**Original Contributions**

**Allosteric Enhancer PD 81,723 Acts by Novel Mechanism to Potentiate Cardiac Actions of Adenosine**

C. Kollias-Baker, J. Ruble, D. Dennis, R.F. Bruns, J. Linden, L. Belardinelli

**Abstract** The 2-amino-3-benzoylthiophene derivative PD 81,723 is an allosteric enhancer of agonist binding to brain A1 adenosine receptors. One aim of this study was to characterize and contrast the effects of PD 81,723 on the A1 receptor-mediated inhibition of adenosine and of a nonmetabolizable and unselective N6-(3-pentyl)adenosine derivative. A second aim was to determine the mechanism of action of PD 81,723. In guinea pig isolated hearts, PD 81,723 potentiated the adenosine and the N6-(3-pentyl)adenosine derivative-induced protranctions of the stimulus–to–His bundle (S-H) interval in a concentration-dependent manner. PD 81,723 (30 μmol/L) decreased the EC50 value for adenosine to prolong the S-H interval by ninefold from 7.4±1.2 to 0.8±0.1 μmol/L, but did not increase the content of adenosine in cardiac effluent. PD 81,723 (30 μmol/L) increased the specific binding of the A1 agonist [3H]cyclopentyl-dipropylxanthine to guinea pig brain membranes by 81% and 72%, respectively. PD 81,723 also increased the fraction of A1 receptors in the high-affinity binding state by an average of 38±13%. The dissociation rate of [3H]CHA from guinea pig brain membranes was decreased in the presence of PD 81,723 (10 μmol/L) from 0.55±0.01/min to 0.35±0.01/min. PD 81,723 did not alter the binding of the A1 antagonist [3H]cyclopentyl-dipropylxanthine to guinea pig brain membranes. The IC50 values for 5'-guanylylimidodiphosphate to reduce specific binding of [3H]CHA to guinea pig cardiac and brain membranes were increased from 1.5±0.2 and 2.0±0.2 μmol/L in the absence of PD 81,723 to 10±3.3 and 18±0.5 μmol/L, respectively, in the presence of PD 81,723 (30 μmol/L). PD 81,723 did not potentiate the coronary vasodilatory actions of the N6-(3-pentyl)adenosine derivative. Specific binding of the A2a agonist [3H]CGS 21680 to brain membranes and the nucleoside transporter ligand [3H]nitrobenzylthioinosine to cardiac membranes was unchanged in the presence of PD 81,723. The results suggest that PD 81,723 specifically potentiates the action of adenosine on A1 receptors by stabilizing receptor–G protein interactions in the presence of agonists. (Circ Res. 1994;75:961-971.)

**Key Words** • isolated heart • radioligand binding • guinea pigs

Adenosine is an autacoid (or local hormone) that modulates numerous functions of the cardiovascular system.1,2 In the heart, in addition to being a potent vasodilator, adenosine slows heart rate (negative chronotropic effect), slows atrioventricular (AV) nodal conduction (negative dromotropic effect), and antagonizes the stimulatory effects (ie, inotropic and arrhythmogenic) of catecholamines.1 These effects of adenosine, which increase the supply of oxygen and decrease cardiac work, are cardioprotective.3 Consistent with this cardioprotective action, adenosine, adenosine receptor agonists, and agents that increase the interstitial concentration of adenosine (eg, nucleoside uptake blockers and adenosine deaminase and adenosine kinase inhibitors) have been shown to reduce myocardial cell damage and dysfunction during hypoxia and ischemia (for extended reference lists, refer to References 1, 3, and 4). Adenosine and the nucleoside uptake inhibitor dipyridamole have been shown to terminate supraventricular and catecholamine-sensitive tachycardias in humans.5–8 Thus, modulation of adenosine metabolism and/or adenosine receptor activation may prove beneficial in the treatment of cardiac arrhythmias and ischemic heart disease.

A number of pharmacologic approaches can be used to modulate the adenosine system (ie, adenosine’s metabolic pathways and receptors). Direct-acting agonists, such as adenosine or synthetic adenosine analogues, mimic the effects of the endogenous nucleoside.6,9 However, adenosine receptors are present in many organ systems; thus, their indiscriminate activation by direct-acting agonists results in numerous systemic effects.10 Inhibitors of the metabolism of adenosine (ie, adenosine deaminase and adenosine kinase inhibitors and nucleoside uptake blockers), by increasing interstitial and plasma levels of the nucleoside, enhance the cardiovascular actions of exogenous and endogenous adenosine.11–13 Allosteric enhancers of the actions of adenosine have recently emerged as alternative agents to direct-acting adenosine agonists and to inhibitors of the metabolism of adenosine.14 The 2-amino-3-benzoylthiophene derivative PD 81,723 has been shown to enhance the binding of adenosine agonists to A1 receptors in brain membranes and therefore has been classified as an allosteric enhancer.14 PD 81,723 has also been shown to specifically potentiate A1 receptor–mediated re-
responses in guinea pig and rat hearts.\textsuperscript{15,16} In contrast to direct-acting adenosine agonists, allosteric enhancers could provide site- and event-specific potentiation of the effects of adenosine.

The aims of the present study were twofold. The first objective was to characterize the potentiation of the negative dromotropic action of adenosine by the allosteric enhancer PD 81,723 in guinea pig hearts and to compare the potentiation by PD 81,723 with that caused by the nucleoside uptake inhibitor drafazine.\textsuperscript{17} The second objective was to characterize the effects of PD 81,723 on radioligand binding to adenosine receptors in cardiac and brain membranes. The results indicate that PD 81,723 potentiates only A\textsubscript{1} receptor-mediated responses of adenosine by selectively enhancing the binding of agonists to A\textsubscript{1} receptors.

**Materials and Methods**

**Chemicals**

(2-Amino-4,5-dimethyl-3-thienyl)-[3-(trifluoromethyl)phenyl]methanone, known as PD 81,723, was a generous gift from Dr R.L. Bruns, Eli Lilly & Co.\textsuperscript{18} Drafazine, a nucleoside uptake inhibitor, was a generous gift from Dr Van Belle, Janssen Pharmaceuticals.\textsuperscript{17} N\textsuperscript{6}-(3-Pentyl)adenosine-cyclopropyl carboxamide (PCPN) was a generous gift from Dr Ray Olsson, University of South Florida. Adenosine, adenosine deaminase, dipyridamole, nase, inhibitor, R.L. carboxamide 81,723 potentiates only negative dromotropic effects of adenosine. The results indicate that 81,723 potentiates only A\textsubscript{1} receptor-mediated effects of adenosine and a 15-minute infusion of all other drugs. During protocols evaluating coronary flow responses (A\textsubscript{2a} adenosine receptor-mediated effect), the coronary perfusion pressure (in millimeters of mercury) was monitored throughout the experiment. Because hearts were perfused at constant flow, the coronary conductance (in millimeters per minute per millimeter of pressure) was calculated as the ratio between the coronary perfusion rate (10 ml/min) and the perfusion pressure (in millimeters of mercury).

**Adenosine Assay**

The concentrations of adenosine in the samples of cardiac effluent collected from the pulmonary artery were determined by reversed-phase high-performance liquid chromatography according to a previously published isocratic method.\textsuperscript{19} Cardiac effluent samples were collected for 15 seconds at predetermined time points and frozen at –80°C until analyzed. Samples were thawed, and 200-μl aliquots were assayed for adenosine content. Adenosine in the samples was identified by retention time and verified by enzymatic degradation to inosine using adenosine deaminase. The concentrations of adenosine in the samples were determined by peak area from experimental samples with those of standard samples containing known quantities of adenosine.

**Membrane Preparation**

Human atrial and guinea pig atrial, ventricular, and brain tissues were separately minced and then homogenized in ice-cold 50 mmol/L Tris-HCl, pH 7.4. Homogenates were filtered through cotton gauze and centrifuged at 48,000g for 15 minutes. The membrane pellets were washed twice by resuspension in fresh buffer and centrifugation. Final pellets were resuspended in 50 mmol/L Tris-HCl, pH 7.4, and frozen at –80°C.

**Isolated Heart Protocols**

**Effects of Allosteric Enhancer PD 81,723 and Nucleoside Uptake Inhibitor Drafazine on Negative Dromotropic Action of Adenosine**

In each of the 10 hearts in this series of experiments, concentration-response relations for adenosine (1 to 10 μmol/L)–induced prolongation of the S-H interval (negative dromotropic effect) were determined in the absence and presence of PD 81,723. In each heart, a control adenosine concentration–response relation (ie, in the absence of PD 81,723) was initially determined. After recording the baseline S-H interval, adenosine was infused at rates to achieve increasingly higher perfusate concentrations until second-degree AV block occurred. After a 15-minute washout of adenosine, PD 81,723 was infused at increasing rates to achieve perfusate concentrations of 2.5, 5, 10, and 30 μmol/L in that order. The
adensine concentration-response relation was determined in the presence of each concentration of PD 81,723. The corresponding S-H intervals were recorded continuously throughout the experiment. In each heart, the effects of a maximum of three concentrations of PD 81,723 on the adenosine concentration-response relation were determined. In a separate group of hearts (n=4), the concentration-response relation for adenosine-induced prolongation of the S-H interval was determined in the absence and presence of drafazine (0.1 μmol/L).

Effects of PD 81,723 and Drafazine on Recovery of Adenosine in Cardiac Effluent

In this series of experiments carried out in eight isolated perfused hearts, the effects of PD 81,723 and drafazine on prolongation of the S-H interval and on recovery of adenosine in cardiac effluent were determined in the absence and presence of adenosine (3 μmol/L). After recording the control S-H interval and collecting three effluent samples, the infusion of adenosine (perfusion concentration, 3 μmol/L) was begun. Once the steady-state effect of adenosine was achieved, three additional cardiac effluent samples were collected, and the corresponding S-H interval was recorded. After a 5-minute washout of adenosine, either PD 81,723 (perfusion concentration, 5 μmol/L) or drafazine (perfusion concentration, 0.1 μmol/L) was infused, followed by adenosine (perfusion concentration, 3 μmol/L). Three more effluent samples were collected, and the corresponding S-H intervals were recorded during the infusions of PD 81,723 and drafazine in the absence and presence of adenosine (3 μmol/L).

Receptor Selectivity of Effect of PD 81,723

Concentration-response relations for A1 and A2 receptor-mediated responses to the nonmetabolizable and nonselective adenosine analogue PCPN were determined in the absence and presence of PD 81,723. In the first series of experiments, carried out in constant-flow (10 mL/min) perfused hearts (n=5), the concentration-response relations for PCPN-induced prolongation of the S-H interval (A1 receptor-mediated effect) and reduction of coronary perfusion pressure (A2 receptor-mediated effect) were determined. In each heart, after the baseline S-H interval was recorded, PCPN was infused at rates to achieve increasingly higher perfuse concentrations until second-degree AV block occurred. The steady-state S-H interval prolongation and maximum decrease in coronary perfusion pressure were recorded at each concentration of PCPN. The coronary conductance was calculated as the ratio between the coronary perfusion rate (10 mL/min) and the perfusion pressure. In a second group of constant-flow perfused hearts (n=5), the steady-state prolongation of the S-H interval and the increase in coronary conductance induced by PCPN were determined in the absence and presence of PD 81,723 (2.5 μmol/L).

Determination of Rank Order of Potency for Negative Dromotropic Effect of A1 Adenosine Receptor Agonists

The potencies (EC50 values) of the adenosine agonists CCPA, CHA, 2-CADO, and R- and S-PIA necessary to prolong the S-H interval were determined in 12 isolated perfused hearts. In each heart after the baseline S-H interval was recorded, an agonist was infused at rates to achieve increasingly higher perfuse concentrations until second-degree AV block occurred. After a 10-minute washout of the agonist, the concentration-response relation for another agonist was determined. A maximum of three agonist concentration-response relations was determined in any one heart. The S-H intervals were recorded continuously throughout the experiment.

Radioligand Binding Protocols

To complement the functional studies described above, the effects of PD 81,723 on the binding of adenosine receptor ligands to cardiac and brain membranes were determined. Assays for A1 and A2 receptors were carried out by using the A1 receptor agonist [3H]CHA, the A1 receptor antagonist [3H]CPX, and the A2 receptor agonist [3H]CGS 21680. In addition, the affinities of the allosteric enhancer PD 81,723 and the nucleoside uptake inhibitors drafazine and dipyridamole for the nucleoside transporter binding site were determined by using the ligand [3H]NBMPR. The incubation solution for binding assays contained 50 mmol/L Tris-HCl buffer (pH 7.4) and adenosine deaminase (2 U/mL). Assays were carried out in triplicate at ambient temperature (19°C to 22°C). Specific binding was determined by subtracting nonspecific binding from total binding. Nonspecific binding values of [3H]CHA, [3H]CPX, [3H]CGS 21680, and [3H]NBMPR were determined by using unlabeled CPA (10 μmol/L), CPT (10 μmol/L), NECA (10 μmol/L), and NBMPR (10 μmol/L), respectively. Incubations were terminated by the addition of 4 mL ice-cold buffer, followed by the collection of membranes onto Whatman GF/C glass fiber filters (Brandel) by vacuum filtration. Filters were washed three times with ice-cold buffer (50 mmol/L Tris-HCl, pH 7.4) to remove unbound ligand. Filter disks containing trapped membrane protein and radioligand were placed in 4 mL Scintiverse II (Fisher Scientific), and the radioactivity was quantified by a liquid scintillation counter.

Effect of PD 81,723 on Submaximal Binding of A1 Agonist [3H]CHA to Cardiac Membranes

A nonsaturating concentration of [3H]CHA (4 nmol/L) was incubated with various concentrations of PD 81,723 (2.5, 5, 10, and 30 μmol/L) and aliquots of human or guinea pig atrial membranes containing 300 to 400 μg of protein in a total incubation volume of 300 μL for 3 hours. Assays were terminated as described above.

Determination of Potency of Adenosine Agonists to Displace Specific Binding of an A1-Selective Agonist to Guinea Pig Brain Membranes

The potencies of the agonists CCPA, CHA, 2-CADO, and R- and S-PIA necessary to displace the specific binding of the A1-selective agonist [3H]CHA to guinea pig forebrain membranes were determined. Increasing concentrations of guinea pig forebrain A1 agonist (from 0.1 to 100 μmol/L) were incubated with [3H]CHA (4 nmol/L) and aliquots of guinea pig forebrain membranes containing 100 to 200 μg of protein in a total incubation volume of 300 μL for 3 hours. Assays were terminated as described above.

Effect of PD 81,723 on Maximum Specific Binding of A1, A2 Agonists and A2 Antagonist to Guinea Pig Brain Membranes

The effects of PD 81,723 on the maximum specific binding (Bmax) and the affinity (Kd) of the A1 agonist [3H]CHA, the A1 antagonist [3H]CPX, and the A2 agonist [3H]CGS 21680 were determined. Increasing concentrations of [3H]CHA (from 0.63 to 40 nmol/L) were incubated with aliquots of guinea pig forebrain membranes containing 100 to 200 μg of protein in the absence and presence of 2.5, 5, 10, and 30 μmol/L PD 81,723 in a total incubation volume of 300 μL for 3 hours. The effects of PD 81,723 (10 μmol/L) on Bmax and Kd of [3H]CPX (0.32 to 20 nmol/L) were determined by using an identical protocol. Similarly, increasing concentrations of [3H]CGS 21680 (from 0.32 to 145 nmol/L) were incubated with aliquots of guinea pig striatal membranes containing 200 to 300 μg of protein in the absence and presence of PD 81,723 (10 μmol/L) in a total incubation volume of 300 μL for 3 hours. Assays were
terminated as described above. The \( B_{max} \) and \( K_d \) values were calculated as described in “Data Analysis.”

**Effect of PD 81,723 on Affinity States of the \( A_1 \) Adenosine Receptor**

The effect of PD 81,723 on \( A_1 \) adenosine receptor affinity states was determined by comparing the potency of the agonist R-PIA to displace the specific binding of the \( A_1 \)-selective antagonist \([\text{H}]\text{CPX} \) to guinea pig forebrain membranes in the absence and presence of PD 81,723 (30 \( \mu \text{mol/L} \)). Increasing concentrations of R-PIA (from 0.01 \( \mu \text{mol/L} \) to 1 \( \mu \text{mol/L} \)) were incubated with \([\text{H}]\text{CPX} \) (1 \( \mu \text{mol/L} \)) and aliquots of guinea pig forebrain membranes containing 100 to 200 \( \mu \text{g} \) of protein in a total incubation volume of 300 \( \mu \text{L} \) for 3 hours. Assays were terminated as described above.

**Effect of PD 81,723 on Modulation of Agonist Binding by Gpp(NH)p**

The effect of PD 81,723 on Gpp(NH)p-induced changes in the specific binding of the agonist \([\text{H}]\text{CHA} \) to \( A_1 \) adenosine receptors was determined in guinea pig ventricular and forebrain membranes. In the presence of increasing concentrations of Gpp(NH)p (from 1 \( \mu \text{mol/L} \) to 1 \( \mu \text{mol/L} \)) and in the absence and presence of PD 81,723 (30 \( \mu \text{mol/L} \)), \([\text{H}]\text{CHA} \) (3 \( \mu \text{mol/L} \)) was incubated separately with aliquots of guinea pig ventricular and forebrain membranes containing 100 or 200 \( \mu \text{g} \) of protein, respectively, in a total incubation volume of 300 \( \mu \text{L} \) for 3 hours. Assays were terminated as described above.

**Effects of PD 81,723 on Binding Kinetics of \( A_1 \) Receptor Agonist and Antagonist**

The effects of PD 81,723 on the association (\( K_{as} \)) and dissociation (\( K_{ds} \)) rates of the \( A_1 \) agonist \([\text{H}]\text{CHA} \) and antagonist \([\text{H}]\text{CPX} \) in guinea pig forebrain membranes were determined. To determine \( K_{as} \), \([\text{H}]\text{CHA} \) (4 \( \mu \text{mol/L} \)) and \([\text{H}]\text{CPX} \) (2 \( \mu \text{mol/L} \)) were incubated separately in a total incubation volume of 250 \( \mu \text{L} \) with aliquots of guinea pig forebrain membranes containing 100 to 200 \( \mu \text{g} \) of protein in the absence and presence of PD 81,723 (10 \( \mu \text{mol/L} \)) for preset periods of time before termination of the assay. To determine \( K_{ds} \), \([\text{H}]\text{CHA} \) (4 \( \mu \text{mol/L} \)) and \([\text{H}]\text{CPX} \) (2 \( \mu \text{mol/L} \)) were incubated separately in a total incubation volume of 250 \( \mu \text{L} \) with aliquots of guinea pig forebrain membranes containing 100 to 200 \( \mu \text{g} \) of protein in the absence and presence of PD 81,723 (10 \( \mu \text{mol/L} \)) for 2 hours. Dissociation of the ligands was initiated by the addition of unlabeled CPA (10 \( \mu \text{mol/L} \)) to displace bound \([\text{H}]\text{CHA} \) and unlabeled CPT (10 \( \mu \text{mol/L} \)) to displace bound \([\text{H}]\text{CPX} \). Assays were terminated at preset periods of time as described above. Kinetic binding parameters were calculated as described in “Data Analysis.”

**Effect of PD 81,723, Draflazine, and Dipyridamole on Nucleoside Transporter Binding Sites**

The potencies of PD 81,723, drazfazine, and dipyridamole necessary to displace the specific binding of \([\text{H}]\text{NBMPR} \) from guinea pig ventricular membranes were determined in competition assays. \([\text{H}]\text{NBMPR} \) (4 \( \mu \text{mol/L} \)) was incubated separately with increasing concentrations of PD 81,723, drazfazine, and dipyridamole (from 1.0 \( \mu \text{mol/L} \) to 30 \( \mu \text{mol/L} \)) and aliquots of guinea pig ventricular membranes containing 200 to 300 \( \mu \text{g} \) of protein in a total incubation volume of 300 \( \mu \text{L} \) for 3 hours. Assays were terminated as described above.

**Data Analysis**

All values are expressed as mean±SEM. Statistical analysis of differences among values in experiments with multiple comparison groups was based on ANOVA followed by Bonferroni testing (interventions versus control values before and after interventions). For experimental protocols having two comparison groups, statistical analysis was performed with a two-tailed \( t \) test (In Stat Mac, GraphPad Software Inc.). The concentration of drug required to cause 50% of the maximal response (eg, \( EC_{50} \) value) for parabolic concentration-response curves was determined by nonlinear regression analysis (Table Curve 3.01, Jandel Corp.). Because values of \( EC_{50} \), equilibrium dissociation constants, and rate constants are log-normally distributed, the data were log-transformed before parametric statistical analyses. Differences between group means and control versus interventions were considered significant at \( P<.05 \).

Equilibrium binding parameters (ie, \( B_{max} \), \( K_d \), and \( IC_{50} \)) were determined with the radioligand binding analysis program LI-GAND 3.0 (Elsevier-Bioskop). The \( K_d \) values were calculated by using the following equation: \( K_d=(IC_{50})(K_d)/[L]+K_c \). Kinetic parameters were determined by fitting the data to either a single-component exponential model (association and antagonist dissociation data) or a two-component exponential model (agonist dissociation data) by using nonlinear regression analysis (Sigma Plot 4.1, Jandel Corp.).

**Results**

**Potentiation of Negative Dromotropic Effect of Adenosine by Allosteric Enhancer PD 81,723 and Nucleoside Uptake Inhibitor Draflazine**

Adenosine caused a concentration-dependent prolongation in the S-H interval (Figs 1 and 2). The \( EC_{50} \) value for this negative dromotropic effect of adenosine was 7.4±1.2 \( \mu \text{mol/L} \) (Fig 1). PD 81,723 in a concentration-dependent manner significantly potentiated this effect of adenosine (Fig 1). The \( EC_{50} \) values necessary for adenosine to prolong the S-H interval in the presence of 2.5, 5, 10, and 30 \( \mu \text{mol/L} \) PD 81,723 were reduced to 5.6±0.4, 3.2±0.3, 1.7±0.2, and 0.8±0.1 \( \mu \text{mol/L} \), respectively (Fig 1). Similarly, drazfazine significantly potentiated the negative dromotropic effect of adenosine (Fig 2). The \( EC_{50} \) value for the S-H interval prolongation induced by adenosine was 6.0±0.21 \( \mu \text{mol/L} \) in the absence and 0.18±0.05 \( \mu \text{mol/L} \) in the presence of 0.1 \( \mu \text{mol/L} \) drazfazine (Fig 2).

Although both PD 81,723 and drazfazine potentiated the negative dromotropic effect of adenosine, only drazfazine caused a concomitant increase (47%) in the concentration of adenosine in the cardiac effluent (Fig 3). PD 81,723 (5 \( \mu \text{mol/L} \)) and drazfazine (0.02 \( \mu \text{mol/L} \))
increased the S-H prolongation caused by 3 μmol/L adenosine from 4.0±0.4 to 20±4.0 and 10.5±1 milliseconds, respectively (Fig 3A). During control conditions (ie, absence of adenosine, PD 81,723, and drafalazine), the concentration of adenosine in the cardiac effluent was 0.32±0.31 μmol/L. The concentration of adenosine in the cardiac effluent in the presence of either 5 μmol/L PD 81,723 or 0.02 μmol/L drafalazine (0.38±0.22 and 0.51±0.38 μmol/L, respectively) was not significantly different from the control value (P>.05). Infusion of adenosine (perfusate concentration, 3 μmol/L) increased the concentration of this nucleoside in the cardiac effluent to 1.5±0.08 μmol/L (Fig 3B). In the presence of PD 81,723 (5 μmol/L), the same perfusate concentration of adenosine (ie, 3 μmol/L) caused a similar (P>.05) increase in the concentration of this nucleoside in the cardiac effluent to 1.24±0.09 μmol/L (Fig 3B). In contrast, in the presence of drafalazine (0.02 μmol/L) the same perfusate concentration of adenosine (3 μmol/L) resulted in a significant increase in the concentration of this nucleoside in the cardiac effluent to 2.5±0.14 μmol/L, a value that is over 1.5-fold higher than that obtained during the infusion of adenosine alone (Fig 3B).

Selective Potentiation by PD 81,723 of A₁ Adenosine Receptor–Mediated Response to PCPN

PD 81,723 potentiated the A₁ receptor–mediated S-H interval prolongation but not the A₂a receptor–mediated increased coronary conductance caused by the N⁶-(3-pentyl)adenosine derivative PCPN (Fig 4). PCPN alone in a concentration-dependent manner prolonged the S-H interval (A₁ response) and increased the coronary conductance (A₂a response), with EC₅₀ values of 8.3±1.1 and 8.0±0.9 nmol/L, respectively (Fig 5). Thus, PCPN was equipotent for A₁ and A₂a receptor–mediated responses. As shown in Fig 4A, the EC₅₀ value for PCPN to prolong the S-H interval was significantly lower in the presence than in the absence of 2.5 μmol/L PD 81,723 (5.6±1.0 nmol/L compared with 8.9±1.5 nmol/L, re-
spectively). In contrast, the concentration-response curve for the A3 receptor-mediated increase in coronary conductance caused by PCPN was not significantly (P > .05) altered by the presence of 2.5 μmol/L PD 81,723 (Fig 4B). Because the concentration-response curves for the A3 receptor-mediated response were incomplete (ie, maximal coronary vasodilation caused by PCPN was not achieved), the EC50 values for PCPN could not be determined.

Increase in Submaximal Specific Binding of A1 Adenosine Agonist to Human and Guinea Pig Atrial Membranes by PD 81,723

PD 81,723 significantly increased the submaximal specific binding of the A1 agonist [3H]CHA to both human and guinea pig atrial membranes in a concentration-dependent manner (Fig 6). In human atrial membranes, 2.5, 5, 10, and 30 μmol/L PD 81,723 increased the specific binding of a nonsaturating concentration of [3H]CHA (4 nmol/L) by 10%, 13%, 15%, and 38%, respectively (Fig 6A). Similarly, in guinea pig atrial membranes, the same concentrations of PD 81,723 increased the specific binding of [3H]CHA (4 nmol/L) by 16%, 37%, 41%, and 78%, respectively (Fig 6B).

A1 Receptor Binding Affinities and Negative Dromotropic Potencies of Adenosine Agonists

To determine if the brain A1 receptor is an appropriate model for the cardiac A1 receptor, the potencies (K) values of various adenosine agonists to displace the specific binding of an A1 receptor agonist to guinea pig brain membranes were compared with the negative dromotropic potencies (EC50 values) of those agonists in isolated hearts (Fig 7). The K values for the adenosine agonists CCPA, CHA, 2-CADO, and R- and S-PIA to displace the specific binding of the A1 receptor agonist [3H]CHA from guinea pig brain membranes were 0.41±0.1, 6.3±1.4, 21.7±5.3, 4.4±1.1, and 112±26 nmol/L, respectively. The EC50 values for these same agonists to prolong the S-H interval were 5.1±1.2, 56±5.4, 145±15.5, 18.2±1.1, and 382±29.2 nmol/L, respectively. The close correlation (r=.97) between the EC50 values and the K1 values of these agonists justifies the use of the brain A1 receptor as a model for the cardiac A1 receptor.

Selective Enhancement of Maximum A1 Receptor Agonist Binding to Guinea Pig Brain Membranes by PD 81,723

PD 81,723 enhanced Bmax of the A1 adenosine agonist [3H]CHA to guinea pig brain membranes (Fig 8) but did...
not affect $B_{\text{max}}$ of the $A_1$ receptor antagonist $[^3H]CPX$ or the $A_2_\alpha$ receptor agonist $[^3H]CGS$ 21680 (Fig 9). As shown in Fig 8, in the absence of PD 81,723 the $B_{\text{max}}$ value of $[^3H]CHA$ binding to guinea pig forebrain membranes was 880±24 fmoles/mg protein. The presence of 2.5, 5, 10, and 30 μmol/L PD 81,723 significantly increased the $B_{\text{max}}$ values in a concentration-dependent manner to 1852±27, 2163±20, 2427±28, and 2646±49 fmoles/mg protein, respectively (Fig 8). PD 81,723 did not significantly ($P>0.05$) affect the affinity of the $A_1$ receptor for the ligand $[^3H]CHA$. The $K_d$ (concentration of the ligand at which half the receptors are occupied) of $[^3H]CHA$ binding in the absence of PD 81,723 was 2.8±0.1 nmol/L, whereas in the presence of 2.5, 5, 10, and 30 μmol/L PD 81,723 the $K_d$ values were 2.5±1.2, 3.3±0.2, 4.1±0.2, and 3.0±1.0 nmol/L, respectively (Fig 8). In contrast to the binding of the $A_1$ agonist, both $B_{\text{max}}$ and $K_d$ of the $A_2_\alpha$ antagonist $[^3H]CPX$ were not significantly ($P>0.05$) different in the absence (3266±56 fmoles/mg protein and 1.7±0.2 nmol/L, respectively) and presence of 10 μmol/L PD 81,723 (3696±35 fmoles/mg protein and 2.0±0.3 nmol/L, respectively) (Fig 9A). Similarly, $B_{\text{max}}$ and $K_d$ of the $A_2_\alpha$ agonist $[^3H]CGS$ 21680 were not significantly ($P>0.05$) different in the absence (1344±43 fmoles/mg protein and 31±10 nmol/L, respectively) and presence of 10 μmol/L PD 81,723 (1371±34 fmoles/mg protein and 40±12 nmol/L, respectively) (Fig 9B).

Fig 8. Graphs showing the effect of PD 81,723 on specific binding of the $A_1$ receptor agonist $[^3H]$cyclohexyladenosine ($[^3H]CHA$) to guinea pig forebrain membranes. The saturation isotherms (A) and Scatchard analysis (B) revealed that PD 81,723 caused a concentration-dependent increase in the maximum specific binding of $[^3H]CHA$. The slopes of the Scatchard plots were not different in the absence and presence of PD 81,723 (B), indicating that PD 81,723 had no significant effect on the affinity of the $A_1$ receptor for $[^3H]CHA$ (see text for $K_d$ values). A, Each data point represents the mean±SEM specific binding of triplicate determinations from three experiments. B, Each data point represents the mean specific binding of triplicate determinations from three experiments.

Fig 9. Graphs showing the lack of effect of PD 81,723 on specific binding of the $A_1$ receptor antagonist $[^3H]$cyclopentylidipropylxanthine ($[^3H]CPX$) (A) to guinea pig forebrain membranes and of the $A_2_\alpha$ receptor agonist $[^3H]CGS$ 21680 (B) to guinea pig striatal membranes. Each data point represents the mean±SEM specific binding of triplicate determinations from three experiments.

**Effect of PD 81,723 on Affinity States of the $A_1$ Adenosine Receptor**

In three of five experiments, the displacement of the specific binding of $[^3H]CPX$ by R-PIA was best fit to a two-site model, indicating the presence of high- and low-affinity binding states (Fig 10). In the presence of PD 81,723 (30 μmol/L), the fraction of the receptors in

Fig 10. Graph showing displacement by R(−)-phenylisopropyl-adenosine (R-PIA) of specific binding of the $A_1$-selective antagonist $[^3H]$cyclopentylidipropylxanthine ($[^3H]CPX$, 1 nmol/L) to guinea pig forebrain membranes in the absence and presence of PD 81,723 (30 μmol/L). PD 81,723 increased the fraction of receptors in the high-affinity ($f_h$) binding state, whereas it decreased the fraction of receptors in the low-affinity ($f_l$) binding state. Each data point represents the mean specific binding of triplicate determinations from a typical experiment. Similar data were obtained in two other experiments. See text for $K_v$ values of $f_h$ and $f_l$ states.
the high-affinity binding state increased an average of 56±13%, whereas the fraction of receptors in the low-affinity state decreased accordingly (n=3, Fig 10). The K_i values of the high- and low-affinity binding states for R-PIA in the absence of PD 81,723 were 4±1.2 and 469±96 nmol/L, respectively; in the presence of the allosteric enhancer, the values were 2.1±0.2 and 197±54 nmol/L, respectively. In the remaining two experiments, [3H]CPX–R-PIA displacement curves were best fit to a one- instead of two-site model. In these experiments, PD 81,723 caused an 11-fold decrease in the K_i value (69.9 [control] versus 6.2 [PD 81,723] nmol/L, n=2) for R-PIA to displace [3H]CPX.

Effect of PD 81,723 on Gpp(NH)p-Induced Inhibition of A_1 Agonist Binding

Gpp(NH)p in a concentration-dependent manner inhibited the specific binding of [3H]CHA to guinea pig ventricular and forebrain membranes, with IC_{50} values of 1.5±0.2 and 2.0±0.2 μmol/L, respectively (Fig 11). PD 81,723 (30 μmol/L) significantly reduced the potency of Gpp(NH)p to inhibit [3H]CHA binding (Fig 11). PD 81,723 (30 μmol/L) increased the IC_{50} values of Gpp(NH)p to inhibit [3H]CHA binding to guinea pig ventricular and forebrain membranes to 10±3.3 and 18±0.5 μmol/L, respectively (Fig 11).

PD 81,723 Decreases Dissociation Rate of A_1 Agonist but Not A_1 Antagonist

PD 81,723 (10 μmol/L), although not affecting K_{dis} of the A_1 receptor agonist [3H]CHA to guinea pig brain membranes (not shown), significantly decreased K_{dis} of the slow phase of agonist dissociation by 36% (Fig 12A). Both in the absence and presence of PD 81,723, K_{dis} of [3H]CHA was 0.12±0.01/min per nanomolar. As shown in Fig 12A, K_{dis} of the fast phase of [3H]CHA dissociation in the absence and presence of PD 81,723 (10 μmol/L) was 0.48±0.1/min and 0.42±0.08/min, respectively (P>0.5). However, K_{dis} of the slow phase of [3H]CHA dissociation in the absence and presence of PD 81,723 (10 μmol/L) was 0.055±0.01/min and 0.035±0.01/min, respectively (P<0.05, Fig 12A). PD 81,723 (10 μmol/L) did not affect either K_{on} (not shown) or K_{dis} of the antagonist [3H]CPX (Fig 12B). K_{on} of [3H]CPX was 0.11/min per nanomolar in the absence and 0.14/min per nanomolar in the presence of PD 81,723 (10 μmol/L). K_{dis} of [3H]CPX was 0.22±0.01/min both in the absence and presence of 10 μmol/L PD 81,723 (Fig 12B).

Lack of Effect of PD 81,723 on Nucleoside Transporter Binding Sites

The allosteric binding enhancer PD 81,723 did not displace the specific binding of [3H]NBMPR from nucleoside transporter binding sites in guinea pig ventricular membranes (Fig 13). In contrast, both the nucleoside uptake inhibitors drafazine and dipyridamole displaced the specific binding of [3H]NBMPR in a
concentration-dependent manner, with $K_i$ values of $17 \pm 2.3$ and $60 \pm 1.3$ nmol/L, respectively (Fig 13).

**Discussion**

The results of the present study demonstrate that PD 81,723 selectively and in a concentration-dependent manner potentiates the $A_1$ receptor-mediated negative dromotropic effect of adenosine and the $N^\alpha$-(3-pentyl)adenosine derivative but does not potentiate the $A_2a$ receptor-mediated coronary vasodilation caused by the agonist. Consistent with these results, PD 81,723 increases the binding of the agonist $[^3H]$CHA to $A_1$ adenosine receptors in human and guinea pig atrial membranes. Furthermore, PD 81,723 increases binding of $[^3H]$CHA but not binding of either the $A_1$ antagonist $[^3H]$CPX or the $A_2a$ agonist [H]CGS 21680 to guinea pig ventricular membranes. Kinetic binding studies showed that PD 81,723 decreases $K_{ma}$ of the agonist $[^3H]$CHA from the $A_1$ receptor but does not affect $K_{ma}$ of the antagonist $[^3H]$CPX from the same receptor. The results suggest that PD 81,723 selectively potentiates the $A_1$ receptor-mediated negative dromotropic effect of adenosine by enhancing the binding of adenosine to $A_1$ receptors.

**Characterization of Potentiation of Negative Dromotropic Effect of Adenosine by PD 81,723**

Recently, our laboratory reported that the 2-amino-3-benzoylthiopine derivative PD 81,723 specifically potentiates the $A_1$ receptor-mediated negative dromotropic effect of adenosine in guinea pig hearts in vitro and in situ. That is, PD 81,723 was shown to specifically potentiate the negative dromotropic effect caused by adenosine and the $A_1$ agonist CPA but not that caused by carbachol or MgCl$_2$. Similarly, Mudumbi et al$^{16}$ reported that PD 81,723 potentiates the negative chronotropic and inotropic effects of adenosine in rat isolated atria. These two studies showed that although the effect of PD 81,723 is mediated through the $A_1$ receptor, PD 81,723 itself does not directly activate the receptor. Previous reports of the effects of PD 81,723 on the binding of adenosine radioligands to brain membranes and on functional responses in isolated perfused hearts suggested that the allosteric enhancer selectively potentiated $A_1$ receptor-mediated effects of adenosine.$^{14,16}$ Selective potentiation of $A_2$ receptor-mediated responses by the allosteric enhancer suggests that PD 81,723 does not act by interfering with adenosine metabolism. In the present study, we have shown directly that PD 81,723 does not bind to the NBMPR-sensitive nucleoside transporter or interfere with adenosine metabolism, so as to increase the concentration of the nucleoside in cardiac effluent (Figs 3 and 13). In contrast to PD 81,723, the potentiation of the negative dromotropic effect of adenosine by nucleoside uptake inhibitors, such as diprydamole$^{12}$ and draflazine (Fig 3), was characterized by an increase in the recovery of this nucleoside in the cardiac effluent. Consistent with these findings, the nucleoside uptake inhibitors draflazine and diprydamole displaced the specific binding of the nucleoside transporter ligand $[^3H]$NBMPR from guinea pig ventricular membranes, with $K_i$ values of $17 \pm 2.2$ and $60 \pm 1.3$ nmol/L, respectively (Fig 13). These results support the conclusion that PD 81,723 potentiates the $A_1$ receptor-mediated negative dromotropic effect of adenosine by a mechanism that is independent of nucleoside uptake inhibition.

The results of the present study also demonstrate for the first time that the enhancement of the negative dromotropic effect of adenosine by PD 81,723 is concentration dependent. That is, incremental increases in the perfusate concentration of PD 81,723 produced significant and parallel leftward shifts of the concentration-response curve for the negative dromotropic effect of adenosine (Fig 1). Furthermore, the results of the present study conclusively show that potentiation of the cardiac effects of adenosine by PD 81,723 is selective for $A_1$ receptor-mediated responses. The cardiac actions of adenosine are mediated by at least two receptor subtypes, $A_1$ and $A_2a$. Activation of $A_1$ receptors is responsible for the negative dromotropic effect of adenosine, whereas the coronary vasodilation caused by adenosine is mediated by the $A_2a$ receptor.$^2$ In the present study, PD 81,723 potentiated the $A_1$ receptor-mediated negative dromotropic effect of the nonspecific adenosine analogue PCPN, which resulted in a leftward shift of the concentration-response curve (Fig 4). In contrast, PD 81,723 did not alter the concentration-response curve for the $A_2a$ receptor-mediated coronary vasodilatory effect of PCPN (Fig 4). Thus, these findings demonstrate that PD 81,723 selectively enhances $A_1$ receptor— but not $A_2a$ receptor—mediated responses of adenosine.

**PD 81,723 Selectively Enhances Binding of Adenosine Agonists to the $A_1$ Receptor**

To determine the biochemical mechanism of the potentiation of the $A_1$ receptor—mediated negative dromotropic effect of adenosine by PD 81,723, a series of radioligand binding studies of human and guinea pig cardiac and guinea pig brain membranes was carried out. The majority of the studies were performed on brain membranes because of the low density of adenosine receptors in heart membranes. Brain membranes have a higher density of adenosine receptors (5- to 10-fold higher) and a lower nonspecific binding than do heart membranes.$^{20,21}$ In addition, the brain $A_1$ receptor appears to be an excellent model for the cardiac $A_1$ receptor. For example, there is a positive and significant
correlation between the rank order of potency of adenosine agonists in slowing the atrial heart rate and AV nodal conduction in rat and guinea pig isolated hearts, respectively, and the A1 adenosine receptor binding affinities of those same agonists in rat22 and guinea pig brain membranes (Fig 7). Furthermore, nearly identical A1 adenosine receptor clones have been isolated from human brain, heart, and kidney cDNA libraries.23

In keeping with the results of the functional studies, PD 81,723 enhanced the binding of the agonist [3H]CHA to human atrial and guinea pig atrial and brain A1 adenosine receptors. The increase in agonist binding was concentration dependent and selective for the A1 receptor and could be demonstrated in both equilibrium and kinetic binding studies. For instance, in equilibrium binding assays, PD 81,723 increased the submaximal and maximal (ie, Bmax) specific binding of the agonist [3H]CHA to A1 adenosine receptors in guinea pig and human atrial and guinea pig brain membranes, respectively (Figs 6 and 8). On the other hand, PD 81,723 did not affect the maximum specific binding of the A2a antagonist [3H]CPX or the A2b, agonist [3H]CGBS 21680 to guinea pig forebrain and striatal brain membranes, respectively (Fig 9). Similarly, the results of the kinetic binding studies confirm a previous report24 that PD 81,723 decreases Kd, of the agonist [3H]CHA but not the antagonist [3H]CPX from A1 adenosine receptors (Fig 12).

The results of the radioligand binding assays described in the present study show that the enhancement by PD 81,723 of agonist binding to A1 adenosine receptors is associated with (1) a decrease in the agonist Kd, from the A2 receptor and (2) an increase in Bmax of agonists to A1 receptors. Slowing of Kd, is a common manifestation of allosteric enhancement.24 For instance, GABA and ropizine have been reported to slow the Kd, of diazepines and [3H]dexamethasone, respectively.25,26 The enhancement of submaximal specific binding of A1 agonists to brain26 and cardiac membranes (Fig 6) by PD 81,723 can be attributed, at least in part, to this mechanism. The observed increase in Bmax of the agonist [3H]CHA by PD 81,723 has not been previously reported. Cardiac and brain A1 adenosine receptors, like other receptors coupled to guanine nucleotide binding proteins (G proteins), are reported to exist in high- and low-affinity binding states.27,28 The linear Scatchard analysis and relatively low Kd, (ie, <10 nM/L) of the saturation isotherms suggest that the agonist [3H]CHA is binding primarily, but not exclusively, to receptors in the high-affinity binding state (Fig 8). The Bmax value of the antagonist [3H]CPX was fourfold higher than the Bmax value of the agonist [3H]CHA in the absence of PD 81,723 (Fig 9). This finding suggests that high-affinity binding detected by the agonist represents only a fraction of the total receptor population. Thus, there are at least two mechanisms whereby PD 81,723 may increase the Bmax value of an agonist: (1) PD 81,723 may improve the coupling of the A1 receptor to the G protein and thereby increase the fraction of receptors in the high-affinity binding state. (2) In addition or alternatively, PD 81,723 may act directly on the A1 receptor to increase the affinity with which agonists bind to the low-affinity state of the receptor. In support of the former hypothesis, PD 81,723 increased the fraction of receptors in the high-affinity binding state by an average of 56±13% (Fig 10). To determine the effect of PD 81,723 on G protein–receptor interactions, the inhibition of agonist binding by the nonhydrolyzable GTP analogue Gpp(NH)p was used as a functional assay for the efficacy of receptor to G protein coupling.29 PD 81,723 reduced the potency of Gpp(NH)p to inhibit the specific binding of [3H]CHA by approximately sixfold and ninefold in cardiac and forebrain membranes, respectively (Fig 11). This finding can be interpreted to indicate that PD 81,723 improves the coupling of A1 adenosine receptors to G proteins. However, additional studies will be required to conclusively establish the involvement of receptor–G protein interactions in the mechanism of action of PD 81,723.

Implications

The nucleoside adenosine has been shown to be cardioprotective during periods of ischemia and hypoxia.4 A similar protective role for adenosine and adenosine analogues has also been shown in brain, liver, and intestine.30-32 This and other potentially beneficial actions of adenosine have led to increased interest in the development of adenosine-related drugs targeted to ameliorate conditions such as myocardial ischemia and stroke.4,30 Because direct-acting receptor agonists will activate A1 adenosine receptors in multiple organ systems, new approaches are needed to achieve event and site specificity. Allosteric enhancers of adenosine may prove to be valuable alternatives to direct-acting adenosine agonists. Such agents should selectively augment the response to adenosine in only those organs or localized areas of a given organ in which production of adenosine is increased. For instance, the cardiac effects of allosteric enhancers will be most prominent in areas of the myocardium where oxygenation is inadequate. These agents will act as site-specific (O2-deprived areas only) and event-specific (ischemia or normoxia) potentiators of the effects of adenosine. In the absence of increased production of this nucleoside (eg, normoxia), allosteric enhancers of adenosine should have little or no effect. In addition, allosteric enhancers may potentiate only those responses mediated by a given receptor subtype, such as the A1 receptor in the case of PD 81,723. Thus, PD 81,723 and similar compounds may prove to be ideal therapeutic approaches to selectively and specifically amplify the actions of endogenous adenosine. In the accompanying study in this issue of Circulation Research,33 we show that PD 81,723 augments A1 but not A2a receptor–mediated actions of endogenous adenosine in isolated and in situ guinea pig hearts.

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References

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C Kollias-Baker, J Ruble, D Dennis, R F Bruns, J Linden and L Belardinelli

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