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Inverse agonism at adenosine A_1 receptors

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Abstract

Inverse agonism at adenosine A_1 receptors was studied in a variety of experimental set-ups. As a read-out, the binding of [^{35}S]GTPγS to membranes of either CHO or COS-7 cells expressing human adenosine A_1 receptors was used. When wild-type receptors were studied, inverse agonism could only be detected at higher levels of receptor expression. However, receptors fused with (mutated) α-subunits of G_i proteins facilitated the detection of inverse agonism, which could be studied in these systems at lower levels of expression. Ligands previously all classified as antagonists behaved differently, even within series of chemical homologues. For instance, in the xanthine class, theophylline and caffeine were neutral antagonists, whereas another xanthine, 1,3-dipropyl,8-cyclopentylxanthine (DPCPX), invariably emerged as a high-affinity inverse agonist. During our investigations, it became apparent that the neutral antagonists identified so far had all modest affinity for the receptor. Therefore, we started a program aimed at the design and synthesis of neutral antagonists with higher affinity. N-0840, N^6-cyclopentyl-9-methyladenine, another neutral antagonist, was used as a lead structure. We succeeded in synthesizing a new series of C8-substituted N-0840 derivatives, many of which had higher affinity than the parent compound. Interestingly, some of the compounds turned out to be inverse agonists, whereas others maintained their neutral antagonistic character.

Keywords: Inverse agonism; Adenosine A_1 receptors; [^{35}S]GTPγS binding; Caffeine; N-0840 derivatives

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1. Introduction

Adenosine receptors belong to the superfamily of G protein-coupled receptors, and are divided into four subtypes: A_1, A_{2A}, A_{2B}, and A_3 receptors. Adenosine A_1 receptors are widely distributed throughout the body with high receptor densities in the central nervous system and fat cells, and lower levels in heart and kidney. They are predominantly coupled to adenylate cyclase via G_i proteins [1].

In 1998, Shryock et al. [2] showed basal adenosine A_1 receptor activity in CHO cells with a high receptor density (~ 6 pmol/mg of protein). Furthermore, the authors identified both inverse agonists and neutral antagonists in [^{35}S]GTPγS binding experiments and cAMP assays on this cell system. Apparently, the adenosine A_1 receptor can be constitutively active upon receptor overexpression.

We used these findings as the starting point for our investigations. For the purpose of this chapter, we focussed on two aspects: (i) to detect and increase inverse agonism at adenosine A_1 receptors through genetic engineering, and (ii) to design and synthesize novel neutral antagonists and inverse agonists with high affinity for the adenosine A_1 receptor.

2. Materials and methods

2.1. Cell systems

In our assays, we used membranes from either CHO (stable receptor expression) or COS-7 cells (transient receptor expression). Two lines of CHO cells were used, one with 'normal' expression of human adenosine A_1 receptors (CHO-A_1: approx. 650 fmol/mg protein [3]), the other with higher expression (CHO-A_1^{++}: approx. 3.4 pmol/mg protein [4]). COS-7 cells were transiently transfected with either the unfused adenosine A_1 receptor, or a fusion protein (A_1–$G_i\alpha$). Cells were grown and harvested under standard conditions [5], and membranes prepared for radioligand binding and [^{35}S]GTPγS activation studies. CHO-A_1 cell membranes were prepared as described previously [5]. Membranes prepared from CHO-A_1^{++} cells were purified by an additional 5-min centrifugation at $1000 \times g$ and 4 °C, before the 30-min centrifugation at $60{,}000 \times g$. Membrane protein concentration was measured with the BCA method [6].

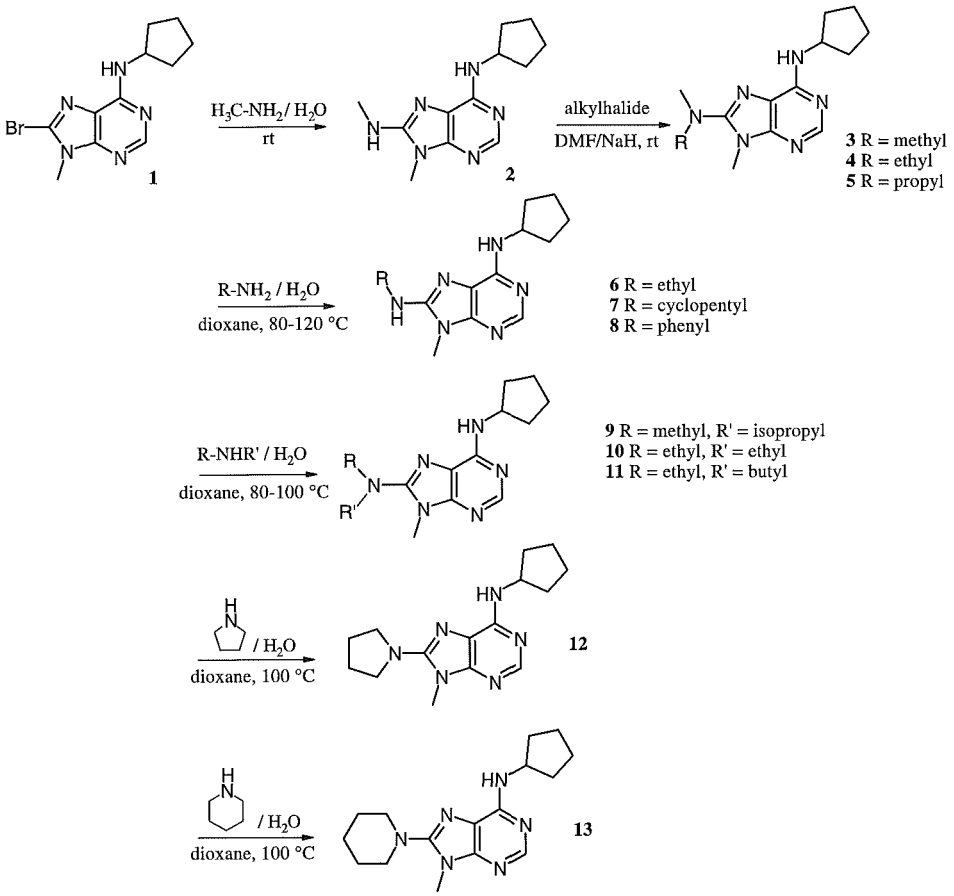
2.2. Radioligand displacement experiments

The adenosine A_1 receptor binding assays were carried out on membranes of CHO-A_1^{++} cells. Membrane aliquots, containing 6 μg of protein and increasing concentrations of the compound, were incubated in 400 μl of 50 mM Tris–HCl, pH 7.4 at 25 °C for 60 min in the presence of ~ 1.6 nM [^{3}H]DPCPX. Non-specific binding was measured in the presence of 10 μM CPA. Incubations were stopped by dilution with the above-mentioned buffer, and bound radioligand was separated by rapid filtration through Whatman GF/B filters using a Brandel harvester. Filters were subsequently washed three times with the

same ice-cold buffer. Bound radioactivity was measured by scintillation spectrometry after the addition of 3.5 ml of Packard Emulsifier Safe.

2.3. [^{35}S]GTPγS binding

The modulation of [^{35}S]GTPγS binding was determined according to the method of Lorenzen et al. [7] with minor modifications. The GDP and NaCl concentrations were 3 or 10 μM and 100 mM, respectively. Incubations were performed at 25 °C for 90 min with 1–5 μg of membrane protein. 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 buffer, 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. Basal [^{35}S]GTPγS binding was set to 100%.



Scheme 1. Synthetic routes for 8-substituted N-0840 derivatives.

2.4. Chemistry

The synthetic routes to the novel chemical entities described in this chapter are depicted in Scheme 1. All final compounds were fully characterised with ^{1}H and ^{13}C NMR, mass spectrometry, and through elemental analysis (within 0.4% of calculated values for C, H, and N). Their purity was estimated to be >99% in all cases.

3. Results

3.1. Experiments with genetically engineered cell systems

3.1.1. Experiments with cells expressing the wild-type adenosine A_1 receptor at normal and high densities

We used two CHO cell membrane preparations, one (CHO-A_1) with receptor densities of approx. 650 fmol/mg protein comparable to those found in mammalian organ preparations (brain cortex, fat cells), the other (CHO-A_1^{++}) with five to six times higher levels of expression, viz. 3.4 pmol/mg protein. First, we compared the two preparations by analysing the effects of the full agonist N^6-cyclopentyladenosine (CPA) and the two partial agonists 5'-methylthio-N^6-cyclopentyladenosine (MeSCPA) and 8-aminobutyl-N^6-cyclopentyladenosine (8-BCPA) [8,9]. Membranes prepared from CHO-A_1 cells had basal levels of [^{35}S]GTPγS binding that were 248 ± 11 cpm/μg of protein. The full agonist CPA increased [^{35}S]GTPγS binding more than 2-fold over basal, while the partial agonists, MeSCPA and BCPA, increased [^{35}S]GTPγS binding to approximately 80% and 60% of CPA's levels, respectively (Fig. 1, left panel). In CHO-A_1^{++} cell membranes, basal levels of [^{35}S]GTPγS binding were significantly higher, that is, 495 ± 50 cpm/ig of protein. CPA, MeSCPA, and 8-BCPA all appeared to be full agonists, increasing [^{35}S]GTPγS binding to

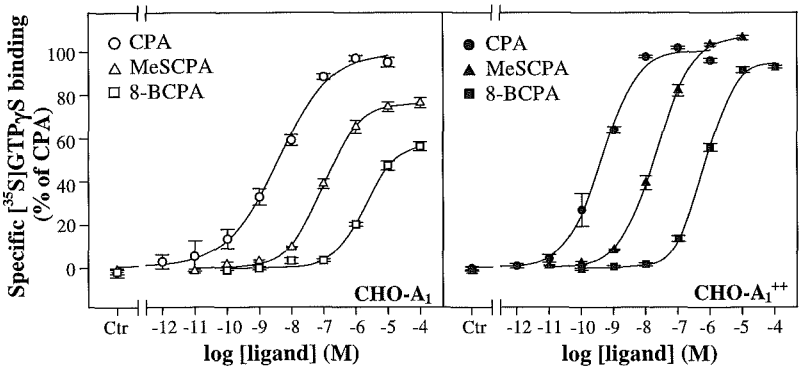


Fig. 1. Modulation of [^{35}S]GTPγS binding by CPA (circles), MeSCPA (triangles), and 8-BCPA (squares) on membranes from CHO-A_1 cells (open symbols, left panel) and CHO-A_1^{++} cells (closed symbols, right panel). Data are expressed as percentage of CPA's effect, which was 224% over basal in CHO-A_1 membranes and 317% over basal in CHO-A_1^{++} membranes. In CHO-A_1 membranes, CPA, MeSCPA, and 8-BCPA had EC_{50} values of 3.75, 102, and 2000 nM, respectively. In CHO-A_1^{++} membranes, EC_{50} values were 0.39, 21, and 673 nM, respectively.

a similar extent, that is, approximately 3-fold over basal values (Fig. 1, right panel). These agonists also showed 3- to 10-fold higher potencies in the experiments with CHO-A_1^{++} membranes. For example, the EC_{50} value for MeSCPA was 102 and 21 nM in CHO-A_1 and CHO-A_1^{++} membranes, respectively.

We then tested the following antagonists, DPCPX, 8-cyclopentyltheophylline (CPT), 1,3-diethyl,8-phenylxanthine (DPX), 3-isobutyl-1-methylxanthine (IBMX), 9-chloro-2-(2-furyl)(1,2,4)triazolo(1,5-*c*)quinazolin-5-amine (CGS15943), xanthine amine congener (XAC), N-0840, theophylline, and caffeine. None of the antagonists significantly changed basal [^{35}S]GTPγS binding to CHO-A_1 cell membranes (Fig. 2, white bars). However, on the CHO-A_1^{++} cell membranes, the antagonists exhibited a variable effect; DPCPX, CPT, DPX, IBMX, CGS 15943, and XAC reduced basal [^{35}S]GTPγS binding to approximately 60–70% of control levels (Fig. 2, grey bars). Thus, these ligands behaved as inverse agonists. On the other hand, N0840, theophylline, and caffeine had no significant effect on basal levels, thus acting as neutral antagonists. For DPCPX, CPT, DPX, and IBMX, full dose–response curves were generated, with IC_{50} values of 3.2, 37, 1100, and 3070 nM, respectively (data not shown).

3.1.2. Experiments with cells expressing fusion proteins of wild-type adenosine A_1 receptor and G_i protein α-subunits

In this experimental set-up, we used chimeric constructs, in which the 3′-end of the human adenosine A_1 receptor was fused (in frame) to the start codon of the rat G_{i1} α-subunit. Due to the proximity of both signalling partners, receptor and G α-subunit, such

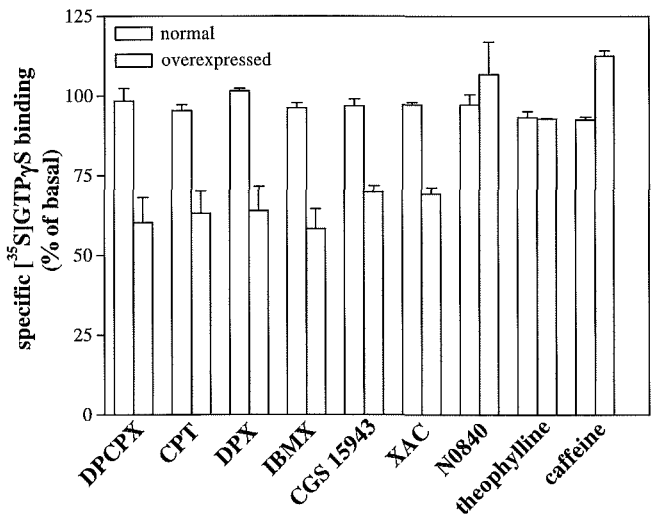


Fig. 2. Effect of different putative antagonists on basal [^{35}S]GTPγS binding to membranes of CHO-A_1 cells (white bars) and CHO-A_1^{++} cell membranes (grey bars). Data are expressed as percentage of basal [^{35}S]GTPγS binding (mean ± S.E.M.). Basal [^{35}S]GTPγS binding was 245 ± 11 cpm/μg of protein (normal) and 495 ± 50 cpm/μg of protein (overexpressed), respectively. Concentrations used were $10 \times K_i$ values of the respective compounds (radioligand binding studies with the same compounds).

fusion proteins may exhibit constitutive receptor activity. We also mutated ^{351}Cys of the G_{i1} α-subunit, primarily to obtain constructs that are insensitive to treatment with pertussis toxin (PTX). In cells treated with PTX, it is highly probable that receptor activation occurs solely via the fusion proteins, and not via endogenously expressed G 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. We used CPT, a more water-soluble analogue of DPCPX, as a reference adenosine A_1 receptor inverse agonist (see Section 3.1.1). Fig. 3 shows the results of [^{35}S]GTPγS binding to COS-7 membranes, expressing the A_1–$G_i\alpha$ fusion protein ^{351}Cys. The agonist CPA increased basal [^{35}S]GTPγS binding more than 7-fold, while CPT decreased it to 52% of the basal level. We used more protein (3 μg of membrane protein) in experiments with CPT than with the agonist CPA (1 μg) to obtain a reasonable window for inhibition of basal [^{35}S]GTPγS binding. 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}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 single concentrations of 1 μM CPT. The results from these [^{35}S]GTPγS binding experiments are summarised in Table 1.

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, that is, 165% stimulation over basal [^{35}S]GTPγS binding, whereas the membrane preparations with selected A_1–$G_i\alpha$ fusion proteins all had larger CPA-induced increases in binding (Table 1).

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 membranes expressing A_1–$G_i\alpha$ fusion protein ^{351}Cys or ^{351}Ile, that is, a reduction to 52% and 51% 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

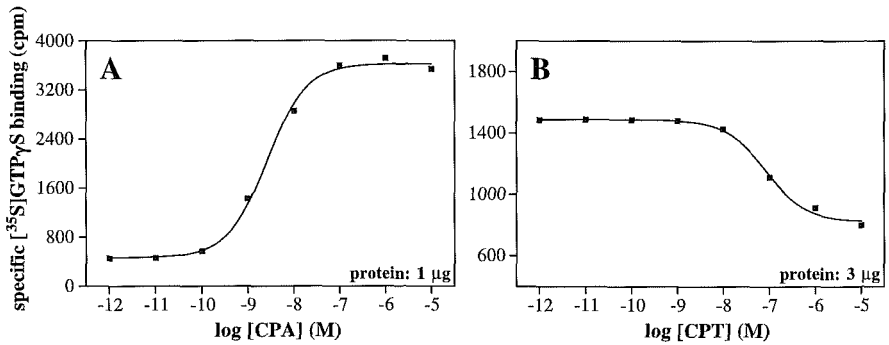


Fig. 3. Stimulation by CPA (panel A) and inhibition by CPT (panel B) of basal [^{35}S]GTPγS binding in membranes prepared from COS-7 cells transfected with the A_1–$G_i\alpha$ fusion protein ^{351}Cys. Data are from a representative experiment performed in duplicate.

Table 1
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 (percentage of basal)	IC_{50} (nM)	Effect (percentage 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}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.
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 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 1). In all cases, the EC_{50} values, for both agonist and inverse agonist, were higher for the fusion proteins than for the unfused wild-type receptor.

3.2. Synthesis and biological evaluation of novel neutral antagonists and inverse agonists

The initial focus in this part of our study was to design novel neutral antagonists with higher affinity for human adenosine A_1 receptors than the reference neutral antagonist N-0840, which was taken as a lead structure. We synthesised a number of compounds of which the 8-substituted amino derivatives are presented here. In Scheme 1, the various methods to generate 8-amino-substituted N^6-cyclopentyl-9-methyladenines (compounds **2–13**) from 8-bromo-N^6-cyclopentyl-9-methyl-adenine (**1**) are detailed [9,10]. Compounds **3–6** were synthesised from N^6-cyclopentyl-8-(N-methylamino)-9-methyladenine (**2**) under mild conditions at room temperature in DMF. The other amines were capable of replacing the 8-bromine atom in **1** directly, resulting in the products **7–13**. For this route, higher temperatures and a different solvent (dioxane) were required.

All compounds were tested in radioligand displacement experiments with the radioligand [^{3}H]DPCPX to determine their affinity for human adenosine A_1 receptors. In Table 2, the results from these radioligand binding experiments have been summarised. Most of the novel derivatives had significantly higher affinity for the adenosine A_1 receptor (7.7–467 nM) than the reference compound N-0840 (852 nM). In general, the secondary amines as substituents (compounds **2**, **6–8**) led to lower adenosine A_1 receptor affinities than the tertiary (compounds **3–5**, **9–11**) and cyclic (compounds **12**, **13**) amines. Moreover, lengthening of the methyl (**3**) to an ethyl group (derivative **10**), and 'ring closure' to a six-membered ring, as in derivative **13**, slightly improved affinity. Apparently, there is sufficient space around the C8-position to accommodate bulky substituents. Derivative **9** with a branched N-methyl-N-isopropylamino substituent on C8 had the best adenosine A_1 receptor affinity in this study, that is, 7.7 nM (Table 2).

Table 2
Affinities of 8-substituted N-0840 analogues at adenosine A_1 receptors expressed as K_i values (in nanomolars ± S.E.M.), and their effect on [^{35}S]GTPγS binding (percentage of basal, i.e. 100%)

Compound	K_i (nM)[a]	Percentage [^{35}S]GTPγS binding[b,c]
DPCPX	2.4 ± 0.1	60 ± 8
N-0840	852 ± 163	107 ± 10
1	43 ± 7	76 ± 4
2	206 ± 27	91 ± 2
3	169 ± 28	85 ± 1
4	89 ± 9	90 ± 1
5	160 ± 23	90 ± 1
6	344 ± 97	72 ± 4
7	2040 ± 200	76 ± 1
8	3560 ± 1530	73 ± 2
9	7.7 ± 1.4	56 ± 2
10	75 ± 8	92 ± 1
11	706 ± 70	99 ± 2
12	403 ± 63	77 ± 3
13	68 ± 6	80 ± 3

[a] Displacement of [^{3}H]DPCPX from CHO-A_1^{++} membranes.
[b] At $10 \times K_i$.
[c] CHO-A_1^{++} membranes (basal [^{35}S]GTPγS binding ~ 450 cpm/μg of protein).

Next, [^{35}S]GTPγS binding was measured on membranes of CHO-A_1^{++} cells. DPCPX was included as a reference inverse agonist and N-0840 as a neutral antagonist. As argued before (see Section 3.1.1), the high receptor expression was necessary to distinguish between neutral antagonists and inverse agonists. DPCPX decreased basal [^{35}S]GTPγS binding on CHO-A_1^{++} membranes to 60% of control (Table 2). The neutral antagonist N-0840 had no (significant) effect on basal [^{35}S]GTPγS binding (107%, Table 2). Five of the newly synthesised compounds (**2**, **4**, **5**, **10**, and **11**) did not drastically change basal [^{35}S]GTPγS binding, thus behaving as neutral adenosine A_1 receptor antagonists. Moreover, four of these products (**2**, **4**, **5**, and **10**) showed a 4- to 10-fold increased adenosine A_1 receptor affinity compared to N-0840. All other compounds decreased basal [^{35}S]GTPγS binding to a varying extent, and acted as (partial) inverse agonists. Compound **9**, with the highest affinity for adenosine A_1 receptors, showed the largest decrease of basal [^{35}S]GTPγS binding, to 56% of control, effectively behaving as a full inverse agonist.

4. Discussion

4.1. Normal and overexpression of wild-type adenosine A_1 receptors

We examined the intrinsic activities of a range of adenosine A_1 receptor ligands in [^{35}S]GTPγS binding studies with membranes prepared from the two CHO cell preparations expressing human adenosine A_1 receptors. The positive intrinsic activity of three agonists, CPA, 8-BCPA, and MeSCPA, was first determined in experiments with CHO-A_1

membranes with 'normal' levels of receptor expression. As anticipated [8,9], both 8-BCPA and MeSCPA were unable to elicit the same full effect as CPA, thus behaving as partial adenosine A_1 receptor agonists. Contrarily, on CHO-A_1^{++} membranes with higher levels of receptor expression, these two partial agonists behaved as full agonists, exhibiting the same effect as CPA. Moreover, the potency to stimulate [^{35}S]GTPγS binding of all three agonists was higher in CHO-A_1^{++} than CHO-A_1 membranes, due to receptor overexpression (Fig. 1). This finding is in good agreement with the classic experiments of receptor alkylation in which full agonists can convert to a more partial nature [11].

We also used CHO-A_1^{++} membranes to determine negative intrinsic activities of putative antagonists. Under our experimental conditions, DPCPX, CPT, DPX, IBMX, CGS 15943, and XAC were shown to reduce [^{35}S]GTPγS binding below basal levels, thus exhibiting inverse agonistic activity. In contrast, N0840, caffeine, and theophylline did not affect basal binding, suggesting that these ligands behaved as neutral antagonists. Previously, DPCPX had also been shown to decrease [^{35}S]GTPγS binding in CHO cells overexpressing the human A_1 receptor [2]. Inverse agonism may have physiological relevance at adenosine A_1 receptors. Ramkumar and Stiles [12] reported enhanced adenylate cyclase activity induced by XAC in rat adipocyte membranes, a rich source of adenosine A_1 receptors. In our hands, XAC was also classified as an adenosine A_1 receptor inverse agonist.

4.2. Fusion proteins between receptor and G protein α-subunit

We investigated the pharmacological characteristics of a number of fusion proteins between the human adenosine A_1 receptor and G_{i1} α-subunits (A_1–$G_i\alpha$). The constructs proved valuable tools in the facilitated detection of inverse agonism. The physical coupling between receptor and the G protein α-subunit, leading to a 1:1 stoichiometric relationship between the two signalling partners, yielded artificial macromolecules with a wide efficacy window. We did not observe 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 (data not shown). In our hands, it thus seemed that this fusion protein, and probably the other ones as well, hardly activated endogenously expressed G α-subunits. Therefore, we did not further use PTX-treated membranes in our experiments. However, mutation of the ^{351}Cys residue also resulted in enhanced receptor (de)activation. For this reason, we investigated whether inverse agonism could be more easily detected with mutated A_1–$G_i\alpha$ fusion proteins.

Similar fusion proteins have been engineered between various receptors (e.g. α_{2A}-adrenergic, adenosine A_1, serotonin 5-HT_{1A} receptor) and different G proteins (e.g. $G_{i1}\alpha$, $G_o\alpha$) (for review, see Ref. [13]). For instance, Kellett et al. [14] showed spontaneous receptor signalling of fusion proteins between the serotonin 5-HT_{1A} receptor and a (mutated) $G_{i1}\alpha$ protein (5-HT_{1A}–$G_{i1}\alpha$). In membranes prepared from HEK293 cells expressing either wild-type (^{351}Cys) or mutated (^{351}Ile) 5-HT_{1A}–$G_{i1}\alpha$ fusion proteins, the increased basal GTPase activity was inhibited by the serotonin 5-HT_{1A} receptor inverse agonist spiperone.

Assuming that the A_1–$G_i\alpha$ fusion proteins resemble a 'precoupled' and, therefore, an active receptor conformation, 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 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 (data not shown), membranes expressing A_1–$G_i\alpha$ fusion proteins showed 7-fold higher basal [^{35}S]GTPγS binding than that found in membranes with the unfused receptor, ~ 600 and ~ 80 cpm/μg of protein, respectively. The latter value also may point to low levels of G proteins present in COS-7 cells. 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 1). The same was observed for inhibition of basal [^{35}S]GTPγS binding by the adenosine A_1 receptor inverse agonist CPT, a more water-soluble analogue of DPCPX. Furthermore, the maximal stimulation and inhibition of basal [^{35}S]GTPγS binding depended on the nature of the amino acid at position 351 of the G_i α-subunit (Ile>Val>Cys>Gly>Pro>unfused). These observations are in line with a report by Bahia et al. [15]. They found a good correlation between the hydrophobicity of the amino acid at this position and the maximal activation of the porcine α_{2A} adrenergic receptor. The authors co-expressed the mutated G_i α proteins and the α_{2A} adrenergic receptor, while we used A_1–$G_i\alpha$ fusion proteins. Nevertheless, we also found larger windows with the hydrophobic amino acids isoleucine and valine than with the more hydrophilic glycine and proline residues.

4.3. Novel inverse agonists and neutral antagonists for the adenosine A_1 receptor

N-0840 is a reference neutral antagonist for adenosine A_1 receptors. However, its affinity, in particular for receptors of human origin, is modest with a K_i value of only 852 nM (Table 2). Therefore, we initiated a program to synthesise higher affinity ligands based on the N-0840 N^6-cyclopentyladenine core. In this chapter, we describe a particular series with an amine function on the C8-position of the molecule (see Scheme 1 for the chemical structures). Among the compounds synthesised were both inverse agonists and (virtually) neutral antagonists as determined in the [^{35}S]GTPγS binding assay on CHO-A_1^{++} membranes. Compound **9** with an *N*-methyl-isopropyl substituent emerged as an inverse agonist with an over 100-fold gain in affinity compared to N-0840 (7.7 nM vs. 852 nM). A chemically close analog of **9** with an *N*-diethyl substituent (compound **10**) only marginally affected basal [^{35}S]GTPγS binding thus behaving as a neutral antagonist, while having over 10-fold higher affinity (75 nM) for the A_1 receptor than N-840. As a consequence, the structure–intrinsic activity relationships for the new compounds appear to be subtle.

5. Conclusion

Inverse agonism at adenosine A_1 receptors is easily demonstrated in systems with enhanced receptor–effector coupling, either by increasing the number of receptors or by physically coupling receptor and G protein α-subunits. With such systems as screening assay, we designed and synthesized novel inverse agonists and neutral antagonists with high affinity for the adenosine A_1 receptor.

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Discussion 8

M. Lohse

This difference in the EC_{50} value, couldn't that reflect the fact that receptors can no longer travel from one G protein to another? So you're reducing the potency of the receptor?

A. IJzerman

That is a good suggestion. The only experimental evidence available is from work by Anna Lorenzen. She co-expressed A_1 receptors with all the α_i subunits that are known to interact with A_1 receptors, i.e. a_{i1}, α_{i2}, α_{i3}, and also α_o to some extent. And interestingly, quite different from the observations by Newman-Tancredi regarding the differences in coupling to the various G proteins, she did not observe any difference in potency. Thus, in that particular system there was no preferential selectivity for a given G protein.

M. Lohse

My suggestion was rather that you have one receptor travelling from one G protein to another to the next, so you have signal amplification which you cannot have in the fusion protein.

T. Schwartz

One comment related to the affinities. Surprisingly when we took the NK_1 receptor and fused it either to G_S or G_Q, and G_Q was the one we really said, "that is the real G protein for this one". It was really the G_S that gave us the high affinity complex. The question is, is the adenosine A_1 receptor really constitutively active? Because you kept on saying that you have to have high receptor expression levels to actually see it, and what should our definition really be for that, I mean, when are we doing something technically to see it, and when is it a real reflection?

A. IJzerman

I presented an engineered system with high receptor expression. There are cells that endogenously express A_1 receptors, for instance the rat thyroid FRTL-5 cell line with approximately 900 fmol/mg protein of A_1 receptor. These cells show constitutive activity at the level of cAMP, which is further enhanced by incubation with DPCPX. Thus, constitutive activity can also be demonstrated in a system of a more physiological nature. Further in vivo evidence may be hard to obtain due to high levels of endogenous adenosine in such systems.

P. Strange

One way to look at the issue of the potency, which may be some kind of change in the receptor-G protein-coupling efficiency, would be to do ligand binding characterisation. Have you done that?

A. IJzerman

We have done that to some extent. We incubated membranes with a saturating concentration of the inverse agonist DPCPX, to see whether comparable levels of receptor expression were observed.

P. Strange

We've done some limited stuff with one fusion protein, and I think we also see that the potencies shift in the direction you see as well.

A. IJzerman

What surprised me also here is that the EC_{50} values shift dependent on the type of mutation in the G protein. That might argue against Lohse's comment, who suggested that one would expect higher EC_{50} values but more or less the same for the various fusion products. Apparently, that is not the case.

M. Lohse

In our case we've also done the ligand binding, and receptor-G protein coupling is not the same as in the unfused, so in that case there is a difference as well.

J.M. Arrang

I just would like to make a comment about the physiological significance of the inverse agonism that you showed for the A_1 receptor. We looked at constitutive activity of various native GPCRs by using the GTPγS binding assay applied to membranes from mouse brain. Interestingly, besides the histamine H_3 receptor, only the A_1 receptor apparently displayed constitutive activity in the brain. Indeed, after treatment of the membranes with adenosine deaminase, an inverse agonist significantly decreased the GTPγS binding at brain membranes.

A. IJzerman

So that was a comment on what really goes on in vivo. Fortunately enough you included the A_1 receptor and the inverse agonist, and you picked it up there.

J.M. Arrang

Yes, only the adenosine A_1 and the histamine H_3 could be detected with that system starting from mouse brain membranes.

A. Newman-Tancredi

A comment on the issue of the term we often use "over-expression of receptors", and I confess I'm not quite clear what this means. Because we often compare expression levels in cell lines to what we see in tissue membrane preparations. But those tissue membrane preparations are mixtures of all sorts of cell types, so is A_1 a neuronal receptor, purely?

A. IJzerman

Neuronal only, I don't know. Fat cells have high A_1 receptor expression on one single cell type.

A. Newman-Tancredi

In CNS tissue, anyway, if we think it's a neuronal receptor, then we can multiply by 5 or 10 the B_{max} values that we observe in tissue homogenates, so you're very easily into 5 pM expression levels without even beginning to discuss, maybe micro-clusters or localisation of the receptor to synapses, which might push the effective expression levels up even much further than that. So if we take all those things into account, then the level of constitutive activity which may actually be present in those tissues could actually be very considerable indeed.

W. Clarke

The notion is that we are probably underestimating how much receptor expression is per unit membrane in cell, in brain or in natural tissues. The A_1 receptor is indeed present on neurones, and certainly hippocampus. We've done electrophysiological studies on neuronal cells, and responsiveness to A_1 agonists can be found in very discrete areas of the cell, so even within a neurone as you mentioned, the local receptor density is probably extraordinarily high.