Adenosine A1 Receptor-Dependent and -Independent Effects of the Allosteric Enhancer PD 81,723

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Accepted for publication August 5, 1998

ABSTRACT

The 2-amino-3-benzylthiophene PD 81,723 has been shown to exhibit allosteric enhancement of adenosine A1 receptor binding and function. The aim of this study was to clarify the mechanism of this effect using membranes purified from rat brain and Chinese hamster ovary (CHO)-A1 cells that stably express the rat adenosine A1 receptor as well as intact CHO-A1 cells. Membranes containing 100 μM magnesium, (2-amino-4,5-dimethyl-3-thienyl) [3-(trifluoromethyl)phenyl]methanone (PD 81,723) significantly increased the affinity of the adenosine A1 receptor agonist, cyclopentyladenosine, for the low-affinity receptor without affecting high-affinity binding or Bmax. In intact cells, PD 81,723 inhibited basal adenylyl cyclase (AC) activity as well as forskolin-, cholera toxin-, and pertussis toxin-stimulated AC activity in CHO-A1 and CHO cells. Basal AC activity was inhibited 49% in CHO and 82% in CHO-A1 cells by 30 μM PD 81,723. In CHO-A1 cells, half-maximal inhibition of forskolin-stimulated AC occurred at 5 μM PD 81,723 compared to 10 μM in CHO cells. Cholera toxin-stimulated AC was reduced 90% in both CHO and CHO-A1 cells by 30 μM PD 81,723. At the same concentration of PD 81,723, pertussis toxin-stimulated AC activity was reduced 86% (CHO-A1) and 77% (CHO). [3H]forskolin was displaced from purified rat liver AC by PD 81,723 with an IC50 of 96 μM. These results demonstrate that two mechanisms appear to contribute to the observed effects of PD 81,723. One mechanism is allosteric enhancement of adenosine A1 receptor function. Results from transfected and nontransfected cells suggest that PD 81,723 also inhibits AC directly by binding to the catalytic unit at or near the forskolin-binding site.

The family of transmembrane receptors and G proteins coupled to adenylyl cyclase (AC) comprise a system of integrated components that function to couple cellular responses to a variety of molecular mediators (Fleming et al., 1992; Hepler and Gilman, 1992). For example, adenosine is produced in the heart in response to hypoxia and acts through G protein-coupled transmembrane adenosine A1 and A2 receptors to protect against tissue damage resulting from inadequate oxygen availability (Fredholm and Sollevi, 1986; Belardinelli et al., 1989). Adenosine's cardioprotective effects result from reduction in oxygen demand by slowing heart rate and nodal conduction, by increasing oxygen supply through coronary vasodilation, and by antagonizing the effects of catecholamines through inhibition of AC (Dobson and Schrader, 1984; Clemo and Belardinelli, 1986; Amoah-Apraku et al., 1993; Romano and Dobson, 1996).

Adenosine A1 receptors are located predominately in coronary arteries. Activation of A1 receptors increases blood flow and oxygen availability to cardiac tissues (Fredholm and Sollevi, 1986; Belardinelli et al., 1989). Inhibitory adenosine A1 receptors are located in atrial and ventricular myocardium and in nodal areas associated with these tissues. Activation of adenosine A1 receptors in the myocardium decreases cardiac oxygen demand through direct and indirect mechanisms. Direct effects include reduction of rate and force of contraction by opening sarcolemmal K+ channels (Belardinelli et al., 1989). Adenosine A1 receptor activation also acts indirectly to antagonize beta adrenergic stimulation by inhibiting the formation of cyclic AMP (cAMP) by AC (Belardinelli et al., 1989).

Allosteric enhancement of adenosine A1 receptor activation could be a potentially useful therapeutic intervention. By selectively enhancing A1 receptor tone near the site of

ABBREVIATIONS: ABA, aminobenzyladenosine; AC, adenylyl cyclase; ADA, adenosine deaminase; CHO, Chinese hamster ovary; CI, confidence interval; CPA, N6-cyclopentyladenosine; [3H]DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; GppNHp, 5’-guanylylimidodiphosphate; IBMX, isobutylmethylxanthine; PBS, phosphate-buffered saline; PD 81,723, (2-amino-4,5-dimethyl-3-thienyl)[3-(trifluoromethyl)phenyl]methanone; TSH, thyroid-stimulating hormone; dhfr, dihydrofolate reductase; FRTL-5, Fisher rat thyroid cells; cpm, counts per minute.
adenosine synthesis, allosteric enhancement should decrease the potential for systemic side effects by alleviating a ubiqui-
tuous systemwide response observed with administration of
adenosine or adenosine agonists. (2-Amino-4,5-dimethyl-3-thienyl)-3-[trifluoromethyl]phenyl)methanone (PD 81,723) has
been reported to exhibit selective allosteric enhancement
of adenosine A1 receptor binding and function in the absence of Mg\(^{2+}\) in membranes obtained from a variety of tissues
(Bruns and Fergus, 1999; Kollias-Baker et al., 1994; Bhatta-
charaya and Linden, 1995) as well as in isolated tissues (Mu-
dumbi et al., 1993). In the present study, we have used
Chinese hamster ovary (CHO) cells expressing an adenosine
A1 receptor derived from rat brain as well as a rat brain
membrane preparation containing native adenosine A1 re-
ceptors to characterize the interaction of PD 81,723 with
adenosine A1 receptor agonists, antagonists, and AC. In
addition, the present study demonstrates the effect of Mg\(^{2+}\) on
these interactions.

Materials and Methods

Materials. Imidazole and adenosine deaminase (in glycerol
(ADA)) were obtained from Boehringer Mannheim Biochemicals
(Indianapolis, IN); carrier-free \(^{125}\)I[Na and \(^{3}H\)forskolin was obtained
from Amersham (Arlington Heights, IL). 8-Cyclopentyl-1,3-cyclopen-
yl adenosine (\(^{3}H\)DPCPX) was purchased from DuPont-NEN (Boston,
MA); Tris, HEPES, sucrose, NaEDTA, forskolin, MgCl\(_2\), 5′-guanylylimidodiphosphate (Gpp[NH]p), polyethyleneimine,
and aminobenzyl adenine (ABA) were purchased from Sigma (St.
Louis, MO). Cholera toxin and pertussis toxin were obtained from
Calbiochem (La Jolla, CA), and \(^{3}H\)-cyclopentyl adenosine (CPA), pep-
statin, and leupeptin were purchased from Research Biochemicals, Inc.
(Natick, MA). PD 81,723 was a gift from Dr. Robert F. Bruns (Ely Lilly,
Indianapolis, IN). The adenosine A1 receptor cDNA (pCDNA-A1) was
kindly provided by Dr. Joel Linden (University of Virginia, Charlotte-
town, VA). Iodinated ABA (\(^{125}\)IABA) was synthesized as described
previously (Linden et al., 1985). All tissue culture reagents were ob-
tained from Sigma or ICN (Costa Mesa, CA). CHO cells and Fisher rat
thyroid (FRTL-5) cells were obtained from the American Type Culture
Collection (Rockville, MD).

Cell Line Generation. Linearized DNA from the adenosine A1
receptor cDNA and from a plasmid coding for dihydrofolate reduc-
tase were cotransfected into CHO cells. The dhfr\(^{-}\) transfectants
were obtained by growing the CHO cells in selection medium (Dul-
becco’s modified Eagle’s medium) supplemented with 10% dialyzed
fetal bovine serum without nucleotides. Three individual clones were
obtained by growing the CHO cells in selection medium (Dul-
becco’s modified Eagle’s medium) supplemented with 10% dialyzed
fetal bovine serum, 100 U/ml penicillin G, 0.1 m
MgCl\(_2\), 5′-guanylylimidodiphosphate, Tris, HEPES, sucrose, Na2EDTA, dimethyl sulfoxide, forskolin, MgCl\(_2\), 5′-guanylylimidodiphosphate, Tris, HEPES, sucrose, Na2EDTA, dimethyl sulfoxide, forskolin, MgCl\(_2\), 5′-guanylylimidodiphosphate, Tris, HEPES, sucrose, Na2EDTA, dimethyl sulfoxide, forskolin, MgCl\(_2\), 5′-guanylylimidodiphosphate, Tris, HEPES, sucrose, Na2EDTA, dimethyl sulfoxide, forskolin, MgCl\(_2\), 5′-guanylylimidodiphosphate, Tris, HEPES, sucrose, Na2EDTA, dimethyl sulfoxide, forskolin, MgCl\(_2\), 5′-guanylylimidodiphosphate, Tris, HEPES, sucrose, Na2EDTA, dimethyl sulfoxide, forskolin, MgCl\(_2\), 5′-guanylylimidodiphosphate, Tris, HEPES, sucrose, Na2EDTA, dimethyl sulfoxide, forskolin, MgCl\(_2\), 5′-guanylylimidodiphosphate, Tris, HEPES, sucrose, Na2EDTA, dimethyl sulfoxide, forskolin, MgCl\(_2\), 5′-guanylylimidodiphosphate, Tris, HEPES, sucrose, Na2EDTA, dimethyl sulfoxide, forskolin, MgCl\(_2\), 5′-guanylylimidodiphosphate, Tris, HEPES, sucrose, Na2EDTA, dimethyl sulfoxide, forskolin, MgCl\(_2\), 5′-guanylylimidodiphosphate, Tris, HEPES, sucrose, Na2EDTA, dimethyl sulfoxide, forskolin, MgCl\(_2\), 5′-guanylylimidodiphosphate, Tris, HEPES, sucrose, Na2EDTA, dimethyl sulfoxide, forskolin, MgCl\(_2\), 5′-guanylylimidodiphosphate, Tris, HEPES, sucrose, Na2EDTA, dimethyl sulfoxide, forskolin, MgCl\(_2\), 5′-guanylylimidodiphosphate, Tris, HEPES, sucrose, Na2EDTA, dimethyl sulfoxide, forskolin, MgCl\(_2\), 5′-guanylylimidodiphosphate, Tris, HEPES, sucrose, Na2EDTA, dimethyl sulfoxide, forskolin, MgCl\(_2\), 5′-guanylylimidodiphosphate, Tris, HEPES, sucrose, Na2EDTA, dimethyl sulfoxide, forskolin, MgCl\(_2\), 5′-guanylylimidodiphosphate, Tris, HEPES, sucrose, Na2EDTA, dimethyl sulfoxide, forskolin, MgCl\(_2\), 5′-guanylylimidodiphosphate, Tris, HEPES, sucrose, Na2EDTA, dimethyl sulfoxide, forskolin, MgCl\(_2\), 5′-guanylylimidodiphosphate, Tris, HEPES, sucrose, Na2EDTA, dimethyl sulfoxide, forskolin, MgCl\(_2\), 5′-guanylylimidodiphosphate, Tris, HEPES, sucrose, Na2EDTA, dimethyl sulfoxide, forskolin, MgCl\(_2\), 5′-guanylylimidodiphosphate, Tris, HEPES, sucrose, Na2EDTA, dimethyl sulfoxide, forskolin, MgCl\(_2\), 5′-guanylylimidodiphosphate, Tris, HEPES, sucrose, Na2EDTA, dimethyl sulfoxide, forskolin, MgCl\(_2\), 5′-guanylylimidodiphosphate, Tris, HEPES, sucrose, Na2EDTA, dimethyl sulfoxide, forskolin, MgCl\(_2\), 5′-guanylylimidodiphosphate, Tris, HEPES, sucrose, Na2EDTA, dimethyl sulfoxide, forskolin, MgCl\(_2\), 5′-guanylylimidodiphosphate, Tris, HEPES, sucrose, Na2EDTA, dimethyl sulfoxide, forskolin, MgCl\(_2\), 5′-guanylylimidodiphosphate, Tris, HEPES, sucrose, Na2EDTA, dimethyl sulfoxide, forskolin, MgCl\(_2\), 5′-guanylylimidodiphosphate, Tris, HEPES, sucrose, Na2EDTA, dimethyl sulfoxide, forskolin, MgCl\(_2\), 5′-guanylylimidodiphosphate, Tris, HEPES, sucrose, Na2EDTA, dimethyl sulfoxide, forskolin, MgCl\(_2\), 5′-guan...
ADA. CPA (0.1 mM) was used for the determination of nonspecific binding. Isotherm and competition assays were performed in duplicate (isotherms) or triplicate (competition) with CPA, and PD 81,723 was added at the same time as radioligand. AC competition experiments using [3H]forskolin (0.1–0.2 μM) and either forskolin or PD 81,723 were performed at room temperature for 15 min in 50 mM Tris containing 8 mM Mg++. All equilibrium binding assays were terminated using a Brandel cell harvester (Gaithersburg, MD) by filtration through Whatman GF/C filter paper presoaked in 0.5% polyethyleneimine. Filtered samples were rinsed free of unbound radioligand with ice-cold 5 mM Tris buffer for adenosine A1 receptor binding or at room temperature with 50 mM Tris containing 8 mM Mg++ for forskolin-binding experiments.

**AMP Determination.** cAMP was measured in intact CHO-A1 and CHO cells using a modification of a previously described method (Daniels et al., 1994). Briefly, cells were grown to near confluence in 96-well plates and the plates were inverted to remove medium. The adherent cells were washed with 200 μl of PBS and incubated at 37°C for 30 min in a total volume of 200 μl of PBS containing 2.5 U/ml ADA, 50 μM forskolin, and various concentrations of CPA and PD 81,723, depending on the assay being performed. For cholera toxin and pertussis toxin experiments, cells were exposed to 10 ng/ml cholera toxin or 100 ng/ml pertussis toxin in cell culture media for 1 h before removal of media and washing with PBS. Reactions were stopped by the addition of 50 μl of 1 N HClO4 followed by neutralization with 2 N KOH. Plates were immediately frozen at −70°C until assayed for cAMP using a previously described radioimmunoassay method (Daniels et al., 1994).

**Data Analysis.** Saturation experiments were analyzed using Lunden 1 Saturation Analysis Software (Cleveland, OH). Competition data were analyzed using the Marquardt algorithm (GraphPAD Software, San Diego, CA). The F statistic was used to compare competition curves for significant improvement of fit by a two-site model versus a one-site model. Data are presented as mean ± S.E.M. or as the geometric mean with 95% CI and were compared using analysis of variance. A P value of less than .05 is considered significant.

### Results

**Effects of Mg++ and PD 81,723 on Radioligand Binding in CHO Cell and Rat Brain Membranes.** The effect of Mg++ on the specific binding of [3H]DPCPX in the presence of 3 μM PD 81,723 was investigated in rat brain membranes. Mg++ decreased the specific binding of radioligand to the adenosine A1 receptor in a dose-dependent manner (Fig. 1A). In addition, the presence of Mg++ decreased the Kd for the agonist CPA, but this decrease was not concentration-dependent (Fig. 1B). We chose 100 μM Mg++ for all subsequent assays utilizing broken cell preparations.

The effect of increasing concentrations of PD 81,723 on specific binding of the agonist [125I]ABA to the adenosine A1 receptor was determined in isolated membranes from rat brain and CHO-A1 cells. In rat brain membranes, agonist-specific binding was significantly increased with increasing concentrations of PD 81,723 (Fig. 2). Maximum increase was seen at 5 to 10 μM PD 81,723, with slight decreases above 10 μM. The calculated Kd for agonist was unaffected by the concentration of PD 81,723 (Fig. 2). Similar results were obtained when CHO-A1 cell membranes were used.

No significant increase in adenosine A1 receptor affinity for agonist or A1 receptor density in the presence of PD 81,723 was observed in equilibrium binding experiments (P > .05, Table 1). In competition experiments, a significant increase in the affinity for agonist was observed for the low-affinity adenosine A1 receptor in CHO-A1 cell membranes and in rat brain membranes in the presence of PD 81,723 when both PD 81,723 and GppNHp were included in the incubation (P < .05, Table 2). No statistically significant effect on high-affinity adenosine A1 receptor binding was observed in either preparation, although a P value equal to .054 was observed for CHO-A1 cell membranes in the presence of PD 81,723 when compared with control.

**Effect of PD 81,723 on Forskolin-Stimulated cAMP Accumulation in CHO-A1 and Nontransfected CHO Cells.** To evaluate the function of the recombinant adenosine A1 receptor, the effects of receptor activation on forskolin-stimulated AC were studied in intact CHO-A1 cells and compared with the response in CHO cells lacking the receptor. Preliminary studies using rat liver membranes enriched for AC had shown that Mg++ inhibited the effect of PD 81,723 on AC in a concentration-dependent manner, but Mg++ was an absolute requirement for enzyme activity. An additional preliminary study in intact cells determined that residual intracellular Mg++ remaining after cell washing was sufficient for AC activity but did not markedly inhibit PD 81,723 effects in these cells. As a result of

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**Figure 1.** Effect of Mg++ on specific binding of [3H]DPCPX (A) and Kd for agonist (CPA) (B) in rat brain membranes in the presence of 3 μM PD 81,723. Binding data are presented as counts per minute (cpm) specifically bound or as percentage of sites bound. Values are mean ± S.E.M. (n = 3).

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**Figure 2.** Effect of PD 81,723 on specific binding of [3H]ABA (A) and Kd for agonist (CPA) (B) in rat brain membranes in the presence of 3 μM PD 81,723. Binding data are presented as counts per minute (cpm) specifically bound or as percentage of sites bound. Values are mean ± S.E.M. (n = 3).
measured in both CHO-A1 and nontransfected CHO cells. Because Cells.

6 statistically significant (1903 \( \pm \) 129 fmol in the presence of 30 nM DPCPX). As

these observations, subsequent cAMP accumulation assays in intact cells were performed in the absence of added Mg\(^{2+}\).

The ability of the adenosine A\(_1\) receptor agonist, CPA, to inhibit forskolin-stimulated cAMP accumulation in the presence and absence of PD 81,723 was determined in transfected CHO-A1 cells (Fig. 3). In these cells, 1.5 nM CPA decreased cAMP concentrations to basal levels with an IC\(_{50}\) of 0.1 nM. When cells were exposed to 3 \( \mu \)M PD 81,723 before the addition of CPA, cAMP production was reduced to 25\% of control values and was decreased to basal levels at 1 nM CPA. The addition of 10 \( \mu \)M PD 81,723, even in the absence of CPA, resulted in a complete inhibition of forskolin-stimulated cAMP accumulation (Fig. 3).

Addition of PD 81,723 resulted in a concentration-dependent inhibition of forskolin-stimulated cAMP accumulation in both CHO-A1 (Fig. 4A) and CHO cells (Fig. 4B). At lower concentrations (2.5–10 \( \mu \)M), the inhibitory effect of PD 81,723 was greater in transfected than in nontransfected cells (Fig. 5). In the absence of PD 81,723, exposure of CHO-A1 cells to 3 nM or 30 nM DPCPX in the presence of 0.1 nM CPA resulted in an absolute increase in AC activity as measured by cAMP accumulation, but these increases were not statistically significant (1903 \( \pm \) 174 versus 2383 \( \pm \) 261 fmol with 3 nM DPCPX, and 2280 \( \pm \) 139 fmol in the presence of 30 nM DPCPX). As shown in Fig. 4A, DPCPX at 3 and 30 nM resulted in a rightward shift of the PD 81,723 concentration response curve in CHO-A1 cells. The addition of 3 nM DPCPX during incubation partially reversed the inhibitory effect of 10 \( \mu \)M PD 81,723 on AC activity from 75% to 50\% of control. At 30 nM DPCPX, a further reversal of PD 81,723 inhibition of cAMP formation to 15\% of control levels was observed.

Identical experiments using the nontransfected CHO cells resulted in a dose-related 45\% decrease in forskolin-stimulated cAMP accumulation at 10 \( \mu \)M PD 81,723 and complete inhibition at 30 \( \mu \)M PD 81,723. DPCPX had no effect on PD 81,723 inhibition of forskolin-stimulated formation of cAMP in nontransfected CHO cells (Fig. 4B). In addition, nontransfected CHO cells exhibited no inhibition of cAMP formation in the presence of CPA (data not shown), confirming the absence of adenosine A\(_1\) receptor expression.

**Effect of PD 81,723 on Basal cAMP Accumulation in Intact Cells.** The effect of PD 81,723 on basal cAMP accumulation was measured in both CHO-A1 and nontransfected CHO cells. Because basal levels of cAMP in these cells were near the lower limit of detection of the assay, it was necessary to measure cAMP accumulation in the presence of the nonselective phosphodiesterase inhib-


tor and adenosine receptor antagonist, isobutylmethylxanthine (IBMX). The use of 500 \( \mu \)M IBMX during the incubation period allowed measurement of changes in basal cAMP levels. As summarized in Table 3, PD 81,723 inhibited basal cAMP accumulation in both cell types. In CHO cells, basal cAMP was reduced 11\% at 10 \( \mu \)M PD 81,723 and 48\% at 30 \( \mu \)M PD 81,723. In CHO-A1 cells, PD 81,723 inhibition of cAMP formation was 30 and 82\% at 10 and 30 \( \mu \)M, respectively. Furthermore, PD 81,723 inhibited both basal and TSH-stimulated cAMP formation in FRTL-5 cells. Basal cAMP accumulation was inhibited by 47\% and TSH-stimulated cAMP accumulation was reduced by 50\% in the presence of 30 \( \mu \)M PD 81,723 (data not shown).

**Effect of PD 81,723 on Cholera Toxin and Pertussis Toxin Activation of Adenylyl Cyclase in CHO-A1 and Nontransfected CHO Cells.** A series of experiments in intact cells was performed using cholera toxin and pertussis toxin to address the question of whether PD 81,723 inhibition of cAMP accumulation was mediated by G proteins. Activation of AC in CHO-A1 cells with either 10 ng/ml cholera toxin or 100 ng/ml pertussis toxin resulted in statistically significant 6.7-fold (cholera toxin) and 3.2-fold (pertussis toxin) increases in basal cAMP accumulation (\( P < .05 \), Table 3). Addition of PD 81,723 resulted in a concentration-dependent inhibition of both pertussis toxin- and cholera toxin-stimulated increases in cAMP accumulation in these cells. PD 81,723 at a concentration of 30 \( \mu \)M reduced cellular cAMP accumulation by 80 to 95\% in both control and toxin-treated cells (Fig. 6A, Table 3).

Similarly, in nontransfected CHO cells toxin pretreatment significantly increased basal cAMP accumulation. A 2.5-fold increase (\( P < .05 \)) was observed in pertussis toxin-treated cells, whereas cholera toxin treatment resulted in a 9.3-fold increase in basal AC activity (\( P < .05 \)). Even in these nontransfected cells, PD 81,723 produced concentration-dependent inhibition of pertussis toxin- and cholera toxin–stimulated increases in cAMP accumulation (Fig. 6B). cAMP accumulation in these cells was reduced 70\% in pertussis toxin-treated and 90\% in cholera toxin-treated cells at 30 \( \mu \)M PD 81,723 as compared to 50\% inhibition in untreated control cells (Table 3).

\([\text{H}]\text{Forskolin Binding to Partially Purified AC in the Absence and Presence of PD 81,723.}\) Competition experiments were performed using partially purified enzyme obtained from rat liver to evaluate the site of PD 81,723 induced inhibition of AC. Rat liver AC was chosen because of its abundance in liver, with established methods for isolation, and because it was not possible to isolate a sufficient quantity of AC from CHO cell membranes. In these experiments, forskolin competitively inhibited the binding of \([\text{H}]\text{forskolin with an IC}_{50}\) of 0.32 \( \mu \)M (95\% CI, 0.29 to 0.36 \( \mu \)M) in two experiments assayed in triplicate (Fig. 7). Using the same liver membrane preparation, PD 81,723 competitively displaced \([\text{H}]\text{forskolin with an IC}_{50}\) of 96 \( \mu \)M (95\% CI, 62 to 130 \( \mu \)M) in three experiments assayed in triplicate.

**Discussion.**

Studies using isolated intact hearts and isolated cardiac atrial and ventricular preparations have suggested a role for adenosine in protection against the effects of hypoxia (Dobson, 1983; Dobson and Schrader, 1984; Clemo and Beillardinelli, 1986; Warner et al., 1992). Under similar experimental conditions, the 2-amino-3-benzothiophene, PD 81,723, has been shown to enhance the effects of adenosine and adenosine analogs in these tissues (Janusz et al., 1991; Amoah-Apraku et al., 1993; Bhattacharya and Linden, 1995; Kollias-Baker et al., 1997). When tested experimentally using membranes obtained from cardiac and other tissues for its effect on adenosine receptor-specific binding and on the calculation of binding parameters, PD 81,723 in the absence of magnesium has generally shown marked increases in
agonsist-specific binding, but no or only modest increases in agonist affinity as defined by $K_i$, $EC_{50}$, and $T_{1/2}$ for dissociation (Bruns and Fergus, 1990; Kollias-Baker et al., 1994, 1997; Bhattacharya and Linden, 1995).

We found, as reported previously by other investigators (Bruns and Fergus, 1990; Janusz et al., 1991; Kollias-Baker et al., 1994; Bhattacharya and Linden, 1995), a concentration-dependent increase in agonist-specific binding to adenosine A$_1$ receptors in crude membrane preparations from rat brain and CHO-A$_1$ cells in the presence of 3 to 30 $\mu$M PD 81,723. In both preparations, this effect was exquisitely sensitive to magnesium ion concentration. At any given concentration of PD 81,723, the observed increase in agonist-specific binding was reduced by increasing concentrations of Mg$^{2+}$. Maximum increases in agonist-specific binding were observed in the absence of this divalent cation. We reasoned, however, that since agonist binding to the adenosine A$_1$ receptor and subsequent functional events would occur in the presence of Mg$^{2+}$ in intact cells, and because AC activation has an absolute requirement for Mg$^{2+}$, it was important to measure the effect of PD 81,723 in the presence of this divalent cation. Therefore, all experiments in this study using isolated membranes were performed in the presence of 100 $\mu$M MgCl$_2$. We also found that different membrane preparations tested under identical conditions produced significant differences in the extent of observed enhancement of agonist-specific binding (data not shown). This effect may reflect differences in the amount of residual Mg$^{2+}$ trapped in the membranes during purification. These observations suggest that the concentration-dependent increases in agonist-specific binding that have been observed in the presence of PD 81,723 may have minimal functional significance for adenosine receptor function within intact biological systems because this measurement is so dependent on the Mg$^{2+}$ concentration used in the binding assay and is maximized in its absence. We were unable, using either CHO-A$_1$ cell or rat brain membranes, to consistently correlate increases in specific binding with an increased affinity of the adenosine A$_1$

### Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CHO-A$_1$ Cells</th>
<th>Rat Brain</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_i$ (nM)</td>
<td>Control</td>
<td>PD 81,723</td>
</tr>
<tr>
<td>$B_{\text{max}}$ (fmol/mg)</td>
<td>Control</td>
<td>PD 81,723</td>
</tr>
<tr>
<td>CHO-A$_1$ cells</td>
<td>0.23 (0.19–0.27)</td>
<td>0.32 (0.27–0.35)</td>
</tr>
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<td>Rat brain</td>
<td>0.41 (0.31–0.49)</td>
<td>0.60 (0.42–0.74)</td>
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### Table 2

<table>
<thead>
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<th>Parameter</th>
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<th>Rat Brain</th>
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<td>$K_i$ (nM)</td>
<td>Control</td>
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</tr>
<tr>
<td>$B_{\text{max}}$ (fmol/mg)</td>
<td>Control</td>
<td>PD 81,723</td>
</tr>
<tr>
<td>CHO-A$_1$ cells</td>
<td>GppNHp</td>
<td>+GppNHp</td>
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<tr>
<td>$K_i$</td>
<td>4.87 (2.84–8.38)</td>
<td>49.9 (38.3–65.1)</td>
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<tr>
<td>$B_{\text{max}}$</td>
<td>52.4 ± 5.2</td>
<td>31.4 ± 9.3</td>
</tr>
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Statistical comparisons are of the effect of PD 81,723 on agonist binding. For clarity, statistical analysis of GppNHp effects are not included.

$a$: Significantly different from control, $-\text{GppNHp}$, $P < 0.05$.

$b$: Significantly different from control, $+\text{GppNHp}$, $P < 0.05$.
receptor for agonist as measured by saturation isotherms. In this study, neither $B_{\text{max}}$ nor $K_d$ was altered significantly in the presence of 3 mM PD 81,723 and 100 mM Mg$^{++}$. This differs somewhat from the results reported by Bhattacharya and Linden (1995), who found no significant increase in affinity for agonist in the presence of 20 mM PD 81,723 using equilibrium binding, but did report significant increases in calculated $B_{\text{max}}$ values. Kollias-Baker et al. reported similar increases in $B_{\text{max}}$ from saturation binding experiments using adenosine A$>_1$ receptor obtained from guinea pig brain (1994) and CHO cells expressing human recombinant A$>_1$ adenosine receptor (1997). The fact that a significant effect on $B_{\text{max}}$ by PD 81,723 was observed in these previous studies, but not in ours, may be explained by the higher concentration of PD used by these investigators and by the fact that their broken cell preparations contained no added Mg$^{++}$, which would have maximized specific binding in their preparations.

The data from competition experiments utilizing [3H]-DPCPX and the adenosine agonist CPA were best fit to a two-site model. The proportion of receptors in high- and low-affinity states was not significantly altered by PD 81,723 and although there was a consistent trend toward an increase in affinity for both high- and low-affinity forms of the receptor, these increases were not significant for $K_{\text{IH}}$. In contrast, Kollias-Baker et al. (1994) have reported that PD 81,723 increased the proportion of receptors in the high-affinity state as well as affinity of agonist for receptor. The competition data reported by Bhattacharya and Linden (1995) were best fit to a single-site curve, but these authors also found a significant increase in agonist affinity in the presence of PD 81,723. Since Mg$^{++}$ is known to act as a promoter of coupling between receptor and G protein (Birnbaumer et al., 1990), the absence of Mg$^{++}$ in their membrane preparations may account for the observed differences. In broken cell preparations in which no Mg$^{++}$ is available, PD 81,723 may function to promote coupling of G proteins to adenosine A$>_1$ receptors. In preparations in which Mg$^{++}$ is present, no additional coupling activity would be observed. Alternatively, it has been suggested recently (Kollias-Baker et al., 1997) that PD 81,723 may potentiate constitutive receptor activity by promoting isomerization of the adenosine A$>_1$ receptor to its active conformation. If interaction between the receptor and PD 81,723 is required for this increase in constitutive receptor activity, our data showing concentration-dependent antagonism by DPCPX in CHO-A$>_1$ cells (Fig.

![Fig. 3. Effect of 3 and 10 μM PD 81,723 on cyclopentyladenosine inhibition of forskolin-stimulated cAMP formation in CHO-A$>_1$ cells. Data are presented as percentage of maximum stimulation in the absence of PD 81,723 and represent five experiments assayed in triplicate for control and 10 μM PD 81,723. The curve for 3 μM PD 81,723 was replicated twice in triplicate. Absolute values ranged from 2400 cpm at maximum adenylyl cyclase stimulation to 100 cpm under basal as well as maximum inhibition conditions. Values are mean ± S.E.M.](image1.png)

![Fig. 4. Effect of the adenosine A$>_1$ receptor antagonist DPCPX on PD 81,723-induced inhibition of forskolin-stimulated cAMP production in CHO-A$>_1$ (A) and nontransfected (B) CHO cells. Values are mean ± S.E.M. (n = 4–5).](image2.png)
4A), but not in nontransfected CHO cells (Fig. 4B), could be explained by this hypothesis. Although significance was not achieved for the high-affinity receptor, the trend of $K_i$ values from analysis of competition curves in the present study suggest a mechanism whereby PD 81,723 acts in the presence of Mg$^{2+}$ to increase the affinity of agonist for both coupled and uncoupled adenosine $A_1$ receptors rather than by converting uncoupled low-affinity receptors back to a coupled, high-affinity conformation (Table 2). Even when GppNHp was used to increase the proportion of low-affinity receptors, the effect of PD 81,723 was on affinity rather than on the proportion of high- versus low-affinity receptors. The observations of Bhattacharya and Linden (1995) that PD 81,723 increased agonist binding in membranes exposed to the uncoupling agent N-ethylmaleimide support the conclusion that PD 81,723 acts to increase the affinity of agonist for both coupled and uncoupled receptors.

PD 81,723 at a concentration of 5 μM has been reported to inhibit forskolin-stimulated cAMP formation in CHO-$A_1$ cells by approximately 50% in the absence of an adenosine agonist (Kollia-Baker et al., 1997). Their results suggest that this direct effect of PD 81,723 on AC is not due to binding at the adenosine receptor or to the presence of endogenous adenosine because agonist, but not PD 81,723, significantly displaced the specific binding of radiolabeled $A_1$ receptor agonist and antagonist in membranes and ADA-attenuated adenosine, but not PD 81,723, inhibition of cAMP formation in intact cells. Our results in intact CHO cells help to clarify the actions of PD 81,723 on adenosine $A_1$ receptors and on AC. Results from this study show direct inhibition of AC by PD 81,723 in both transfected and nontransfected CHO cells. Half-maximal inhibition of forskolin-stimulated cAMP production occurs at 10 μM PD in nontransfected CHO cells. This effect is not $A_1$ receptor-mediated, as these cells do not express adenosine receptors. Because half-maximal inhibition of AC in CHO-$A_1$ cells was detected at 5 μM PD, it is tempting to suggest that approximately 50% of the effect of PD 81,723 on forskolin-stimulated cAMP production is mediated by the adenosine $A_1$ receptor and that the balance is an independent effect in which PD 81,723 acts directly on AC to inhibit the formation of cAMP. We recognize that there may be differences in AC isoforms in rat liver and CHO cells; nonetheless, the competitive displacement of $[^3H]$forskolin from semipurified rat liver AC by PD 81,723 is consistent with a direct effect on AC.

In addition to determining the effect of PD 81,723 on forskolin-stimulated cAMP formation, we measured its effect on basal levels of cAMP. Basal AC activity as measured by cAMP accumulation in the presence of 500 μM IBMX was reduced nearly 80% in CHO-$A_1$ cells exposed to 30 μM PD 81,723. Under identical conditions, basal cAMP accumulation was reduced by 50% in nontransfected CHO cells and also in FRTL-5 cells, which are an entirely different cell line. The percentage of inhibition in nontransfected CHO cells differs from the results obtained when the cells were stimulated with forskolin. Under conditions of forskolin stimulation, PD 81,723 reduced cAMP levels by 90%. As discussed previously, however, to measure a reduction in basal activity, the cells must be cultured in the presence of IBMX to prevent degradation of cAMP by phosphodiesterase. Under these experimental conditions, it was possible to measure differences in low levels of cAMP. Because no IBMX was present in the forskolin experiments, a 50% change in the very low basal levels of cAMP in the presence of 30 μM PD 81,723 would not have been detected.

PD 81,723 retains its inhibitory effect on AC activity in the absence of normal G protein function. Cholera toxin is known to increase AC activity through the cholera toxin-catalyzed ADP-ribosylation of the stimulatory $G_s$ protein (Jacquemin et al., 1986). This covalent modification of $G_s$ maintains it in a permanently activated state and production of cAMP by AC is continuously stimulated. Pertussis toxin, on the other hand, activates AC by abolishing $G_i$-mediated inhibition of the enzyme (Hsia et al., 1984). This effect is achieved through covalent modification of the inhibitory $G_i$ protein by pertussis toxin. The abolition of inhibitory modulation of AC by $G_i$ leaves the cell with no mechanism to limit the constitutive formation of cAMP. The inhibition of cholera toxin- and pertussis toxin-stimulated AC activity by PD 81,723 in both transfected and nontransfected CHO cells provides further evidence for a direct interaction with AC.

The results of our study are consistent with at least two

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<tr>
<td>Effect of PD 81,723 (30 μM) on basal, cholera toxin-, and pertussis toxin-stimulated adenylyl cyclase activity in CHO and CHO-$A_1$ cells</td>
</tr>
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| Conditions as described in Materials and Methods. All values are expressed in femtomoles cAMP (mean ± S.E.M., n = 3–4) or as percentage of control. |

<table>
<thead>
<tr>
<th></th>
<th>Basal</th>
<th>Cholera Toxin</th>
<th>Pertussis Toxin</th>
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<tbody>
<tr>
<td><strong>Control</strong></td>
<td><strong>PD 81,723</strong></td>
<td><strong>Control</strong></td>
<td><strong>PD 81,723</strong></td>
</tr>
<tr>
<td>CHO</td>
<td>297 ± 56</td>
<td>153 ± 28</td>
<td>2774 ± 776</td>
</tr>
<tr>
<td>CHO-$A_1$</td>
<td>458 ± 67</td>
<td>82 ± 29</td>
<td>3054 ± 967</td>
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*Significantly different from control, P < .05.
*Significantly different from basal control, P < .05.
mechanisms by which PD 81,723 inhibits AC. As previously reported by others (Bruns et al., 1990; Mudumbi et al., 1993; Bhattacharya and Linden, 1995; Kollias-Baker et al., 1997), it enhances interaction of adenosine A1 receptor agonists with the receptor. The precise nature of that interaction remains to be defined. PD 81,723 also has direct inhibitory effects on AC activity that are independent of the adenosine A1 receptor. Because forskolin is believed to bind intracellularly to the catalytic unit of AC (Ho and Shi, 1984) and can be displaced by PD 81,723 from membrane preparations enriched for AC activity, PD 81,723 may inhibit AC activation by interfering directly with the function of the catalytic subunit. This binding site may be identical with or near the intracellular P-site, which has been shown by others (Bushfield and Johnson, 1990) to be involved in nonreceptor-mediated inhibition of AC activity by adenosine.

A possible mechanism of dual action by PD 81,723 is that it may bind intracellularly at a single site on AC and allosterically effect both receptor function and cyclase catalytic function simultaneously. Alternatively, the two effects may result from binding of PD 81,723 at two (or more) intracellular sites, with direct AC inhibition separate from adenosine A1 receptor enhancement. Finally, PD 81,723 may enhance adenosine A1 receptor binding and function of agonist by binding extracellularly at a site near but not at the receptor, whereas the direct inhibition of AC occurs through an intracellular site on the catalytic unit.

In summary, PD 81,723 has an allosteric effect to enhance adenosine A1 receptor activity. In addition to this indirect effect, PD 81,723 also exhibits a direct inhibitory effect on AC activity. In cells expressing adenosine A1 receptors and in the presence of agonist, the combined indirect and direct inhibitory effects are additive and completely abrogate AC activity at low microgram concentrations of PD 81,723.

Acknowledgments

We thank Dr. Robert F. Bruns and Joel Linden for their generous gifts of PD 81,723 and the cDNA for the rat brain adenosine A1 receptor, respectively.

References


Clemo HF and Belardinelli L (1986) Effect of adenosine on atrioventricular conduc-

![Fig. 6. Effect of PD 81,723 on basal, pertussis toxin- (100 ng/ml), and cholera toxin- (10 ng/ml) stimulated cAMP accumulation in CHO-A1 (A) and nontransfected (B) CHO cells. Experimental conditions were as outlined in Materials and Methods. Curves represent four to five experiments assayed in triplicate and are mean ± S.E.M.](image)

![Fig. 7. Representative curve of [3H]forskolin versus forskolin or PD 81,723 competition in semipurified rat liver membranes. Experiments were replicated twice for forskolin and three times for PD 81,723. Each assay was performed in triplicate.](image)

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