Available online at www.sciencedirect.com
SCIENCE DIRECT
European Journal of Pharmacology 510 (2005) 1–8
www.elsevier.com/locate/ejphar

A "locked-on," constitutively active mutant of the adenosine A_1 receptor

Rianne A.F. de Ligt[a], Scott A. Rivkees[b], Anna Lorenzen[c], Rob Leurs[d], Ad P. IJzerman[a,*]

[a]Division of Medicinal Chemistry, Leiden/Amsterdam Center for Drug Research, Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands
[b]Yale University School of Medicine, Division of Pediatric Endocrinology, USA
[c]Institute of Pharmacology, University of Heidelberg, D-69120 Heidelberg, Germany
[d]LACDR, Vrije Universiteit, Amsterdam, The Netherlands

Received 3 January 2005; accepted 7 January 2005

Abstract

We studied the wild-type human adenosine A_1 receptor and three mutant receptors, in which the glycine at position 14 had been changed into an alanine, a leucine, or a threonine residue. All receptors were characterized in radioligand binding experiments, the wild-type and the $Gly^{14}Thr$ mutant receptor in greater detail. Both receptors were allosterically modulated by sodium ions and PD81,723 (2-amino-4,5-dimethyl-3-thienyl-[3(trifluoromethyl)-phenyl]methanone), although in a different way. All mutant receptors appeared to be spontaneously or "constitutively" active in a [^{35}S]GTPγS binding assay, the first demonstration of the existence of such CAM (constitutively active mutant) receptors for the adenosine A_1 receptor. The $Gly^{14}Thr$ mutant receptor was also constitutively active in another functional assay, i.e., the inhibition of forskolin-induced cAMP production in intact cells. Importantly, this mutant displayed a peculiar "locked-on" phenotype, i.e., neither agonist nor inverse agonist was capable of modulating the basal activity in both the GTPγS and the cAMP assay, unlike the wild-type and the two other mutant receptors.

Keywords: Adenosine A_1 receptor; Inverse agonism; Constitutive activity; G14T mutant; "Locked-on" phenotype

1. Introduction

G protein-coupled receptors (GPCRs) may occur in at least two conformational states, one inactive (R), the other active (R*) (Leff, 1995). To study these different receptor states, constitutively active mutant (CAM) receptors have been engineered as a research tool. CAM receptors are thought to have an increased proportion of their population in the R* conformation, i.e., they mimic the active state of the receptor. Consequently, they show a higher level of spontaneous receptor activity, even in the absence of an agonist (De Ligt et al., 2000; Leurs et al., 1998; Milligan et al., 1997; Parnot et al., 2002). However, a CAM adenosine A_1 receptor has not been reported yet.

Other strategies to discriminate between the various receptor states include the use of appropriate modulators of ligand binding. Examples of such modulators are GTP, sodium ions, and, specific for the adenosine A_1 receptor, PD81,723 (2-amino-4,5-dimethyl-3-thienyl-[3(trifluoromethyl)-phenyl]methanone), which is an allosteric enhancer of agonist binding. All three modulators influence ligand binding to the adenosine A_1 receptor by intervening with the existing receptor equilibrium in a different manner. First, GTP uncouples the G protein from the receptor, and shifts the receptor equilibrium to the R conformation. This results in a decreased affinity of agonists. Secondly, sodium ions also shift the equilibrium to the inactive receptor state (R). They probably exhibit their effect by interacting with a highly conserved aspartate residue in the second transmembrane helix of virtually all GPCRs, Asp^{55} in the human adenosine A_1 receptor (Barbhaiya et al., 1996). Finally, PD81,723 enhances adenosine A_1 agonist binding (Bruns and Fergus, 1990). It presumably acts by increasing the number of adenosine A_1 receptors in the R* conformation, and so it increases the affinity of adenosine receptor agonists. The shifts in ligand binding properties induced by these three modulators can also be used to discriminate

* Corresponding author. Tel.: +31 71 527 46 51; fax: +31 71 527 45 65.
E-mail address: ijzerman@lacdr.leidenuniv.nl (A.P. IJzerman).

0014-2999/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2005.01.007

between the different classes of ligands, i.e., agonists, inverse agonists and neutral antagonists. For instance, GTP has been used to discriminate between full and partial agonists (Van der Wenden et al., 1995) or neutral antagonists and inverse agonists (Van Calenbergh et al., 2002).

In the present study we focussed on the wild-type human adenosine A_1 receptor and a mutant receptor previously described by us (Rivkees et al., 1999). In this receptor the glycine residue at position 14 had been changed into a threonine ($Gly^{14}Thr$ A_1). Adenosine A_1 receptor agonists, e.g., N^6-cyclopentyladenosine (CPA), displayed a higher affinity for this $Gly^{14}Thr$ A_1 mutant than for the wild-type adenosine A_1 receptor. In retrospect we reasoned this is an indication that the $Gly^{14}Thr$ A_1 receptor might be spontaneously active and behave as a CAM receptor. For comparison, we also introduced two new mutations at this position, an alanine ($Gly^{14}Ala$) or a leucine ($Gly^{14}Leu$) residue.

Various methods were used to establish the pharmacological effects of the Gly^{14}-mutations. Saturation analyses revealed differences in R:R* ratios for the $Gly^{14}Thr$ A_1 receptor compared to the wild-type adenosine A_1 receptor. Allosteric modulation of ligand binding provided further insights into this conformational equilibrium. Finally, receptor activation and functionality of the various (mutant) adenosine A_1 receptors was measured with [^{35}S]GTPγS binding studies and cAMP determinations. We discovered that all three mutants display constitutive activity and that the $Gly^{14}Thr$ mutant receptor possesses a very peculiar "locked-on" phenotype.

2. Materials and methods

2.1. Chemicals

[^{35}S]GTPγS (1250 Ci/mmol), [^{3}H]DPCPX (1,3-dipropyl,8-cyclopentylxanthine, 112 Ci/mmol), [^{3}H]CCPA (2-chloro,N^6-cyclopentyladenosine, 55 Ci/mmol) and [^{3}H]cAMP (25 Ci/mmol) were obtained from Perkin Elmer Life Sciences (Dreieich, Germany). Adenosine deaminase (ADA), DEAE dextran, chloroquine, and dithiothreitol were purchased from Sigma. EDTA, $MgCl_2$, GDP, and GTPγS were obtained from Boehringer (Mannheim, Germany). CPA, DPCPX and 8-cyclopentyltheophylline (CPT) were purchased from Research Biochemicals Inc. (Natick, USA). A fraction containing protein kinase A (PKA) was isolated from bovine adrenal glands according to Leurs et al. (1994). Except for foetal calf serum (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 wild-type A_1 or the $Gly^{14}Thr$ A_1 receptor were subcloned into the mammalian expression vector pcDNA3 (Rivkees et al., 1999), whereas the cDNAs encoding either the $Gly^{14}Ala$ or $Gly^{14}Leu$ A_1 receptor were subcloned into pcDNA6 (both Invitrogen, San Diego, USA).

2.2. Cell culture

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% foetal calf serum, penicillin (50 IU/ml), and streptomycin (50 μg/ml). Human embryonal kidney (HEK293) cells were cultured under similar conditions in DMEM supplemented with 10% new-born calf serum and antibiotics.

2.3. Transfections

COS-7 cells were transiently transfected with either the wild-type or one of the three mutant A_1 receptors, or mock-transfected with the expression vector pcDNA3 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 supplemented with 2% foetal calf serum and 100 μM chloroquine, 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 cell medium, and incubated at room temperature for 2–10 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.

HEK293 cells were transiently transfected with 10–15 μg DNA (per 12-well plate) using calcium phosphate precipitation. Briefly, HEK293 cells were subcultured (1:5) into 12-well plates 2 h prior to transfection. After 2 h, 50 μl of a calcium phosphate–DNA coprecipitate mixture was added to each well and the plates were incubated overnight at 37 °C and 7% CO_2. The next day, transfection solution was replaced by normal culture medium and the cells were grown for another 24 h.

2.4. 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 homogenized 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 centrifugation steps at 4 °C: a 10-min centrifugation at $1000\times g$ and the obtained supernatant for 30 min at $60{,}000\times g$. The final pellet was resuspended in 3 ml ice-cold 50 mM Tris HCl

buffer (pH 7.4), supplemented with adenosine deaminase (ADA, 2 U/ml). Protein concentrations were measured with the bicinchoninic acid method with bovine serum albumin as a standard (Smith et al., 1985).

2.5. 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 0.8 nM [^{3}H]DPCPX, and increasing concentrations of CPA and DPCPX in a total volume of 400 μl. To study the modulatory effects of 1 M NaCl, 10 μM PD81,723, and 1 mM GTP, the indicated concentrations were added when appropriate. 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.

2.6. [^{35}S]GTPγS binding

[^{35}S]GTPγS binding was measured in 100 μl containing 50 mM Tris HCl (pH 7.4), 1 mM EDTA, 1 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% 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate (CHAPS), and 0.5% bovine serum albumin. Incubations were started by addition of the membrane suspension (6–10 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.

2.7. cAMP assays

HEK293 cells were used in cAMP experiments on the third day after transfection. Normal growth medium was replaced by HEPES-buffered DMEM (pH 7.4), supplemented with ADA (5 U/ml), cilostamide (50 μM), and rolipram (50 μM). After a 30-min incubation at 37 °C CPA or DPCPX (both at 100 nM) was added for another 10 min. Subsequently, forskolin (1 μM) was added. After 15 min, the incubations were stopped by aspirating the assay medium and by adding 300 μl ice-cold 0.1 N HCl to the cells.

The amount of cAMP was determined by competition with [^{3}H]cAMP for protein kinase A (PKA). Briefly, sample or cAMP standard (0–16 pmol), approximately 1.8 nM [^{3}H]cAMP, and 100 μl PKA-solution were incubated on ice for 2.5 h. The incubations were stopped by rapid dilution with 2 ml of ice-cold 50 mM Tris HCl buffer (pH 7.4), and bound radioactivity was immediately recovered by filtration through Whatman GF/C filters using a Brandel harvester. Filters were washed twice with 1 ml of the same buffer, and retained radioactivity was measured after 6 h by liquid scintillation counting (LKB Wallac, 1219 Rackbeta).

2.8. Data and statistical analysis

Competition binding data were analysed and IC_{50} and K_i values were calculated using a nonlinear regression computer program (Prism 3.0, Graphpad Software, San Diego, CA, USA). K_d values of [^{3}H]DPCPX at the wild-type adenosine A_1 receptor used were 1.8 nM (control, Table 2), 1.0 nM (+NaCl), 2.6 nM (+GTP), and 2.4 nM (+PD81,723), respectively. The corresponding K_d values at the Gly^{14}Thr A_1 receptor were 2.2 nM (Table 2), 0.95 nM, 3.7 nM, and 2.1 nM, respectively (data not shown).

All data shown are expressed as mean±S.E.M. of at least three independent experiments, all performed in duplicate. Statistical analysis was carried out by Student's *t* test, and *P* values<0.05 were considered to be statistically significant. In the text, *n* refers to the number of independent experiments.

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

As a start we determined IC_{50} values of CPA and DPCPX for the wild-type and three Gly^{14}-mutated receptors (Table 1). Compared to the wild-type adenosine A_1 receptor, the IC_{50} value of CPA was similar for the Gly^{14}Ala and Gly^{14}Leu A_1 receptor. Interestingly, the IC_{50} value of CPA for the Gly^{14}Thr A_1 receptor was significantly lower than for the three other constructs, namely 5.04 nM versus 436 (wild-type), 459 (Gly^{14}Ala), and 551 (Gly^{14}Leu) nM, respectively. On the other hand, DPCPX had only slightly different IC_{50} values for all four adenosine A_1 receptors. The Gly^{14}Thr A_1 receptor was further analysed in radioligand binding experiments, because of its remarkably increased affinity for CPA compared to both the wild-type

Table 1
Affinities (IC_{50}±S.E.M.) of CPA and DPCPX for the wild-type and three Gly^{14}-mutated adenosine A_1 receptors, as determined in displacement experiments with [^{3}H]DPCPX on transfected COS-7 cell homogenates

Receptor	IC_{50} (nM)	
	CPA	DPCPX
Wild type	436±118	2.96±0.34
Gly^{14}Ala	459±87	3.70±0.50
Gly^{14}Leu	551±46	5.23±0.62[a]
Gly^{14}Thr	5.04±1.40[a]	4.45±0.87

Data are from three independent experiments, performed in duplicate.
[a] $P<0.05$, statistical difference versus wild type.

adenosine A_1 receptor and the two other Gly^{14}-mutated A_1 receptors.

3.1. Saturation analysis

First, K_d and B_{max} values for both [^{3}H]DPCPX and [^{3}H]CCPA binding to the wild-type adenosine A_1 and the Gly^{14}Thr A_1 receptor were determined (Table 2), yielding similar K_d values for either radioligand on the two receptors.

Interestingly, the B_{max} values varied significantly between the different receptors and the radioligand used. The tritiated agonist, [^{3}H]CCPA, revealed a low density of wild-type adenosine A_1 receptors, namely 285 fmol/mg protein. The B_{max} value of Gly^{14}Thr A_1 receptors labelled with [^{3}H]CCPA was twofold higher, 577 fmol/mg protein. The same number of Gly^{14}Thr A_1 receptors was recognised by the radiolabelled 'antagonist' [^{3}H]DPCPX. On the other hand, the total number of wild-type adenosine A_1 receptors, as obtained with [^{3}H]DPCPX, was sixfold higher than the B_{max} value determined with [^{3}H]CCPA.

3.2. Displacement experiments

We next determined the affinities of CPA and DPCPX for the wild-type adenosine A_1 and the Gly^{14}Thr A_1 receptor in [^{3}H]DPCPX displacement studies. The K_d values given in the 'Materials and methods' section were used to calculate the K_i values that are presented in Table 3.

As mentioned above we found a significant, 80-fold increase in affinity of CPA at the Gly^{14}Thr A_1 receptor compared to the wild-type adenosine A_1 receptor, with K_i values of 3.45 nM and 289 nM, respectively (Fig. 1, Table 3). The affinity of DPCPX was only slightly affected by the mutation, with a 1.5-fold decrease in affinity. All curves, including displacement by CPA, were fitted best according to a one-site rather than a two-site competition binding model.

Table 2
B_{max} (in fmol/mg of protein) and K_d (in nM) values of [^{3}H]DPCPX and [^{3}H]CCPA derived from saturation experiments on homogenates of COS-7 cells transfected with either the wild-type or the Gly^{14}Thr adenosine A_1 receptor

	[^{3}H]DPCPX		[^{3}H]CCPA	
	Wild type	Gly^{14}Thr	Wild type	Gly^{14}Thr
B_{max}	2180 (±133)	571 (±270)	285 (±95)	577 (±155)
K_d	1.8 (±0.3)	2.2 (±0.5)	0.93 (±0.16)	1.3 (±0.1)

B_{max} and K_d (±S.E.M.) values were determined in three independent experiments, performed in duplicate.

Table 3
K_i values±S.E.M. of CPA and DPCPX at the wild-type adenosine A_1 and the Gly^{14}Thr A_1 receptor in the presence or absence of 1 M NaCl, 10 μM PD81,723, or 1 mM GTP (n=3–7)

		Wild-type A_1		Gly^{14}Thr A_1	
		K_i (nM)	Shift	K_i (nM)	Shift
CPA	Control	289±70		3.45±0.90	
	+NaCl	720±76	2.5[b]	17.0±4.4	4.9[a]
	+PD81,723	81±12	0.28[a]	2.74±0.88	0.79
	+GTP	472±52	1.6	4.50±0.28	1.3
DPCPX	Control	2.00±0.22		3.08±0.53	
	+NaCl	0.87±0.06	0.44[b]	0.94±0.26	0.31[a]
	+PD81,723	3.08±0.58	1.5	6.00±0.68	1.9[a]
	+GTP	2.75±0.29	1.4[a]	2.95±0.24	0.96

Significant shift compared to unity, [a]$P<0.05$, [b]$P<0.01$.
K_i values were calculated using the K_d values mentioned in the Materials and methods section, and the shifts by dividing K_i (in the presence of modulator) by K_i (control).

3.3. Allosteric modulation by sodium ions, PD81,723 and GTP

We observed a more than twofold decrease in affinity of CPA at the wild-type adenosine A_1 receptor in the presence of 1 M NaCl. The reverse was seen for the binding of DPCPX, since its affinity increased twofold. At the Gly^{14}Thr A_1 receptor the nature of the modulation of CPA and DPCPX binding by sodium ions was similar. However, the observed changes in affinity were more pronounced, i.e., a fivefold decrease for CPA and a threefold increase of the affinity of DPCPX (Table 3).

Furthermore, PD81,723 induced a threefold increase in the affinity of CPA at the wild-type adenosine A_1 receptor. Contrarily, the affinity of CPA at the Gly^{14}Thr A_1 receptor

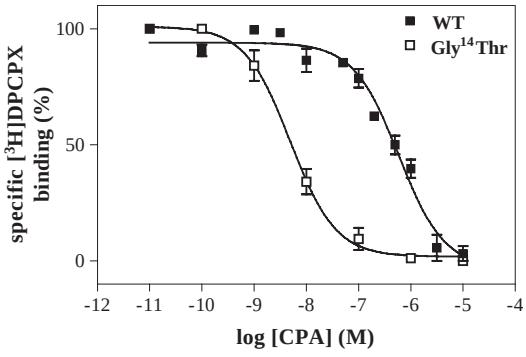

Fig. 1. Displacement of [^{3}H]DPCPX by CPA from COS-7 cell homogenates expressing the wild-type (■) or the Gly^{14}Thr (□) A_1 receptor in the absence of any modulator. All data points were calculated as a percentage of specific binding in the absence of CPA, and are presented as mean±S.E.M of 3 independent experiments, performed in duplicate. Both curves were fitted best with one-site competition analysis.

was not significantly changed in the presence of 10 μM PD81,723. Moreover, at the Gly^{14}Thr A_1 receptor PD81,723 also seemed to affect DPCPX binding, decreasing its affinity almost twofold (Table 3). Lastly, we investigated the effect of 1 mM GTP on binding properties. In our hands, using homogenates of transfected COS-7 cells, we did not observe pronounced effects of GTP (Table 3).

3.4. [^{35}S]GTPγS binding experiments

To compare the interaction of the wild-type and the Gly^{14}Thr A_1 receptor with the G proteins present in our COS-7 cell membrane preparation, we performed [^{35}S]GTPγS binding experiments. In these studies we also included the Gly^{14}Ala and Gly^{14}Leu A_1 receptor. On the wild-type adenosine A_1 receptor basal [^{35}S]GTPγS binding (100%) was further increased by CPA (EC_{50}=0.21 (0.16–0.30) nM) to 165% and decreased to 74% by both DPCPX (at 1 μM) and CPT, a close analogue of DPCPX (EC_{50}=15 (10–23) nM). Intriguingly, modulation of basal [^{35}S]GTPγS binding by either CPA, DPCPX or CPT could not be detected in membranes expressing the Gly^{14}Thr A_1 receptor. Over a range of concentrations (10^{-12} M to 10^{-6} M) neither CPA nor CPT or DPCPX induced any significant effect. Basal [^{35}S]GTPγS binding, however, was substantially higher in membranes transfected with the Gly^{14}Thr A_1 receptor, compared to membranes expressing the wild-type adenosine A_1 receptor, at ~3000 and ~800 cpm per 10 μg of membrane protein, respectively (Fig. 2).

In addition, the two other mutations at this position (Gly^{14}Ala and Gly^{14}Leu) also resulted in higher basal [^{35}S]GTPγS binding than the wild-type adenosine A_1 receptor, ~2200 and ~3000 cpm per 10 μg of COS-7 cell membrane protein, respectively. However, in these two cases both CPA and CPT were able to modulate this basal [^{35}S]GTPγS binding to similar levels as observed for the wild-type receptor. The maximal stimulation induced by 1 μM CPA was 165%, 204% and 192% for the wild-type, Gly^{14}Ala, and Gly^{14}Leu receptor, respectively, whereas the maximal inhibition induced by 1 μM CPT was 26%, 23%, and 32%, respectively.

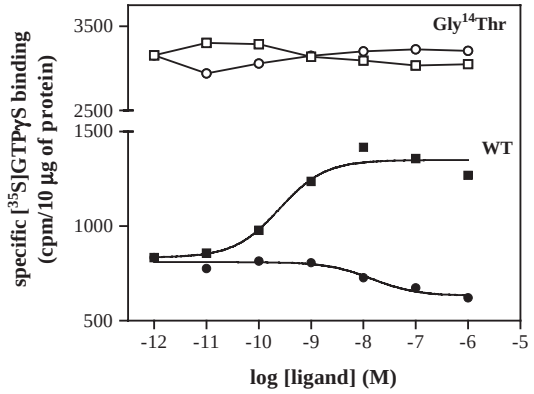

Fig. 2. Modulation of basal [^{35}S]GTPγS binding by CPA (■,□) and CPT (●,○) at COS-7 cell membranes expressing either wild-type (■,●) or Gly^{14}Thr A_1 receptors (□,○). Represented a typical experiment, performed in duplicate.

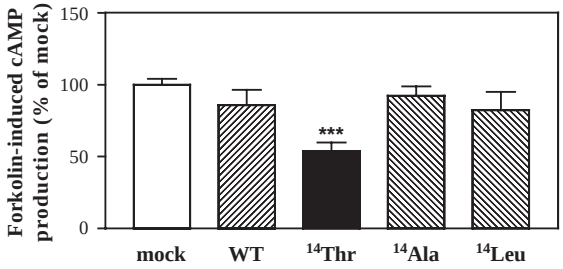

Fig. 3. cAMP production induced by forskolin (1 μM) in HEK293 cells expressing wild-type, Gly^{14}Ala, Gly^{14}Leu (hatched bars) or Gly^{14}Thr A_1 receptors (black bar), represented as percentage of forskolin-induced cAMP production in mock-transfected HEK293 cells (white bar) and corrected for amounts of protein. Data are the mean±S.E.M from 4 independent experiments, performed in duplicate. (***P<0.001 versus mock).

3.5. cAMP determinations

Finally, we determined the cAMP content in intact transfected HEK293 cells to check whether the Gly^{14}-mutated A_1 receptors were functionally active. Since basal cAMP production was low, we stimulated transfected HEK293 cells with 1 μM forskolin to directly activate endogenously expressed adenylate cyclase. The white bar in Fig. 3 represents the cAMP level in mock-transfected HEK293 cells induced by forskolin (set at 100% as control value). This forskolin-induced cAMP production was not inhibited upon expression of either wild-type, Gly^{14}Ala, or Gly^{14}Leu A_1 receptors (hatched bars, Fig. 3). On the other hand, HEK293 cells transfected with Gly^{14}Thr A_1 receptors showed a significant inhibition of the forskolin-induced cAMP production to approximately 50% of control values (P<0.001, black bar, Fig. 3). Please note that we used identical amounts of DNA for expression purposes.

Next, the effects of CPA and DPCPX on forskolin-induced cAMP formation were tested (Table 4). None of the ligands significantly changed basal cAMP production in HEK293 cells transfected with the empty vector (mock) or the Gly^{14}Thr A_1 receptor. However, CPA (100 nM)

Table 4
Forskolin-induced cAMP production in HEK293 cells expressing wild-type or Gly^{14}-mutated adenosine A_1 receptors

Construct	Forskolin (1 μM)		
	Control	CPA	DPCPX
Mock	100±4	89±6	107±2
Wild type	86±11	49±11[a]	125±13[a]
Gly^{14}Ala	92±7	69±9[a]	102±5
Gly^{14}Leu	82±13	43±8[a]	125±11[a]
Gly^{14}Thr	54±6[b]	61±6	39±11

cAMP production is given as percentage of control in mock-transfected HEK293 cells. Data are the mean±S.E.M. from at least 4 independent experiments.

[a] P<0.05 versus control.

[b] P<0.001 versus mock.

decreased forskolin-induced cAMP production in HEK293 cells transfected with wild-type, $Gly^{14}Ala$, or $Gly^{14}Leu$ A_1 receptors to 49, 69, and 43%, respectively ($P<0.05$). In addition, 100 nM DPCPX moderately increased forskolin-induced cAMP production in HEK293 cells, expressing wild-type or $Gly^{14}Leu$ A_1 receptors, while cells transfected with the $Gly^{14}Ala$ A_1 receptor were unaffected (Table 4).

4. Discussion

Mutation of the glycine residue at position 14 (Gly^{14}) of the human adenosine A_1 receptor into a threonine (as present in the human adenosine A_{2A} receptor) had been shown to increase agonist affinity (Rivkees et al., 1999). Since this finding might be indicative for the receptor's constitutive activity, we now analysed the $Gly^{14}Thr$ A_1 receptor in more detail. For comparison, two other mutations at this position, $Gly^{14}Ala$ and $Gly^{14}Leu$ were included in this study. The rationale for this choice was that both alanine and leucine are of similar size to glycine and threonine, respectively, while lacking the hydrophobic nature (hydroxy group) of threonine.

The Gly^{14}-mutation is located in helix I near the extracellular N-terminal domain. It is also in close proximity of a conserved glutamate at position 16 (Glu^{16}). The latter may interact with a histidine in helix VII (His^{278}), and both amino acids are believed to play a role in receptor activation (IJzerman et al., 1996). Most of the mutations inducing constitutive receptor activity, as thoroughly reviewed by Pauwels and Wurch (1998) and Parnot et al. (2002), are found within the transmembrane domains of the receptor and in the intracellular loops, e.g., the E/DRY motif (intracellular loop 2) and the 'BBXXB' sequence (intracellular loop 3, B is a basic amino acid residue: Arg, Lys, or His, and X a non-basic residue). There is one comparable mutation known in the yeast α-factor receptor in which Asn^{46} at the top of TM1, when mutated to serine, induces constitutive activity (Parrish et al., 2002). The Gly^{14}-mutated adenosine A_1 receptors studied indeed show characteristics of CAM receptors, despite the uncommon site of mutation.

4.1. Radioligand binding studies

We performed most studies on the $Gly^{14}Thr$ A_1 receptor, in view of its unusual features. Apparently, $Gly^{14}Thr$ A_1 receptors are expressed at lower density than wild-type adenosine A_1 receptors (Table 2). Nevertheless, virtually all $Gly^{14}Thr$ A_1 receptors occur in an active receptor state, R*. Such a high proportion of R* for the mutant receptor might be indicative of the receptor's constitutive activity, as is its low receptor density. In general, CAM receptors show lower expression levels than wild-type receptors (Alewijnse et al., 2000; Hjorth et al., 1998). The small proportion of R* of the wild-type adenosine A_1 receptor explains the apparent discrepancy between the affinities of [^{3}H]CCPA and its closely related analogue CPA, 0.93 and 289 nM, respectively. Because the displacement of [^{3}H]DPCPX by CPA was best described by a one-site competition model, the apparent K_i value of 289 nM represented mostly the low affinity of CPA for the wild-type adenosine A_1 receptor. Moreover, the K_d value of [^{3}H]CCPA was similar for both wild-type and $Gly^{14}Thr$ A_1 receptors. Thus, agonists do not have increased affinities for the $Gly^{14}Thr$ A_1 receptor, rather this mutation reveals their high affinity.

The results from the radioligand binding experiments, including the modulatory effects of sodium ions and PD81,723, agree with predictions from the two-state receptor model. Sodium ions regulate agonist binding to adenosine receptors by interfering with the existing receptor equilibrium (Goodman et al., 1982; Green, 1984). Modulation induced by sodium ions was pronounced in the present study. In the presence of a high concentration of NaCl (1 M) the affinity of DPCPX increased significantly, consistent with the suggestion that DPCPX might be regarded as an inverse agonist (Shryock et al., 1998; De Ligt et al., 2004). The affinity of the agonist CPA was significantly decreased in the presence of sodium ions, as had also been found by Barbhaiya et al. (1996). Both these observations are in good agreement with a shift in receptor equilibrium towards the R conformation. Interestingly, sodium ions displayed even more pronounced effects on ligand binding at $Gly^{14}Thr$ A_1 receptors. Since virtually all $Gly^{14}Thr$ A_1 receptors exist in the R* conformation, sodium ions thus seem capable of inducing an even larger shift towards R than at wild-type adenosine A_1 receptors, which have only 13% as R* (Table 2). As a consequence, this led to larger changes in K_i values.

The effect of PD81,723 on ligand binding can be rationalised as follows. PD81,723 is an allosteric enhancer of adenosine A_1 agonist binding, and shifts the receptor equilibrium towards R* (Bhattacharya and Linden, 1995; Bruns and Fergus, 1990). The increase in affinity of CPA at the wild-type adenosine A_1 receptor is thus readily explained. Also the lack of change in CPA binding to $Gly^{14}Thr$ A_1 receptors was anticipated. Since the mutant receptors already occur in the R* conformation mostly, PD81,723 does not influence the equilibrium in favour of this conformation any further. The effect of PD81,723 on DPCPX binding appeared in line with the latter ligand's nature of an inverse agonist. In the presence of 10 μM PD81,723 wild-type adenosine A_1 receptors had equal affinity for DPCPX as $Gly^{14}Thr$ A_1 receptors under control conditions, approximately 3 nM. Apparently, PD81,723 induces a shift towards R*. An inverse agonist, however, prefers the R conformation over R*, and "likes" the wild-type receptor in the absence of PD 81,723 most. Differences in affinity are small however with K_i values only varying between 2 and 6 nM.

In our hands, the observed effects of GTP on ligand binding were modest. A possible reason may be found in the experimental conditions. In general, COS-7 cells possess a

relatively limited pool of G proteins. Furthermore, our use of cell homogenates may have resulted in the presence of residual endogenous GTP, which would then reduce the effect of the added GTP.

4.2. Functional studies

Changes in G protein interaction induced by the Gly^{14}-mutation were studied in [^{35}S]GTPγS binding experiments. Wild-type adenosine A_1 receptors showed a moderate spontaneous activity in this assay, amenable to modulation by both CPA, DPCPX and CPT. The results from [^{35}S]GTPγS binding studies with membranes expressing the Gly^{14}Thr A_1 receptor were intriguing. Despite a lower expression of Gly^{14}Thr A_1 receptors, we noticed an almost fourfold increase in basal [^{35}S]GTPγS binding of Gly^{14}Thr-membranes compared to membranes expressing wild-type adenosine A_1 receptors. However, the inverse agonists CPT and DPCPX were unable to decrease basal [^{35}S]GTPγS binding, nor could the agonist CPA increase it. In addition, [^{35}S]GTPγS binding experiments with COS-7 membranes expressing either Gly^{14}Ala or Gly^{14}Leu A_1 receptors showed strongly increased basal G protein activation for both membrane preparations, while these two mutants had expression levels comparable to the wild-type adenosine A_1 receptor (data not shown). Remarkably, both CPA and CPT were able to alter basal [^{35}S]GTPγS binding to cell membranes expressing these mutant A_1 receptors.

The functionality of the Gly^{14}-mutated A_1 receptors was further studied by assessing their influence on the amount of cAMP in intact HEK293 cells. Transfected HEK293 rather than COS-7 cells were used in cAMP assays. The former cells possess higher amounts of G proteins leading to a better efficiency of receptor–effector coupling. To increase basal cAMP production, transfected HEK293 cells were stimulated with 1 μM forskolin, which submaximally activated endogenously expressed adenylate cyclases (data not shown). Because the HEK293 cells were not fully stimulated, DPCPX was able to show inverse agonistic behaviour, i.e., to further increase cAMP production.

HEK293 cells transfected with wild-type, Gly^{14}Ala, or Gly^{14}Leu A_1 receptors, all had similar forskolin-induced cAMP levels as mock-transfected HEK293 cells (Fig. 3). Accordingly, in HEK293 cells these (mutated) adenosine A_1 receptors, did not demonstrate significant constitutive receptor activity. The Gly^{14}Thr A_1 receptor, on the other hand, decreased forskolin-induced cAMP production to 54% compared to mock-transfected HEK293 cells, consequently behaving as a CAM adenosine A_1 receptor, also in this test system. Interestingly, Shryock et al. (1998) were able to show an inhibition of forskolin-induced cAMP production in CHO cells expressing human wild-type adenosine A_1 receptors at a high density (4–8 pmol per mg of membrane protein). Therefore, in their study the human adenosine A_1 receptor appeared spontaneously active, probably due to the significant receptor overexpression.

Moreover, in HEK293 cells expressing wild-type adenosine A_1 receptors, CPA (100 nM) inhibited forskolin-induced cAMP production to 49% of control (forskolin-induced cAMP production, Table 4). In transfected COS-7 cells, however, CPA only modestly reduced basal cAMP production (77% of basal, data not shown). This observation indicated, again, a relatively poor receptor–G protein coupling in COS-7 cells, and supported the use of HEK293 cells in cAMP determinations.

Table 4 summarises the effects of CPA and DPCPX on forskolin-induced cAMP production in the various transfected HEK293 cells. Two mutant receptors, Gly^{14}Ala and Gly^{14}Leu A_1, behaved as the wild-type adenosine A_1 receptor, i.e., CPA caused an inhibition of basal cAMP levels. Interestingly, effects of both CPA and DPCPX were absent in cells transfected with the Gly^{14}Thr A_1 receptor just as observed in the [^{35}S]GTPγS binding experiments. This very peculiar phenotype has been reported on one other occasion. Prioleau et al. (2002) mutated the tyrosine residue in the conserved NPXXY motif in TM VII of the serotonin 5-HT_{2C} receptor. They found the Tyr^{368}Asn mutation to display a "locked-on" constitutively active phenotype in which neither agonists nor antagonists/inverse agonists were capable of influencing the (enhanced) production of inositol phosphates as second messengers. The tested compounds, however, retained their capacity to bind to the receptor. Interestingly, receptor agonists for the 5-HT_{2C} receptor proved over 100-fold more active on this mutant than on the wild-type receptor, just as we observed for the K_i value of CPA on the Gly^{14}Thr adenosine A_1 receptor. Other Tyr^{368} mutant 5-HT_{2C} receptors also displayed constitutive activity, but in these cases both agonists and inverse agonists were capable of modulating inositol phosphate production, just as we noticed for the Gly^{14}Ala and Gly^{14}Leu adenosine A_1 receptor.

5. Conclusions

In conclusion, in this study we described, for the first time, three CAM (Gly^{14}Ala, Gly^{14}Leu, and Gly^{14}Thr) adenosine A_1 receptors, as found in [^{35}S]GTPγS binding experiments. Moreover, it seemed that each mutant A_1 receptor had distinctive characteristics. Although the Gly^{14}Thr A_1 receptor was able to bind ligands, such as CPA and DPCPX (Tables 1–3), it was not susceptible to further receptor (de)activation, as measured with [^{35}S]GTPγS binding (Fig. 2) or cAMP production (Table 4). Particularly, its basal receptor activity could not be counteracted with an inverse agonist. Both CPT and DPCPX, used in [^{35}S]GTPγS binding studies, and DPCPX, applied in cAMP determinations, were unable to shift the receptor equilibrium towards R. Therefore, it appeared that the Gly^{14}Thr A_1 receptor is 'locked-on' in an active state, or, in other words, that it is physically trapped in its R* conformation.

Acknowledgements

The authors (R.d.L., R.L., and A.P.IJ.) acknowledge financial support from the EU BIOMED2 programme "Inverse agonism. Implications for drug design" (#BMH4-CT97-2152).

References

Alewijnse, A.E., Timmerman, H., Jacobs, E.H., Smit, M.J., Roovers, E., Cotecchia, S., Leurs, R., 2000. The effect of mutations in the DRY motif on the constitutive activity and structural instability of the histamine H_2 receptor. Mol. Pharmacol. 57, 890–898.

Barbhaiya, H., McClain, R., IJzerman, A.P., Rivkees, S.A., 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.

Bhattacharya, S., Linden, J., 1995. The allosteric enhancer, PD81,723, stabilizes human A_1 adenosine receptor coupling to G proteins. Biochim. Biophys. Acta 16, 15–21.

Brakenhoff, R.H., Knippels, E.M., Van Dongen, G.A., 1994. Optimization and simplification of expression cloning in eukaryotic vector/host systems. Anal. Biochem. 218, 460–463.

Bruns, R.F., Fergus, J.H., 1990. Allosteric enhancement of adenosine A_1 receptor binding and function by 2-amino-3-benzoylthiophenes. Mol. Pharmacol. 38, 939–949.

De Ligt, R.A.F., Kourounakis, A.P., IJzerman, A.P., 2000. Inverse agonism at G protein-coupled receptors: (Patho)physiological relevance and implications for drug discovery. Br. J. Pharmacol. 130, 1–12.

De Ligt, R.A.F., Van der Klein, P.A., Von Frijtag Drabbe Kuenzel, J.K., Lorenzen, A., Ait El Maate, F., Fujikawa, S., Van Westhoven, R., Van den Hoven, T., Brussee, J., IJzerman, A.P., 2004. Synthesis and biological evaluation of disubstituted N^6-cyclopentyladenine analogues: the search for a neutral antagonist with high affinity for the adenosine A_1 receptor. Bioorg. Med. Chem. 12, 139–149.

Goodman, R.R., Cooper, M.J., Gavish, M., Snyder, S.H., 1982. Guanine nucleotide and cation regulation of the binding of [^{3}H]cyclohexyladenosine and [^{3}H]diethylphenylxanthine to adenosine A_1 receptors in brain membranes. Mol. Pharmacol. 21, 329–335.

Green, R.D., 1984. Reciprocal modulation of agonist and antagonist binding to inhibitory adenosine receptors by 5′-guanylylimidodiphosphate and monovalent cations. J. Neurosci. 4, 2472–2476.

Hjorth, S.A., Orskov, C., Schwartz, T.W., 1998. Constitutive activity of glucagon receptor mutants. Mol. Endocrinol. 12, 78–86.

IJzerman, A.P., Von Frijtag Drabbe Künzel, J.K., Kim, J., Jiang, Q., Jacobson, K.A., 1996. Site-directed mutagenesis of the human adenosine A_{2A} receptor. Critical involvement of Glu13 in agonist recognition. Eur. J. Pharmacol. 310, 269–272.

Leff, P., 1995. The two-state model of receptor activation. Trends Pharmacol. Sci. 16, 89–97.

Leurs, R., Smit, M.J., Menge, W.M.B.P., 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.

Leurs, R., Smit, M.J., Alewijnse, A.E., Timmerman, H., 1998. Agonist-independent regulation of constitutively active G protein-coupled receptors. Trends Biochem. Sci. 23, 418–422.

Milligan, G., MacEwan, D.J., Mercouris, M., Mullaney, I., 1997. Inverse agonism at adrenergic and opioid receptors: studies with wild type and constitutively active mutant receptors. Recept. Channels 5, 209–213.

Parnot, C., Miserey-Lenkei, S., Bardin, S., Corvol, P., Clauser, E., 2002. Lessons from constitutively active mutants of G protein-coupled receptors. Trends Endocrinol. Metab. 13, 336–343.

Parrish, W., Eilers, M., Ying, W., Konopka, J.B., 2002. The cytoplasmic end of transmembrane domain 3 regulates the activity of the *Saccharomyces cerevisiae* G-protein-coupled alpha-factor receptor. Genetics 160, 429–443.

Pauwels, P.J., Wurch, T., 1998. Review: amino acid domains involved in constitutive activation of G-protein-coupled receptors. Mol. Neurobiol. 17, 109–135.

Prioleau, C., Visiers, I., Ebersole, B.J., Weinstein, H., Sealfon, S.C., 2002. Conserved helix 7 tyrosine acts as a multistate conformational switch in the 5HT2C receptor. Identification of a novel "locked-on" phenotype and double revertant mutations. J. Biol. Chem. 277, 36577–36584.

Rivkees, S.A., Barbhaiya, H., IJzerman, A.P., 1999. Identification of the adenine binding site of the human A_1 adenosine receptor. J. Biol. Chem. 274, 3617–3621.

Shryock, J.C., Ozeck, M.J., 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.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C., 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150, 76–85.

Van Calenbergh, S., Link, A., Fujikawa, S., De Ligt, R.A.F., Vanheusden, V., Golisade, A., Blaton, N.M., Rozenski, J., IJzerman, A.P., Herdewijn, P., 2002. 5′-Deoxy congeners of 9-(3-amido-3-deoxy-β-D-xylofuranosyl)-N^6-cyclopentyladenine: new adenosine A_1 receptor antagonists and inverse agonists. J. Med. Chem. 45, 1845–1852.

Van der Wenden, E.M., Von Frijtag Drabbe Künzel, J.K., Mathôt, R.A., Danhof, M., IJzerman, A.P., Soudijn, W., 1995. Ribose-modified adenosine analogues as potential partial agonists for the adenosine receptor. J. Med. Chem. 38, 4000–4006.