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

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Abstract

We studied fusion proteins between the human adenosine A_1 receptor and different ^{351}Cys-mutated G_{i1} α-subunits (A_1–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 nine different A_1–G_iα fusion proteins in radioligand binding studies. In addition, we performed [^{35}S]GTPγS binding experiments to study receptor and G protein activation of selected A_1–G_iα fusion proteins. Compared to unfused adenosine A_1 receptors, the affinity of N^6-cyclopentyladenosine (CPA) at wild-type A_1–G_iα fusion proteins (^{351}Cys) increased more than eightfold, while the affinity of 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) did not change significantly. Furthermore, we showed that the allosteric enhancer of agonist binding, PD81,723 (2-amino-4,5-dimethyl-3-thienyl-[3-(trifluoromethyl)-phenyl]methanone), elicited similar effects on ligand binding; i.e. CPA binding to the A_1–G_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 A_1–G_iα fusion proteins, presumably because they exhibit their effect through uncoupling of the R–G complex. From [^{35}S]GTPγS binding experiments, we learned that all the A_1–G_iα fusion proteins tested had a higher basal receptor activity than the unfused adenosine A_1 receptor, thereby providing improved conditions to observe inverse agonism. Moreover, the maximal CPA-induced stimulation of basal [^{35}S]GTPγS binding was increased for the five A_1–G_iα fusion proteins tested, whereas the inhibition induced by 8-cyclopentyltheophylline (CPT) was more pronounced at ^{351}Cys, ^{351}Ile, and ^{351}Val A_1–G_iα fusion proteins. Thus, the maximal receptor (de)activation depended on the amino acid at position 351 of the G_i α-subunit. In conclusion, A_1–G_iα fusion proteins, especially with ^{351}Cys and ^{351}Ile, can be used as research tools to investigate inverse agonism, due to their increased readout window in [^{35}S]GTPγS binding experiments.

Keywords: Adenosine A_1 receptor; G protein; Fusion protein; Allosteric modulation; Constitutive activity; Inverse agonism

1. Introduction

Adenosine A_1 receptors belong to the superfamily of G-protein-coupled receptors (GCPRs). They preferentially interact with G_i and G_o proteins (Fredholm et al., 2001). Various strategies have been employed to study the interaction between receptor and G protein. One method is

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the construction of so-called fusion proteins, in which both signalling partners, GPCR and G α-subunit, are physically linked to each other. In 1994, the first fusion protein (between β_2-adrenoceptor and α_s-subunit) was described and found to be functional in both ligand binding experiments and cAMP determinations (Bertin et al. 1994).

The same strategy has been applied to adenosine A_1 receptors. Waldhoer et al. (1999) analysed the kinetic behaviour of the adenosine A_1 receptor linked to both α_i- and α_o-subunits. The authors also introduced a fusion protein in which the α_i-subunit (of rat α_{i1}) was mutated at position ^{351}Cys. This cysteine residue is known to be ADP-ribosylated by pertussis toxin (PTX; Milligan, 1988); changing it for another amino acid (glycine or isoleucine in this case) renders the fusion protein insensitive to the toxin. 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 (Wise et al., 1999) and G protein inhibitors such as suramin and derivatives (Kudlacek et al., 2002). Curiously, Bevan et al. (1999) introduced a "trivalent" fusion protein between adenosine A_1 receptor, green fluorescent protein, and α_i-subunit, which behaved very much as the wild-type receptor in terms of ligand binding and G protein activation.

In the present study, we performed a further analysis of such fusion proteins with two aims that have not yet been subject of investigation. Firstly, we wondered whether the constructs can still be influenced by allosteric modulators. In our laboratory, we have been investigating allosteric modulation of adenosine receptors by, e.g. sodium ions and PD81,723 (Van der Klein et al., 1999; Gao and IJzerman, 2000; Kourounakis et al., 2001).

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 Ligt et al., 2000). 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.

2. Materials and methods

2.1. Chemicals

CPA, DPCPX, CPT, and PD81,723 were purchased from Research Biochemicals (Natick, USA). Adenosine deaminase (ADA), diethylaminoethyl (DEAE) dextran, chloroquine, and dithiothreitol were purchased from Sigma, while EDTA, $MgCl_2$, GDP, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate (CHAPS), and GTPγS were obtained from Boehringer (Mannheim, Germany). [^{3}H]DPCPX (120 Ci/mmol), [^{3}H]CCPA (2-chloro-N^6-cyclopentyldenosine, 55 Ci/mmol), and [^{3}H]cAMP (25 Ci/mmol) were purchased from NEN (DuPont Nemours, 's-Hertogenbosch, NL). [^{35}S]GTPγS (1250 Ci/mmol) was obtained from NEN (Cologne, Germany). BSA and BCA protein assay reagent were purchased from Pierce Chemical Company (Rockford, IL, USA). 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. (1994). Except for foetal calf serum (FCS, Greiner, Netherlands), all cell culture materials were taken from laboratory stocks. All other chemicals were obtained from standard sources, and were of the highest purity commercially available. The cDNAs encoding either the unfused adenosine A_1 receptor or a fusion protein between the human adenosine A_1 receptor and rat G_{i1} α-subunit were produced by one of us (G.M.). Full fusion constructs were excised from pCR-Script with *Eco*RI and *Xho*I and ligated into the mammalian expression vector pcDNA3 according to the instructions supplied by the vendor (Invitrogen, San Diego, USA).

2.2. Cell culture and transfection

African green monkey kidney (COS-7) cells were maintained at 37 °C in a humidified atmosphere with 7% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% FCS, penicillin (50 IU/ml), and streptomycin (50 μg/ml). COS-7 cells were transiently transfected with either the unfused adenosine A_1 receptor, or a fusion protein (A_1–$G_i\alpha$) using a slightly modified protocol of the 'in suspension' DEAE dextran method previously described by Brakenhoff et al. (1994). In short, COS-7 cells were subcultured 1 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 μg/10^6 cells) and DEAE dextran (400 μg/ml) were mixed in a total volume of 4 ml RSC, and incubated at room temperature for 2 min. 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 $1000\times g$ for 5 min, resuspended in normal growth medium, and seeded in the appropriate plates.

2.3. Membrane preparation

After 48 h, transiently transfected COS-7 cells were harvested with a cell scraper, and recovered by a 5-min centrifugation at $1000\times g$. Cells were then homogenised in ice-cold 50 mM Tris–HCl buffer (pH 7.4) with a polytron homogeniser (5 s, speed 8), and used for radioligand binding studies. To measure [^{35}S]GTPγS binding, the cell homogenates were purified with two additional centrifuga-

tion steps at 4 °C: a 10-min centrifugation at 1000×*g* and the obtained supernatant for 30 min at 60,000×*g* (Beckman L8-50 M/E Ultracentrifuge). The final pellet was resuspended in 3 ml ice-cold 50 mM Tris–HCl buffer (pH 7.4), supplemented with ADA (2 U/ml). Protein concentrations were measured with the bicinchoninic acid method with BSA as a standard (Smith et al. 1985).

2.4. Radioligand binding studies

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

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

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

2.5. [^{35}S]GTPγS binding

[^{35}S]GTPγS binding was measured in 100 μl containing 50 mM Tris–HCl (pH 7.4), 1 mM EDTA, 5 mM $MgCl_2$, 10 μM GDP, 1 mM dithiothreitol, 100 mM NaCl, 5 U/ml ADA, 0.3 nM [^{35}S]GTPγS (~50,000 cpm), 0.004 % CHAPS, and 0.5 % BSA. Incubations were started by addition of the membrane suspension (1–3 μg protein/tube) to the test tubes, and carried out in duplicate for 90 min at 25 °C. They were stopped by rapid filtration through Whatman GF/B filters, pre-soaked in 50 mM Tris–HCl, 5 mM $MgCl_2$ (pH 7.4) containing 0.02 % CHAPS. The filters were washed twice with 4 ml of the buffer mentioned before, and retained radioactivity was measured using liquid scintillation counting. Non-specific binding of [^{35}S]GTPγS was measured in the presence of 10 μM unlabelled GTPγS, and subtracted from total bound radioactivity.

2.6. Data and statistical analysis

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

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

3. Results

First, we established B_{max} and K_d values in saturation experiments with [^{3}H]DPCPX on COS-7 membranes expressing either the adenosine A_1 receptor alone or fusion proteins (A_1–$G_i\alpha$) between the adenosine A_1 receptor and three (mutated) $G_i\alpha$ proteins (^{351}Cys (=wild type), ^{351}Gly, and ^{351}Ile). Saturation experiments were performed both in the absence and presence of 1 M NaCl (Table 1).

The affinity of [^{3}H]DPCPX for these A_1–$G_i\alpha$ fusion proteins was similar to its affinity for the unfused adenosine A_1 receptor. Moreover, the A_1–$G_i\alpha$ fusion proteins were

Table 1
K_d and B_{max} values from saturation experiments with [^{3}H]DPCPX on COS-7 cell homogenates transfected with the human adenosine A_1 receptor or various (mutated) A_1–$G_i\alpha$ fusion proteins (^{351}Cys (=wild type $G_i\alpha$), ^{351}Gly, and ^{351}Ile)

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

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.

[a] n=2.

[b] p<0.005, significant difference versus control.

[c] p<0.001, significant difference versus control.

expressed at similar receptor densities, which were lower than those for the adenosine A_1 receptor alone. The other A_1–$G_i\alpha$ fusion proteins (^{351}Phe, ^{351}His, ^{351}Pro, ^{351}Arg, ^{351}Ser, and ^{351}Val) were tested in a single saturation experiment with [^{3}H]DPCPX in the absence of sodium ions. They showed K_d values in a similar range as observed for the adenosine A_1 receptor alone (1.2–2.1 nM), while receptor expression varied to some extent (0.4–1.0 pmol/mg of protein).

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

The results from radioligand binding experiments with the adenosine A_1 receptor agonist, CPA, are listed in Table 2. CPA had significantly higher affinities for five of the A_1–$G_i\alpha$ fusion proteins (^{351}Cys, ^{351}Gly, ^{351}Ile, ^{351}Ser, and ^{351}Val) than for the unfused adenosine A_1 receptor. In the presence of 1 M NaCl, CPA's affinity for the unfused adenosine A_1 receptor decreased threefold. Contrarily, the A_1–$G_i\alpha$ fusion proteins did not seem to be affected very much by sodium ions, exhibiting no significant shifts in the presence of 1 M NaCl, except for ^{351}Arg and ^{351}Pro. In these two cases, the affinity of CPA decreased five- and eightfold, respectively, and resembled the affinity of CPA for the unfused adenosine A_1 receptor in the presence of sodium ions (Table 2). Furthermore, in the presence of 10 μM PD81,723, all A_1–$G_i\alpha$ fusion proteins exhibited an increased affinity for CPA. This PD81,723-induced enhancement of CPA binding was also observed at unfused adenosine A_1 receptors, which had a fourfold higher affinity for CPA in the presence of 10 μM PD81,723. Yet, under these conditions, all nine A_1–$G_i\alpha$ fusion proteins had a higher affinity for CPA (13–76 nM) than the unfused adenosine A_1 receptor (111 nM, Table 2).

Table 2
IC_{50} values (nM) for CPA binding to COS-7 cell homogenates transfected with the human adenosine A_1 receptor or different ^{351}Cys-mutated A_1–$G_i\alpha$ fusion proteins

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–G_i ^{351}Ser	135±23	121±33	0.90	22±7	0.19[a]
A_1–Gi ^{351}Val	87±21	84±5	0.97	16±4	0.18[a]

The experiments were carried out in the presence or absence of 1 M NaCl or 10 μM PD81,723. Shifts were calculated by dividing the IC_{50} (in the presence of modulator) by IC_{50} (control). Data are expressed as means± S.E.M. from at least three independent experiments performed in duplicate (n=3–5).

[a] Significant shift compared to unity, $p<0.005$.
[b] Significant shift compared to unity, $p<0.05$.
[c] Significant shift compared to unity, $p<0.001$.

Table 3
IC_{50} values (nM) for DPCPX binding to COS-7 cell homogenates transfected with the human adenosine A_1 receptor or different ^{351}Cys-mutated A_1–$G_i\alpha$ fusion proteins

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–Gi ^{351}Cys	4.5±0.92	5.1±0.44	1.13	8.8±2.4	1.96
A_1–Gi ^{351}Gly	4.6±0.94	3.3±0.37	0.72	5.4±0.64	1.17
A_1–Gi ^{351}Ile	4.5±0.66	4.6±1.12	1.02	3.3±1.1	0.73
A_1–Gi ^{351}Phe	3.7±0.80	1.7±0.34	0.46[a]	5.1±0.53	1.38
A_1–Gi ^{351}His	2.3±0.58	1.4±0.14	0.61	4.1±0.72	1.78
A_1–Gi ^{351}Pro	4.3±0.95	1.4±0.17	0.33[a]	4.4±0.45	1.02
A_1–Gi ^{351}Arg	3.6±0.31	1.3±0.21	0.36[b]	6.1±0.99	1.69[a]
A_1–G_i ^{351}Ser	6.4±0.69	1.8±0.07	0.28[b]	6.0±0.29	0.94
A_1–Gi ^{351}Val	4.7±0.74	1.5±0.21	0.32[b]	7.9±1.8	1.68

The experiments were carried out in the presence or absence of 1 M NaCl or 10 μM PD81,723. Shifts were calculated by dividing the IC_{50} (in the presence of modulator) by IC_{50} (control). Data are expressed as means± S.E.M. from at least three independent experiments performed in duplicate (n=3–5).

[a] Significant shift compared to unity, $p<0.05$.
[b] Significant shift compared to unity, $p<0.005$.

The results from radioligand displacement studies with the adenosine A_1 receptor antagonist/inverse agonist DPCPX are presented in Table 3. The A_1–$G_i\alpha$ fusion proteins ^{351}Ile, ^{351}Ser, and ^{351}Val had slightly, but significantly lower affinities for DPCPX than the unfused adenosine A_1 receptor, while the other constructs showed statistically similar affinities for DPCPX. Moreover, sodium ions increased the affinity of DPCPX for the unfused adenosine A_1 receptor approximately twofold. The binding of DPCPX to the A_1–$G_i\alpha$ fusion proteins ^{351}Phe, ^{351}Pro, ^{351}Arg, ^{351}Ser, and ^{351}Val also increased two- to threefold, while DPCPX binding to the other A_1–$G_i\alpha$ fusion proteins (^{351}Cys, ^{351}Gly, ^{351}Ile, and ^{351}His) was unaffected. In the presence of 10 μM PD81,723, the IC_{50} value of DPCPX at A_1–$G_i\alpha$ fusion protein ^{351}Arg increased almost twofold ($p<0.05$). However, the affinity of DPCPX for the other A_1–$G_i\alpha$ fusion proteins, as well as for the unfused adenosine A_1 receptor, was not significantly changed in the presence of 10 μM PD81,723. Since we used 8-cyclopentyltheophylline (CPT) in our GTPγS binding studies (see below), we also determined its affinity for one of the fusion proteins. CPT displayed an IC_{50} value of 30±12 nM on the A_1–G_i ^{351}Ile product in the absence of any modulator, being sevenfold less active than DPCPX.

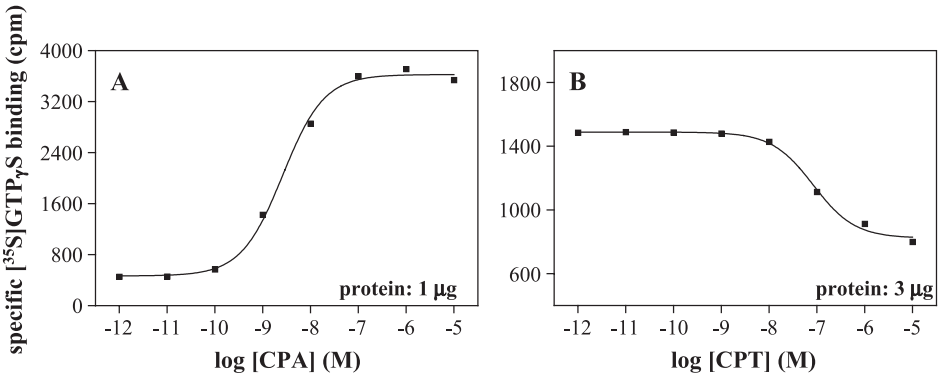

Fig. 1. Stimulation by CPA (panel A) and inhibition by CPT (panel B) of basal [^{35}S]GTPγS binding in membranes prepared from COS-7 cells transfected with the A_1–$G_i\alpha$ fusion protein ^{351}Cys. Data are from a representative experiment performed in duplicate.

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

We performed similar experiments with COS-7 membranes expressing the unfused adenosine A_1 receptor (10 µg of protein) and the A_1–$G_i\alpha$ fusion proteins ^{351}Cys, ^{351}Gly, ^{351}Ile, ^{351}Pro and ^{351}Val (1–3 µg of protein). For the latter two A_1–$G_i\alpha$ fusion proteins, we tested only a single concentration of 1 µM CPT. It should be noted that less protein was needed in experiments with the A_1–$G_i\alpha$ fusion proteins to give similar basal [^{35}S]GTPγS binding. The results from these [^{35}S]GTPγS binding experiments are summarised in Table 4.

First, the extent of the CPA-induced stimulation of basal [^{35}S]GTPγS binding differed for the various membrane preparations. Membranes expressing the unfused adenosine A_1 receptor showed only a modest effect of CPA, i.e. 165 % stimulation over basal [^{35}S]GTPγS binding, whereas the membrane preparations with the selected A_1–$G_i\alpha$ fusion proteins all had larger CPA-induced increases in basal [^{35}S]GTPγS binding (Table 4).

Similar observations were made when the inverse agonist, CPT, was present. In all cases, CPT decreased basal [^{35}S]GTPγS binding, and to a different extent depending on the A_1–$G_i\alpha$ membrane preparation used. The CPT-induced decrease of basal [^{35}S]GTPγS binding was most prominent in experiments with COS-7 cell membranes expressing A_1–$G_i\alpha$ fusion protein ^{351}Cys or ^{351}Ile, i.e. a reduction to 52% and 51% (49% in the presence of PTX) of the basal levels, respectively. On membranes with the A_1–$G_i\alpha$ fusion protein ^{351}Val, CPT lowered basal [^{35}S]GTPγS binding to 55% of control values. Finally, the inhibitory effect of CPT on basal [^{35}S]GTPγS binding measured at the A_1–$G_i\alpha$ fusion proteins, ^{351}Gly and ^{351}Pro, or at unfused adenosine A_1 receptors, was less pronounced (Table 4).

Table 4
Modulation by CPA or CPT of basal [^{35}S]GTPγS binding to the unfused adenosine A_1 receptor and various A_1–$G_i\alpha$ fusion proteins (^{351}Cys, ^{351}Gly, ^{351}Ile, ^{351}Pro, and ^{351}Val)

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

EC_{50}/IC_{50} values are expressed with 95% confidence limits (n=3). Effects of CPA and CPT are expressed as percentage of basal [^{35}S]GTPγS binding. Basal [^{35}S]GTPγS binding on membranes with the A_1–$G_i\alpha$ fusion proteins was ~600 and ~1600 cpm in experiments with CPA and CPT, respectively. Basal [^{35}S]GTPγS binding on membranes expressing the unfused adenosine A_1 receptor was ~800 cpm for both CPA and CPT.
n.d.=not determined.

4. Discussion

In 1994, the first fusion protein between the β_2-adrenoceptor and its cognate G_s α-subunit was described (Bertin et al., 1994). 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, 2000). 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., 1993 and references therein; Leff, 1995). 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 (PD 81,723) or inhibited (e.g. by sodium ions) in an allosteric manner (Van der Klein et al., 1999; Kourounakis et al., 2001; and references therein). 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" (Shryock et al., 1998). 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. (2003), R–G_α fusion proteins might also couple to and activate endogenously expressed G proteins. Treatment with pertussis toxin (PTX) assures that receptor activation arises solely through a mutated G α_i-subunit of the fused R–G construct, since the α-subunits of endogenously expressed G proteins are ADP-ribosylated by the toxin, and hence inactivated. Interestingly, we did not find significant differences in modulation of [^{35}S]GTPγS binding between PTX-treated and untreated membranes of COS-7 cells transfected with A_1–$G_i\alpha$ (^{351}Ile) fusion proteins (Table 4). In our hands, it thus seemed that the fusion proteins hardly activated endogenously expressed G α-subunits, if at all. Therefore, we did not use PTX-treated membranes in our further experiments.

Saturation experiments with the radiolabelled antagonist/inverse agonist [^{3}H]DPCPX taught us that its affinity was similar for the fusion proteins and the adenosine A_1 receptor itself (see Table 1 and text in Results). Consequently, we found no significant differences in the IC_{50} values of DPCPX determined in radioligand binding displacement studies with COS-7 membranes expressing the various A_1–$G_i\alpha$ fusion proteins (control column in Table 3). 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 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 expression of endogenous G proteins in COS-7 cell membranes, such that a precoupled complex hardly occurs.

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

In the presence of 1 M NaCl, CPA's affinity for the unfused adenosine A_1 receptor decreased threefold (Table 2). Sodium ions act through an aspartate residue in transmembrane helix II, which is highly conserved in GPCRs (Horstman et al., 1990). Their interaction with this aspartate residue, Asp^{55} in the human adenosine A_1 receptor, presumably induces a change in receptor conformation towards the inactive R state. Moreover, mutation of this residue into an asparagine ($Asp^{55}Asn$) diminished the sodium-induced decrease in agonist affinity (Barbhaiya et al., 1996). In the latter study, it was also shown that concentrations of NaCl lower than 1 M also affect agonist binding, although less outspoken. Remarkably, the affinity of CPA for most of the A_1–$G_i\alpha$ fusion proteins was unaffected by sodium ions. Although the adenosine A_1 receptor was not mutated in the A_1–$G_i\alpha$ fusion proteins, sodium ions appeared unable to elicit their effect. It may be that the physical connection between receptor and G_i α-subunit causes a conformational change, which, in turn, prevents the sodium ions from binding to the adenosine A_1 receptor. Alternatively, sodium ions may promote the formation of an inactive state (R) of the receptor (Leff, 1995), which cannot or only partly be achieved in receptors already fused with a G protein. 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 (Tables 1 and 3). Apparently, the binding of the antagonist/inverse agonist DPCPX remains sensitive to sodium ions. Surprisingly, sodium ions were able to decrease CPA binding at ^{351}Pro and ^{351}Arg A_1–$G_i\alpha$ fusion proteins (Table 2). Although speculative, the distinct characteristics of proline (ring structure) and arginine (positive charge) residues may have more profound effects on the conformation of the G_i α-subunit, and of the fusion protein as a whole.

The effects of 10 μM PD81,723 on ligand binding were rather similar for both the unfused adenosine A_1 receptor and the different A_1–$G_i\alpha$ fusion proteins. In the presence of PD81,723, CPA's affinity for the unfused receptor increased fourfold. The allosteric modulator increased the affinity of CPA for the various A_1–$G_i\alpha$ fusion proteins to a varying extent, namely two- to sixfold (Table 2). It should be noted

here that in the presence of 10 μM PD81,723, the affinity of CPA for the unfused receptor was lower compared to its affinity for the different A_1–$G_i\alpha$ fusion proteins under similar conditions. Thus, PD81,723 is still able to enhance CPA's binding to the adenosine A_1 receptor when the G_i α-subunit is physically linked to it. The molecular mechanism of action for PD81,723 has not been resolved yet, but these findings suggest that PD81,723 has a direct effect on the receptor itself and, subsequently, shifts the receptor equilibrium towards a more activated form (R*) of the receptor (Leff, 1995). The binding of DPCPX (Table 3) was not very much affected by PD81,723, suggesting that the antagonist/inverse agonist binding is relatively insensitive to the presence of an allosteric enhancer of agonist binding.

The second objective of this research was to investigate whether these A_1–$G_i\alpha$ fusion proteins may be used to detect inverse agonism at adenosine A_1 receptors more easily. To observe inverse agonistic activity of, e.g. DPCPX, a sufficient basal receptor activity is required (De Ligt et al., 2000). Possibilities to increase basal receptor activation include receptor overexpression (Shryock et al., 1998), or the design of constitutively active mutant receptors (Samama et al., 1993). Assuming that these A_1–$G_i\alpha$ fusion proteins resemble a 'precoupled' and, therefore, an active receptor conformation, we reasoned that basal receptor activity of A_1–$G_i\alpha$ fusion proteins should be higher than the basal activity of unfused adenosine receptors. We indeed found far higher basal [^{35}S]GTPγS binding for all A_1–$G_i\alpha$ fusion proteins tested in [^{35}S]GTPγS binding experiments. Despite lower receptor densities (see Table 1 and text in Results), membranes expressing A_1–$G_i\alpha$ fusion proteins showed more than sevenfold higher basal [^{35}S]GTPγS binding than that found in membranes with the unfused receptor, approximately 600 and 80 cpm/μg of protein, respectively. Moreover, for all A_1–$G_i\alpha$ fusion proteins tested, the maximal stimulation of basal [^{35}S]GTPγS binding was significantly larger than the maximal CPA-induced effect with unfused adenosine A_1 receptors (Table 4). For instance, CPA caused a 14-fold increase [(1014−100)/(165−100)] in stimulation on the ^{351}Ile fusion protein compared to the unfused receptor. The same was observed for inhibition of basal [^{35}S]GTPγS binding by the adenosine A_1 receptor inverse agonist CPT. This compound decreased basal [^{35}S]GTPγS binding to the ^{351}Ile and ^{351}Cys fusion proteins very effectively (−49/48% vs. −25% for the unfused receptor expressed in COS-7 cells). Differences in absolute numbers are also substantial. We used up to 10-fold less protein when studying the fusion constructs, implicitly showing the enormous increase in "window".

The maximal stimulation and inhibition of basal [^{35}S]GTPγS binding appeared to depend on the nature of the amino acid at position 351 of the G_i α-subunit (Ile>Val>Cys>Gly>Pro>unfused receptor, for agonist activation). These observations are in line with the report by Bahia et al. (1998). They found a good correlation between the hydrophobicity of the amino acid at this position and the maximal activation of the porcine α_{2A}-adrenoceptor. Note that the authors co-expressed the mutated $G_i\alpha$ proteins and the α_{2A}-adrenoceptor, while we used A_1–$G_i\alpha$ fusion proteins. Here, we show that their conclusions regarding the correlation mentioned above were also applicable to our mutated A_1–$G_i\alpha$ fusion proteins.

5. Conclusions

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

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

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

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

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