 'fusion' receptor equilibrium further towards R*G.

We also demonstrated that fusion proteins between the human adenosine A₁ receptor and various ³⁵¹Cys-mutated rat G_iα subunits can be used as a research tool to investigate inverse agonism at adenosine A₁ receptors. Despite lower receptor densities, basal [³⁵S]GTPγS binding to COS-7 membranes expressing A₁-G_iα fusion proteins was higher than in membranes with the unfused adenosine A₁ receptor. In addition, proper substitution of ³⁵¹Cys, for instance into an isoleucine residue, increased the readout window of CPA-stimulated [³⁵S]GTPγS binding substantially. The same fusion protein (³⁵¹Ile A₁-G_iα) also showed increased inhibition of basal [³⁵S]GTPγS binding induced by CPT.

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

References

1. Fredholm BB, IJzerman AP, Jacobson KA, Klotz KN, Linden J. (2001) International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol. Rev.* 53:527-552.
2. Bertin B, Freissmuth M, Jockers R, Strosberg AD, Marullo. (1994) Cellular signaling by an agonist-activated receptor/ G_s α fusion protein. *Proc. Natl. Acad. Sci. USA* 91:8827-8831.
3. Waldhoer M, Wise A, Milligan G, Freissmuth M, Nanoff C. (1999) Kinetics of ternary complex formation with fusion proteins composed of the A_1 -adenosine receptor and G protein α -subunits. *J. Biol. Chem.* 274:30571-30579.
4. Milligan G. (1988) Techniques used in the identification and analysis of function of pertussis toxin-sensitive guanine nucleotide binding proteins. *Biochem. J.* 255:1-13.
5. Wise A, Sheehan M, Rees S, Lee M, Milligan G. (1999) Comparative analysis of the efficacy of A_1 adenosine receptor activation of $G_{i/o}$ G proteins following coexpression of receptor and G protein and expression of A_1 adenosine receptor - $G_{i/o}$ fusion proteins. *Biochemistry* 38:2272-2278.
6. Kudlacek O, Waldhoer M, Kassack MU, Nickel P, Salmi JAI, Freissmuth M, Nanoff C. (2002) Biased inhibition by a suramin analogue of A_1 -adenosine receptor/G protein coupling in fused receptor/G protein tandems: the A_1 -adenosine receptor is predominantly coupled to G_o in human brain. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 365:8-16.
7. Bevan N, Palmer T, Drmota T, Wise A, Coote J, Milligan G, Rees S. (1999) Functional analysis of a human A_1 adenosine receptor/green fluorescent protein/ $G_{i1}\alpha$ fusion protein following stable expression in CHO cells. *FEBS Lett.* 462:61-65.
8. Van der Klein PA, Kourounakis AP, IJzerman AP. (1999) Allosteric modulation of the adenosine A_1 receptor. Synthesis and biological evaluation of novel 2-amino-3-benzoylthiophenes as allosteric enhancers of agonist binding. *J. Med. Chem.* 42:3629-3635.
9. Gao ZG, IJzerman AP. (2000) Allosteric modulation of A_{2A} adenosine receptors by amiloride analogues and sodium ions. *Biochem. Pharmacol.* 60:669-676.
10. Kourounakis A, Visser C, De Groote M, IJzerman AP. (2001) Differential effects of the allosteric enhancer (2-amino-4,5-dimethyl-trienyl)[3-(trifluoromethyl) phenyl]methanone (PD81,723) on agonist and antagonist binding and function at the human wild-type and a mutant (T277A) adenosine A_1 receptor. *Biochem. Pharmacol.* 61:137-144.
11. De Ligt RAF, Kourounakis AP, IJzerman AP. (2000) Inverse agonism at G protein-coupled receptors: (patho)physiological relevance and implications for drug discovery. *Br. J. Pharmacol.* 130:1-12.
12. Leurs R, Smit MJ, Menge WMBP, Timmerman H. (1994) Pharmacological characterization of the human histamine H_2 receptor stably expressed in chinese hamster ovary cells. *Br J Pharmacol.* 112:847-854.
13. Brakenhoff RH, Knippels EM, Van Dongen GA. (1994) Optimization and simplification of expression cloning in eukaryotic vector/host systems. *Anal. Biochem.* 218:460-463.
14. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. (1985). Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 150:76-85.
15. Milligan G. (2000) Insights into ligand pharmacology using receptor-G-protein fusion proteins. *Trends Pharmacol. Sci.* 21:24-28.
16. Lefkowitz RJ, Cotecchia S, Samama P, Costa T. (1993). Constitutive activity of receptors coupled to guanine nucleotide regulatory proteins. *Trends Pharmacol. Sci.* 14:303-307.
17. Leff P. (1995) The two-state model of receptor activation. *Trends Pharmacol. Sci.* 16:89-97.
18. Shryock JC, Ozeck MJ, Belardinelli L. (1998). Inverse agonists and neutral antagonists of recombinant human A_1 adenosine receptors stably expressed in Chinese hamster ovary cells. *Mol. Pharmacol.* 53:886-893.
19. Molinari R, Ambrosio C, Riitano D, Sbraccia M, Grò MD, Costa T. (2003). Promiscuous coupling at receptor-G α fusion proteins: the receptor of one covalent complex interacts with the α -subunit of another. *J. Biol. Chem.* 278:15778-15788.
20. Horstman DA, Brandon S, Wilson AL, Guyer CA, Cragoe EJ, Jr., Limbird LE. (1990) An aspartate conserved among G protein receptors confers allosteric regulation of α_2 -adrenergic receptors by sodium. *J. Biol. Chem.* 265:21590-21595.
21. Barbhaiya H, McClain R, IJzerman AP, Rivkees SA. (1996) Site-directed mutagenesis of the human A_1 adenosine receptor: influences of acidic and hydroxy residues in the first four transmembrane domains on ligand binding. *Mol. Pharmacol.* 50:1635-1642.

22. Samama P, Cotecchia S, Costa T, Lefkowitz RJ. (1993) A mutation-induced activated state of the β_2 -adrenergic receptor. Extending the ternary complex model. *J. Biol. Chem.* 268:4625-4636.
23. Bahia DS, Wise A, Fanelli F, Lee M, Rees S, Milligan G. (1998). Hydrophobicity of residue 351 of the G protein G_{i1} α determines the extent of activation by the α_{2A} -adrenoceptor. *Biochemistry* 37:11555-11562.

