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Differential effects of the allosteric enhancer (2-amino-4,5-dimethyl-trienyl)[3-(trifluoromethyl) phenyl]methanone (PD81,723) on agonist and antagonist binding and function at the human wild-type and a mutant (T277A) adenosine A_1 receptor

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Abstract

The 2-amino-benzoylthiophene derivative PD81,723 [(2-amino-4,5-dimethyl-trienyl)[3-(trifluoromethyl) phenyl]methanone] has been shown to allosterically enhance agonist binding and function at the adenosine A_1 receptor. The aim of the present study was to elucidate the effects of PD81,723 both as an allosteric enhancer and as an antagonist on the adenosine A_1 receptor. We investigated its effect on the human wild-type in relation to a mutant (T277A) adenosine A_1 receptor for which agonists have a greatly diminished affinity. Binding (saturation and displacement experiments) and functional adenosine 3′,5′-cyclic monophosphate studies were performed, and differential effects of allosteric enhancer PD81,723 on agonists and antagonists were observed on the wild-type (wt) and mutant adenosine A_1 receptor. Our results showed opposite effects of PD81,723 on the binding of agonists and antagonists. Within the concept of a simplified two-state receptor model, it is possible that the effects of PD81,723 are mainly "allosteric", enhancing the binding of adenosine A_1 agonists and inhibiting the binding of antagonists/inverse agonists. However, the suggestion that PD81,723 acts as an allosteric inhibitor of DPCPX (1,3-dipropyl-8-cyclopentylxanthine) binding cannot be confirmed by kinetic studies, since PD81,723 does not seem to affect the dissociation kinetics of [^{3}H]DPCPX. Nevertheless, our results show that the action of PD81,723 on DPCPX binding is due to more than mere competitive antagonistic activity, i.e. binding to the ligand-binding site and competing with the binding of DPCPX, as suggested previously. The effect of PD81,723 on the mutant receptor was much less pronounced. Mutation of Thr277 to Ala not only decreased agonist affinity but also inhibited the effects of PD81,723. Insensitivity of the mutT277A to PD81,723 may be linked to the fact that this mutant appears to be uncoupled from G proteins. It further supported a differential binding mode of PD81,723 compared to competitive antagonists for the adenosine A_1 receptor.

Keywords: G protein-coupled receptors; Allosteric; Modulation; PD81, 723; Adenosine A_1; Mutant

1. Introduction

The actions of adenosine in the mammalian body are mediated by four adenosine receptor subtypes, A_1, A_{2A}, A_{2B}, and A_3 [1]. The adenosine A_1 receptor is interfaced with a G_i protein that is negatively coupled to the adenylate cyclase–cAMP signal transduction pathway, and thus, upon activation, leads to a reduction in intracellular cAMP levels. The adenosine A_1 receptor is highly and widely expressed in the CNS, but also in other tissues such as fat cells, bladder, and heart [1]. A variety of adenosine-mediated effects (hypotension, inhibition of lipolysis, analgesia) occurs via the adenosine A_1 receptor, rendering it an important target for pharmacological intervention. Due to their ubiquitous presence, the ability to selectively modulate adenosine A_1 receptors within specific target tissues is of great therapeutic importance. Such an opportunity of intervention is provided by the concept of allosteric modulation of G-

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Abbreviations: CCPA, 2-chloro-N^6-cyclopentyladenosine; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; CPA, N^6-cyclopentyladenosine, R-PIA, N^6-[-(*R*)-1-methyl-2-phenylethyl]adenosine; NECA, 5′-(N-ethyl)-carboxamidoadenosine; N-0840, N^6-cyclopentyl-9-methyladenine; theophylline, 1,3-dimethylxanthine; PD81,723, (2-amino-4,5-dimethyl-trienyl)[3-(trifluoromethyl) phenyl]methanone; BCA, bicinchoninic acid; BCS, bovine calf serum; cAMP, adenosine 3′,5′-cyclic monophosphate; DMEM, Dulbecco's modified Eagle's medium; CHO, Chinese hamster ovary; and wt, wild-type.

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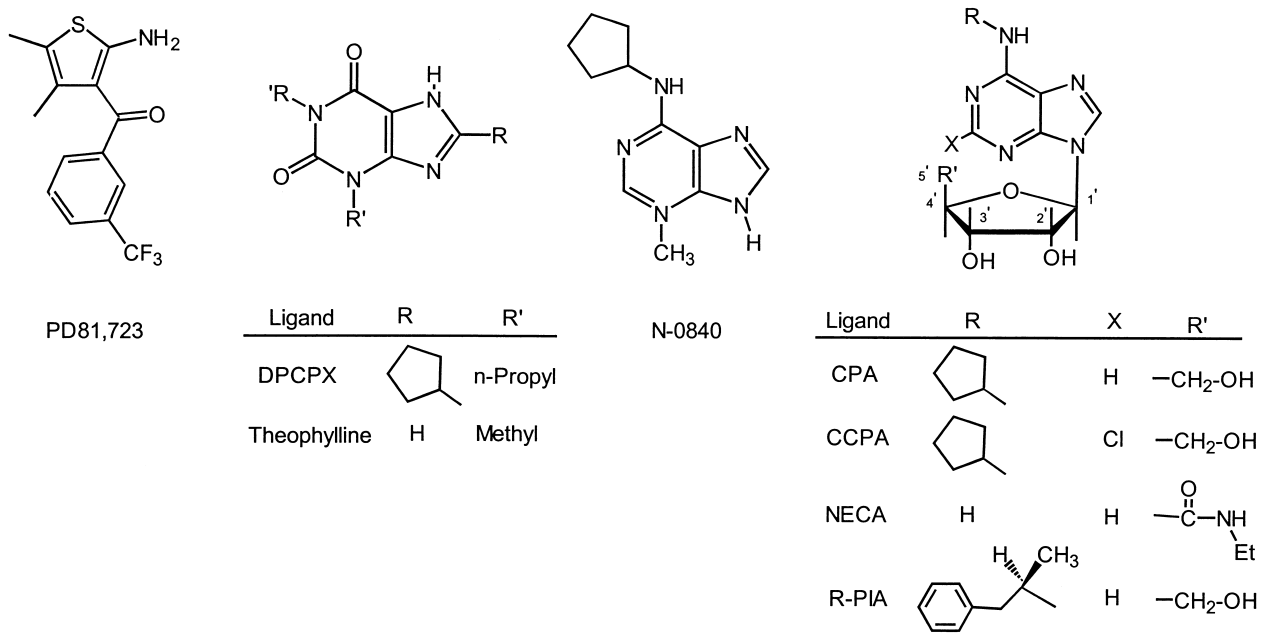

Fig. 1. Structures of PD81,723 and adenosine A_1 receptor ligands.

protein-coupled receptors (GPCRs). This concept, although more extensively investigated in other classes of GPCRs, such as the muscarinic receptor [2,3], has also been demonstrated for the adenosine A_1 receptor. A relatively new class of compounds, 2-amino-benzoylthiophene derivatives, has been shown to allosterically enhance agonist binding and function at the adenosine A_1 receptor [4–7].

One potent derivative, PD81,723, (Fig. 1) has been more extensively studied [4,8–13]. It may indeed offer the therapeutically desired selectivity by enhancing the actions of endogenously released adenosine *in vivo*. Although there is considerable interest in understanding how PD81,723 interacts with the adenosine A_1 receptor, no knowledge is yet available concerning its "allosteric" site of binding. The simultaneous antagonistic activity that PD81,723 and derivatives exhibit for the adenosine A_1 receptor has been attributed to competition for the ligand-binding site [4–6]. The precise molecular mechanism through which PD81,723 exerts its effects is thus not known. However, a large number of available data so far support the notion that PD81,723 stabilizes a high-affinity agonist-preferring state of the adenosine A_1 receptor [4,7].

Site-directed mutagenesis is a useful technique for elucidating ligand-receptor interactions by testing the role of several amino acids within the receptor. Mutation of Thr277 to Ala (mutT277A) on the adenosine A_1 receptor has been shown to greatly decrease the affinity of agonists without influencing the binding of antagonists [14–17]. Investigation of the effect of this mutation on the binding and action of PD81,723 would be of interest to potentially elucidate its mechanism of action. Thus, the aim of the present study was to better characterize the effects of PD81,723 both as an allosteric enhancer and as an antagonist on the adenosine A_1 receptor by investigating its effect on the human wt in relation to the mutT277A receptor.

2. Materials and methods

2.1 Materials

CPA, DPCPX, 8-cyclopentyltheophylline, and N-0840 were obtained from RBI. Adenosine deaminase was obtained from Boehringer Mannheim, while NECA, R-PIA, forskolin, theophylline, and BSA were from Sigma. cAMP was purchased from Serva, while PD81,723 was a gift from Parke-Davis. [^{3}H]DPCPX (specific activity 120 Ci/mmol) and [^{3}H]CCPA (specific activity 30 Ci/mmol) were purchased from NEN. BCA and BCA protein assay reagent were purchased from Pierce Chemical Co. BCS was obtained from HyClone Laboratories Inc., streptomycin/penicillin from Ducheva, cilostamide from Tocris Cookson Ltd., rolipram was a gift from Schering AG, and all other cell culture reagents were obtained from GIBCO Laboratories. CHO cells stably transfected with the human A_1 adenosine receptor (wild-type [$CHOA_1$-wt] and mutant [$CHOA_1$-mutT277A]) were obtained from the Garvan Institute of Medical Research [18]. All other chemicals were obtained from standard sources.

2.2. Cell culture

Cells were grown in a 1:1 mixture of DMEM and Ham's F12 medium containing 10% BCS, streptomycin (50 μg/mL), and penicillin (50 IU/mL) at 37° in 5% CO_2. Cells were subcultured (1:10) weekly.

Table 1
Saturation parameters of [^{3}H]DPCPX and [^{3}H]CCPA on CHOA$_1$-wt and of [^{3}H]DPCPX on CHOA$_1$-mutT277A cell membranes

		Control	+PD81,723	+Theophylline
	[^{3}H]CCPA			
	B_{max} (fmol/mg)	138 (± 9)	137 (± 8)	128 (± 6)
	K_d (*nM*)	1.8 (± 0.2)	1.2 (± 0.3)	3.3 (± 0.6)*
CHOA$_1$-wt				
	[^{3}H]DPCPX			
	B_{max} (fmol/mg)	650 (± 71)	633 (± 74)	779 (± 251)
	K_d (nM)	1.6 (± 0.1)	8.8 (± 1.4)**	6.8 (± 2.0)*
	[^{3}H]DPCPX			
CHOA$_1$-mutT277A	B_{max} (fmol/mg)	55.8 (± 2.7)	64.4 (± 9.1)	67.7 (± 3.8)*
	K_d (nM)	0.2 (± 0.0)	0.5 (± 0.1)*	2.1 (± 0.7)*

Saturation parameters of [^{3}H]DPCPX and [^{3}H]CCPA on CHOA$_1$-wt and of [^{3}H]DPCPX on CHOA$_1$-mutT277A cell membranes in the absence or presence of 10 μM PD81,723 or 10 μM theophylline. Values are the means (± SEM) of three experiments performed in duplicate.
Significance from control value * $P < 0.05$.; ** $P < 0.01$.

2.3. Membrane preparation

CHOA$_1$ cell membranes were prepared as described previously [17]. Membrane protein concentration was measured by the BCA method [19].

2.4. Receptor binding assays

Membrane aliquots, containing 40 μg (CHOA$_1$-wt membranes) or 80 μg (CHOA$_1$-mutT277A membranes) protein, were incubated in 400 μL of 50 mM Tris–HCl, pH 7.4 at 25° for 90 min in the presence or absence of PD81,723 (10 μM) and relevant (radio)ligands. For saturation experiments, concentrations of [^{3}H]DPCPX or [^{3}H]CCPA ranged from 0.1 to 12 nM. Displacement experiments were carried out with 1.6 nM [^{3}H]DPCPX. Non-specific binding was measured in the presence of 10 μM CPA or 8-cyclopentyltheophylline. Displacement experiments on intact CHOA$_1$ cells were carried out with 0.4×10^6 cells/sample. Separation of bound from free radioligand was performed by rapid filtration through Whatman GF/B filters that were subsequently washed three times with ice-cold buffer. Filter-bound radioactivity was measured by scintillation spectrometry after the addition of 3.5 mL of Packard Emulsifier Safe.

2.5. cAMP assay

CHOA$_1$ cells were harvested, using a trypsin solution (0.25% in PBS/EDTA) and, after centrifugation, resuspended in medium and plated in 24-well plates at 400 μL or 2×10^4 cells/well. After 24 hr, the medium was removed and the cells were washed three times with 500 μL DMEM, containing 50 mM HEPES, pH 7.4. Subsequently, the cells were incubated in 300 μL DMEM + HEPES for 45 min (37°, 5% CO_2) with rolipram and cilostamide (10 μM each), adenosine deaminase (10 U/mL), and various concentrations of ligands with or without PD81,723 (10 μM). After the addition of 100 μL forskolin (10 μM), cells were incubated for an additional 15 min (37°, 5% CO_2). The assay was terminated by removing the supematant, and cells were lysed by the addition of 200 μL of 0.1 M cold HCl. The cell lysate was resuspended and stored at −20°. For determination of the amount of cAMP produced, protein kinase A (PKA) was incubated for 3 hr on ice in a final volume of 200 μL containing 100 μL PKA in buffer (K_2HPO_4, 150 mM; EDTA, 10 mM; BSA, 0.2%), 50 μL [^{3}H]cAMP (2 μM) in K_2HPO_4/EDTA buffer (K_2HPO_4, 150 mM; EDTA, 10 mM), 20 μL of the cell lysate, and 30 μL 0.1 M HCl or 50 μL of cAMP solution (concentration range of 0–16 pmol/200 μL for the generation of a standard curve). Bound radioactivity was separated by rapid filtration through Whatman GF/C filters and washed once. Filter-bound radioactivity was measured by scintillation spectrometry after the addition of 3.5 mL of Packard Emulsifier Safe.

2.6. Calculations

All binding data were analysed using the non-linear regression curve fitting program GraphPad Prism v. 2.01 (GraphPad). 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. Inhibitory binding constants (K_i values) were derived from the IC_{50} values according to the Cheng and Prusoff equation $K_i = IC_{50}/(1 + [C]/K_d)$ where [C] is the concentration of the radioligand and K_d its dissociation constant [20]. All values obtained are means of at least three independent experiments performed in duplicate.

3. Results

3.1. Saturation assays

On the CHOA$_1$-wt cell membranes (Table 1, Fig. 2A), the K_d value of [^{3}H]DPCPX increased approx. 5-fold in the

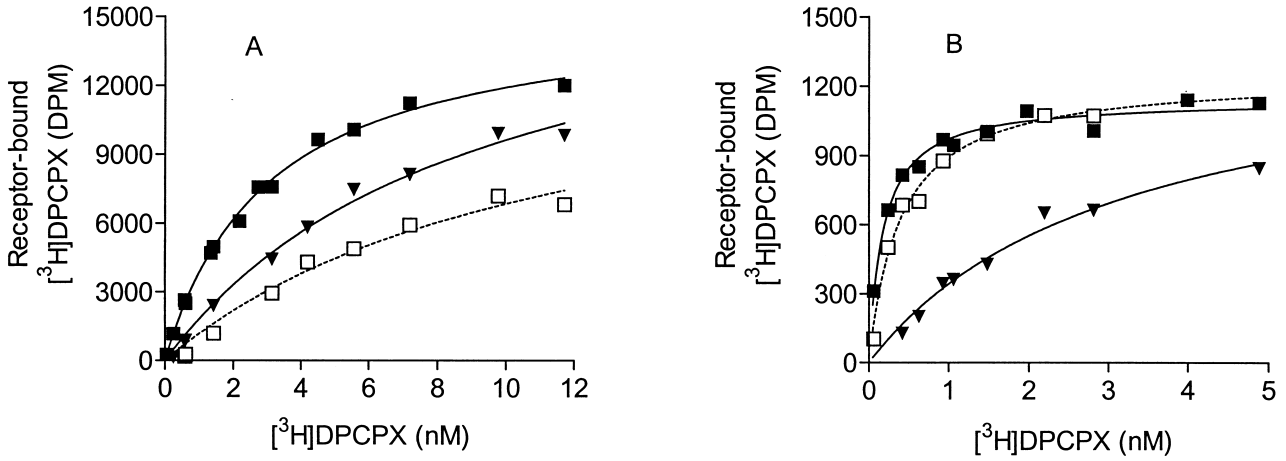

Fig. 2. Saturation curves for the binding equilibrium of [^{3}H]DPCPX to CHOA$_1$-wt (A) and CHOA$_1$-mutT277A (B) cell membranes. Control curve (■), in the presence of 10 μM PD81,723 (□) or 10 μM theophylline (▼). Representative graphs from one experiment performed in duplicate (see Table 1 for K_d and B_{max} values).

presence of 10 μM PD81,723, with no significant influence on B_{max}. Under similar conditions, 10 μM of the competitive antagonist theophylline (K_i = 8 μM, data not shown) influenced the K_d of [^{3}H]DPCPX in a comparable way, although to a lesser extent (approx. 4-fold). For agonist [^{3}H]CCPA binding on CHOA$_1$-wt cell membranes, the reverse was observed. In the presence of 10 μM PD81,723, a slight increase in affinity was observed, while theophylline (10 μM) increased the K_d value of [^{3}H]CCPA approx. 2-fold. On CHOA$_1$-mutT277A cell membranes (Table 1, Fig. 2B), PD81,723 had a relatively minor effect, increasing the K_d value of [^{3}H]DPCPX approx. 2-fold. Theophylline in this case increased the K_d value approx. 10-fold.

3.2. Displacement assays

Displacement experiments of [^{3}H]DPCPX on CHOA$_1$-wt or mutT277A cell membranes by various adenosine A$_1$ receptor agonists were carried out in the presence or absence of 10 μM PD81,723. Affinities of the tested ligands under these conditions are shown in Table 2, and are expressed as apparent K_i values as derived by a one-site competition analysis of the binding data. On the CHOA$_1$-wt cell membranes, affinities of all agonists increased 2- to 3-fold in the presence of PD81,723, the larger affinity shift being observed for R-PIA. On the CHOA$_1$-mutT277A cell membranes, affinities of agonists (CPA, NECA, and R-PIA) were greatly reduced from the nM (wt) to μM range (Table 2, Fig. 3). The lowest affinity for this mutant receptor was shown for NECA, with a 22,000-fold decrease in its K_i compared to the wt receptor, followed by CPA (5,000-fold decrease) and R-PIA (500-fold decrease). PD81,723 in this case did not have any effect on the affinities of the agonists, since K_i values in its presence remained virtually unchanged and within the μM range (Table 2).

3.3. cAMP assays

Assays were carried out to determine the effect of PD81,723 in various ligands on cAMP production in CHOA$_1$-wt and CHOA$_1$-mutT277A cells. In both cases, cells were stimulated with forskolin (10 μM), and cAMP production by various ligands was recorded as a percentage of forskolin-stimulated cAMP (=100%). CHOA$_1$-wt cells (receptor density approx. 650 fmol/mg protein) were incu-

Table 2
Affinities of various agonists in the absence or presence of PD81,723 in displacement experiments with 1.6 nM [^{3}H]DPCPX on CHOA$_1$ cell membranes (wild-type or mutant)

	CHOA$_1$-wt membranes			CHOA$_1$-mutT277A membranes		
	K_i (nM)	K_i (nM) +PD81,723	Shift[a]	K_i (μM)	K_i (μM) +PD81,723	Shift[a]
CPA	2.29 (± 0.11)	1.17 (± 0.61)	1.96*	11.31 (± 5.00)	19.10 (± 4.01)	0.59
NECA	7.82 (± 0.66)	3.11 (± 0.94)	2.51*	176.0 (± 48.5)	123.9 (± 14.1)	1.43
R-PIA	3.09 (± 0.57)	0.93 (± 0.25)	2.87*	1.73 (± 1.20)	2.36 (± 1.74)	0.73

Affinities of various agonists in the absence or presence of 10 μM PD81,723 in displacement experiments with 1.6 nM [^{3}H]DPCPX on CHOA$_1$ cell membranes (wild-type or mutant). Values are the means (± SEM) of three experiments performed in duplicate.

[a] Shift is expressed as the ratio of K_i values of ligand in the absence and presence of 10 μM PD81,723.

* $P < 0.05$.

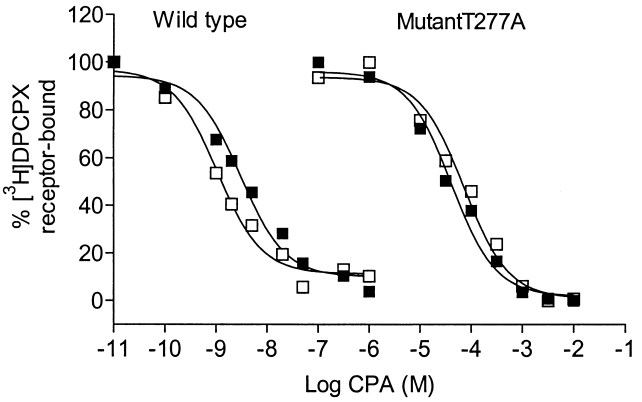

Fig. 3. Displacement of [^{3}H]DPCPX by CPA from human wild-type and mutant adenosine A_1 receptors in the absence (■) or presence (□) of PD81,723 (10 μM). Representative graphs from one experiment performed in duplicate (see Table 2 for affinity values).

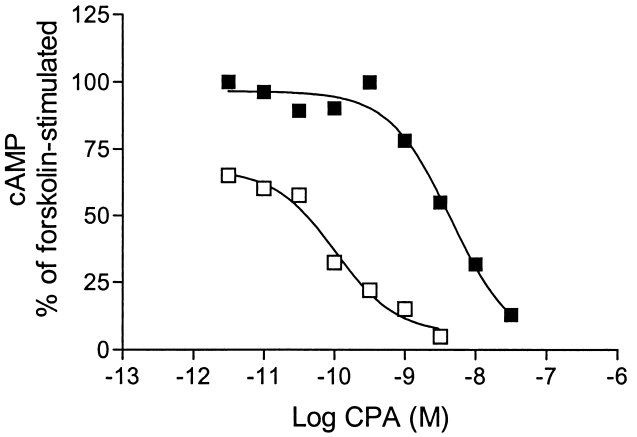

Fig. 5. Forskolin-stimulated cAMP production of $CHOA_1$ cells after addition of CPA in the absence (□) or presence (■) of 10 μM PD81,723. Representative graph from one experiment performed in triplicate.

bated with 1 nM CPA, 0.16 μM DPCPX, or 21 μM N-0840 in the absence or presence of PD81,723 (10 μM) as well as with PD81,723 alone. $CHOA_1$-mutT277A cells were incubated with 10^{-4} M CPA or DPCPX in the presence or absence of PD81,723.

On $CHOA_1$-wt cells, PD81,723 (10 μM) alone inhibited cAMP production to approx. 70–80% of forskolin-stimulated levels. The agonist CPA decreased cAMP production to 70% at a concentration of 1 nM, while PD81,723 further decreased the cAMP production in combination with CPA (Fig. 4). DPCPX increased the cAMP production by approx. 30%. In the additional presence of PD81,723 cAMP production decreased to forskolin levels (87% of forskolin-stimulated). Antagonist N-0840 had no influence on cAMP production either in the absence or presence of PD81,723. For the agonist CPA, a dose–response curve was recorded in the absence and presence of 10 μM PD81,723 (Fig. 5). EC_{50} values obtained were 4.17 (± 0.62) nM and 0.55 (± 0.47) nM (N = 3) in the absence and presence of PD81,723, respectively. Both CPA and DPCPX, at much higher concentrations (10^{-4} M), failed to influence cAMP production beyond forskolin-stimulated levels on $CHOA_1$-mutT277A cells. PD81,723 also failed to have an effect, either alone or in combination with CPA or DPCPX.

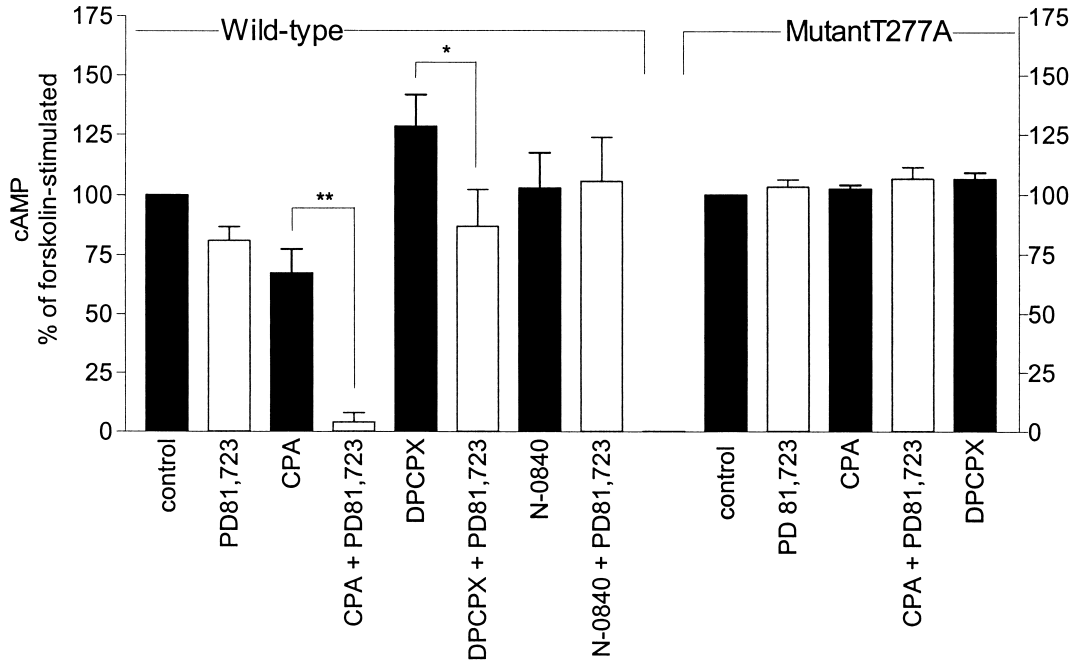

Fig. 4. cAMP production by $CHOA_1$ (wild-type and mutant T277A) cells after addition of CPA (1 nM), DPCPX (0.16 μM), or N-0840 (21 μM) in the absence or presence of PD81,723 (10 μM). cAMP production was also determined for 10 μM PD81,723 alone. Values are the means (± SEM) of 3–4 experiments performed in duplicate. $^*P < 0.05$, $^{**}P < 0.005$.

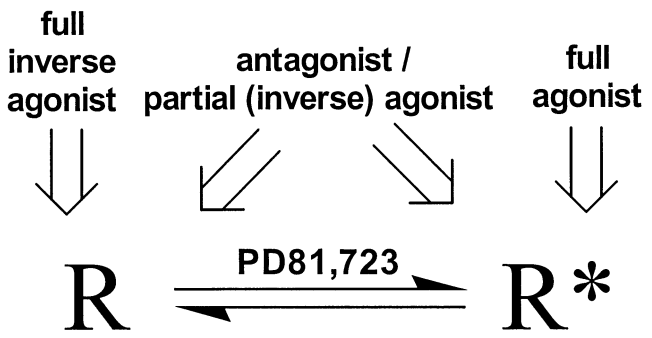

Fig. 6. Simplified model representing the two conformational states of a receptor (inactive R, and active R*) and their differential affinities for ligands.

4. Discussion

We would like to focus on two aspects of our findings: i) the diverse effects of PD81,723 on agonist and antagonist binding on the human wt adenosine A_1 receptor; and ii) the diverse effects of allosteric enhancer PD81,723 on human versus mutant T277A adenosine A_1 receptor. *On the human adenosine A_1 wt receptor*, we observed opposite effects of PD81,723 on the binding of agonist [^{3}H]CCPA and antagonist [^{3}H]DPCPX (Table 1). The increase in affinity of agonist [^{3}H]CCPA by PD81,723, although not significant compared to the 2- to 3-fold increase in affinity of agonists (CPA, R-PIA, NECA) in displacement experiments (Table 2), is in accordance with the hypothesis that PD81,723 tends to stabilise a "high affinity for agonists" receptor state [21]. Within the concept of a simplified two-state receptor model (Fig. 6), this same state is a "low-affinity" state for inverse agonists. On the assumption that DPCPX is an inverse agonist as has been recently suggested [22], this may explain the significant decrease in affinity of [^{3}H]DPCPX in the presence of PD81,723. Although it has been previously reported [5,6] that PD81,723 possesses competitive antagonistic activity by binding to the ligand-binding site and competing with the binding of DPCPX, it may be that PD81,723 also acts as an allosteric inhibitor of DPCPX binding. However, in kinetic experiments in which the dissociation of [^{3}H]DPCPX was followed over time, no influence of PD81,723 could be established. Both in the absence and presence of 10 μM PD81,723 the dissociation rate constant of DPCPX remained unchanged (results not shown). The distinct properties of PD81,723 as an allosteric enhancer versus a competitive antagonist were further demonstrated here by the verification that PD81,723 did not increase the K_d of agonist [^{3}H]CCPA (if anything it reduced it) like a classical competitive antagonist. Theophylline, a competitive antagonist with similar apparent affinity as PD81,723 for the adenosine A_1 receptor, under the same experimental conditions, as expected, increased the K_d of [^{3}H]CCPA approx. 2-fold, showing an opposite effect compared to PD81,723.

In an attempt to identify whether different agonists were all equally sensitive to PD81,723 on the human adenosine A_1 receptor, we evaluated the binding (in the presence or absence of PD81,723) of CPA, NECA, and R-PIA. These compounds are all agonists with high affinities for the adenosine A_1 receptor but with potentially different binding modes, as suggested from adenosine A_1 receptor mutation/binding data [23]. Although the increase in binding by PD81,723 of all agonists on the $CHOA_1$-wt cell membranes ranged from 2- to 3-fold, with R-PIA exhibiting the larger increase in affinity, the effect of PD81,723 did not seem to be significantly different for the various agonists. In our view, this is another indication that PD81,723 binds to a site distinct from the ligand-binding site and exerts its effect without directly affecting the binding for agonists. This is also in line with the view that PD81,723 lacks competitive antagonistic activity.

Activation of adenosine A_1 receptors inhibits adenylate cyclase activity. To obtain a significantly high control level of cAMP, assays were carried out in the presence of forskolin. The effects of PD81,723 on these functional studies (cAMP production) were consistent with its behavior in binding studies. Alone, it inhibited cAMP production to some extent, an effect that is attributed to the promotion of the adenosine A_1 receptor to its active conformation (R*) and thus potentiation of constitutive receptor activity, as has also been suggested elsewhere [13]. The activity of CPA (at a concentration approx. equal to its K_i) in reducing cAMP levels on $CHOA_1$-wt cells was enhanced in the presence of PD81,723. This has also been shown previously for cAMP production mediated by the rat adenosine A_1 receptor [24]. Further, the increase in cAMP by DPCPX (at a concentration equal to 100 times its K_i) is indicative of its inverse agonistic behavior. This has also been observed on CHO cells overexpressing the human adenosine A_1 receptor (~8 pmol/mg protein) [22]. In the same study, antagonist N-0840 did not have any effect, although neither DPCPX nor N-0840 was evaluated in the presence of PD81,723. Although not significant compared to control values, the effect of PD81,723 in combination with DPCPX (inverse agonist) and N-0840 (neutral antagonist) may be indicative of the differential preference of these two ligands for the two conformational states (R vs R*) of the receptor, the equilibrium between which is altered by PD81,723. By shifting the receptor towards the R* state, PD81,723 inhibits the binding and thus the function, i.e. the increase in cAMP production, of DPCPX.

On the human adenosine A_1 mutant T277A receptor, a different picture emerged. The effect of PD81,723 on [^{3}H]DPCPX binding to this receptor was much less pronounced. Since the effect of PD81,723 was distinct in this case from the effect of the competitive antagonist theophylline, which caused a 10-fold increase in K_d, we propose different binding modes to this receptor of the two compounds. The binding of PD81,723 at the potential "allosteric" site is disturbed or else no conformational changes are induced by PD81,723. In addition, the differential effect (or binding modes) of PD81,723 and theophylline on this re-

ceptor further supports an "allosteric" interaction of PD81,723 on the wt adenosine A_1 receptor versus "competitive antagonism" with DPCPX.

The lack of effect of PD81,723 on agonist binding on this mutant receptor (Table 2) may also be explained by the possibilities mentioned above. Since the affinity of agonist CPA for this mutant receptor remained unchanged in the presence of 1 mM GTP (results not shown), it seems that this mutant receptor is uncoupled. This uncoupled state of the mutT277A receptor may also account for its insensitivity to PD81,723. One of the suggested modes of action of PD81,723 is the increase in the fraction of receptors coupled to the G protein. The action of PD81,723 in this case may be insufficient to override the inability of the mutant receptor to couple to the G protein. The conserved affinity of this receptor for antagonists (e.g. DPCPX, theophylline) but not for agonists indicates that this mutant receptor may be comparable to the low-affinity (R) state of the adenosine receptor according to the simplified two-state model (Fig. 6). Thus, this mutant may be compared with a receptor permanently "locked" in a low-affinity state for agonists, which neither allosteric modulators such as PD81,723 nor agonists are able to "unlock". Although a more pronounced effect (very low affinity) on this mutant has been reported for NECA [14], we found greatly reduced affinities for all tested agonists (CPA, NECA, and R-PIA). This somewhat contradicts the proposed specific interaction of Thr277 with the 5′ ribose substituent of NECA [14], but indicates a more general role of this residue in interacting with agonists.

No information on the function of this mutant receptor has yet been reported. The lack of effect of CPA or PD81,723 on forskolin-stimulated cAMP production on these cells may either be due to the non-functionality of this receptor (e.g. its inability to couple to the G protein) or to the extensive loss of agonist binding and activation. PD81,723 has recently been reported to directly inhibit adenylate cyclase, i.e. independently of its interaction with the adenosine A_1 receptor [24]. However, this effect was not observed in the case of the $CHOA_1$-mutT277A cell line.

In conclusion, binding and functional studies were performed and differential effects of allosteric enhancer PD81,723 on agonists and antagonists were observed on the wt and mutant adenosine A_1 receptor. Within the concept of a simplified two-state receptor model, it is possible that the effects of PD81,723 are mostly "allosteric", enhancing the binding of adenosine A_1 agonists and inhibiting the binding of inverse agonists. Mutation of Thr277 to Ala not only decreases agonist binding to the receptor, but also abolishes the effects of PD81,723.

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