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Effects of Long-Term Treatment with the Allosteric Enhancer, PD81,723, on Chinese Hamster Ovary Cells Expressing Recombinant Human A_1 Adenosine Receptors

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SUMMARY

In this study, desensitization and down-regulation of A_1 adenosine receptors (A_1AR) by the allosteric enhancer PD81,723 (PD) and by N^6-cyclopentyladenosine (CPA) were investigated after 24-hr pretreatment of CHO-K1 cells stably expressing recombinant human A_1AR. Pretreatment with 20 μM PD and 10 μM CPA caused a 1.5- and 4.0-fold, respectively, desensitization (reduced potency of CPA to lower cAMP). Pretreatment with PD and/or CPA did not modify the acute effect of PD to increase (5-fold) the potency of CPA. Radioligand binding was used to measure receptor down-regulation in cell membranes and in intact cells. Pretreatment of cells with PD had no effect on the number of membrane binding sites for the agonist [^{125}I]N^6-(3-iodo-4-aminobenzyl)adenosine or for the antagonist, [^{3}H]8-cyclopentyl-1,3-dipropylxanthine, but the binding of these radioligands to intact cells was modestly reduced (20–37%), possibly reflecting an effect of pretreatment on receptor subcellular distribution. Pretreatment of cells with CPA produced large (>40%) reductions in the binding of radioligands to both membranes and intact cells. Pretreatment of cells with CPA also increased the number of presumed internalized receptors measured as [^{3}H]8-cyclopentyl-1,3-dipropylxanthine binding sites in intact cells insensitive to blockade by the charged antagonist 8-sulfophenyltheophylline. The relatively small degree of functional desensitization and down-regulation of A_1AR caused by long term exposure of cells to PD is considered to be encouraging in terms of the therapeutic potential of the allosteric enhancer class of compounds.

The purine nucleoside adenosine produces numerous physiological actions that are mediated by G protein-coupled receptors (1). The adenosine receptors have been subclassified as A_1, A_{2A}, A_{2B} and A_3 based on molecular cloning, pharmacological, and functional characteristics (2, 3). A series of 2-amino-3-benzoylthiophene compounds, including PD, have been found to increase agonist radioligand binding selectively to A_1AR in membranes prepared from brain (4), heart (5), and transfected cells (6) and to enhance the functional effects of A_1AR activation in rat brain, FRTL-5 thyroid cells (7), rat and guinea pig heart (8, 9), and CHO-K1 cells transfected with recombinant human A_1AR (6). PD apparently acts by binding to an allosteric site distinct from the orthostatic adenosine binding site to stabilize agonist/A_1AR/G protein complexes (6). PD has no effect on ligand binding to rat or canine A_{2A}AR, canine A_3AR, or δ-opioid, M_2-muscarinic, or α_2-adrenergic receptors (4, 10).

The allosteric compounds are of interest because of their unusual mechanism of action and because of the potential clinical benefits of selectively enhancing the activity of A_1ARs at ischemic tissue sites where adenosine is produced locally. A_1AR activation has been shown to produce protective effects in cardiovascular (11, 12), renal, gastrointestinal, immune, and nervous tissues (13–15). Possible beneficial effects of treating patients prone to ischemia with A_1AR agonists may be limited by side effects and receptor desensitization (16–19). In this study, we sought to determine whether long term exposure of cells to PD causes desensitization. The results indicate that in comparison to CPA, PD produces minimal desensitization of the cellular response to CPA or down-regulation of A_1ARs. Also, the allosteric action of PD to enhance the cellular actions of CPA does not desensitize.

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ABBREVIATIONS: AR, adenosine receptor; ABA, N^6-(4-aminobenzyl)adenosine; CPA, N^6-cyclopentyladenosine; CPX, 8-cyclopentyl-1,3-dipropylxanthine; DMSO, dimethylsulfoxide; HEPES, 4-(2-hydroxyethyl)1-piperazineethanesulfonic acid, PD, 2-amino-4,5-dimethyl-3-thienyl-[3(trifluoromethyl)-phenyl]methanone (PD81,723 or LY202472); *R*-PIA, *R*-phenyl-isopropyladenosine; 8-SPT, 8-sulfophenyltheophylline; GABA, γ-aminobutyric acid.

Materials and Methods

Chemicals and supplies. HEPES, Tris, $MgCl_2$, and forskolin were purchased from Sigma Chemical Co. (St. Louis, MO); *R*-PIA, CPA, and CPX from Research Biochemicals (Natick, MA); adenosine deaminase from Boehringer Mannheim (Mannheim, Germany); DMSO from Fisher Scientific (Columbia, MD); and [^{3}H]CPX (110 Ci/mmol) from DuPont-NEN (Boston, MA). PD was a gift from Robert F. Bruns (Lilly Research Lab, Eli Lilly, Indianapolis, IN). Human A_1AR stably transfected in CHO-K1 cells (American Type Culture Collection, Rockville, MD) was generated in our laboratory by Anna Robeva. ^{125}I-ABA was synthesized as described previously (20). Tissue culture reagents were obtained from GIBCO (Grand Island, NY).

Cell culture. CHO-K1 cells stably expressing human A_1AR were grown to confluence onto 100-mm plates (Falcon Plastics, Oxnard, CA) in F12 medium (GIBCO-BRL) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 0.5 mg/ml G-418 (GIBCO-BRL) in an atmosphere of 95% air/5% CO_2 at 37°. Cells were seeded at 0.2–1 × 10^4 cells/cm^2 and subcultured after detachment with the use of trypsin/EDTA (0.05%/0.5 mM).

Pretreatment protocol. Intact cells were pretreated with vehicle (0.3% DMSO), 20 μM PD, and/or 10 μM CPA for 24 hr in Ham's F12 medium containing 1 unit/ml of adenosine deaminase. Cells were then washed, and intact cells or membranes were prepared for binding or functional assays as described below. To ensure that the compounds added during the pretreatment period were completely washed out, cells attached to tissue culture plates were washed four times. Intact cells in suspension or membranes were then prepared and washed two more times. In some instances, 10 μM CPA was added to cells just before washing to allow evaluation of the effectiveness of the washing protocol. This failed to alter radioligand binding to membranes, suggesting that the added CPA was effectively removed. It is possible, however, that long term pretreatment results in the uptake of some CPA and/or PD into a cellular compartment that is resistant to wash-out.

Membrane preparation. CHO-K1 cells expressing human A_1AR were harvested from culture dishes and membranes were prepared as described previously (21). Membranes were resuspended in HEPES/EDTA buffer (10 mM HEPES, 1 mM EDTA, pH 7.4) supplemented with 10% w/v sucrose and stored in aliquots (1 mg membrane protein/ml) at −20°. Protein was measured with the use of fluorescamine fluorescence with bovine serum albumin standards (22).

Radioligand binding. For equilibrium binding assays, 0.01–0.1 mg of membrane protein was incubated in HEPES/EDTA buffer with adenosine deaminase (1 unit/ml) and radioligand (^{125}I-ABA or [^{3}H]CPX) for 2.5 hr at 21° in a volume of 100 μl. The bound and free radioligands were separated with filtration through Whatman GF/C filter paper using a Brandel tissue harvester (Gaithersburg, MD). Binding assays were performed in triplicate and nonspecific binding determined by adding 1 μM CPX either before or at the same time as radioligand. To measure dissociation kinetics, ^{125}I-ABA was bound to equilibrium, and then 20 μM R-PIA was added alone or in combination with PD for various times before filtration. The dissociation rate was accelerated by raising the incubation temperature to 37°. For whole-cell binding, CHO-K1 cells expressing A_1AR were pretreated with PD and/or CPA for 24 hr, washed four times with ice-cold PBS, and detached with cold PBS containing 5 mM EDTA. The cells were then washed twice with cold PBS and resuspended in cold Dulbecco's modified Eagle's medium containing 20 mM HEPES. Cells (100,000–150,000) were incubated with adenosine deaminase (1 unit/ml) and radioligands for 2.5 hr on ice in a volume of 200 μl.

cAMP assays. cAMP determinations were made on cells in suspension after their removal from plates by treatment with 5 mM EDTA in PBS for 5–10 min. Cells were washed twice with PBS and resuspended at room temperature in serum-free Dulbecco's modified Eagle's medium supplemented with 20 mM HEPES, pH 7.4, and 10 units/ml adenosine deaminase and pipetted into test tubes (50,000–70,000 cells/0.2 ml, 21°). Compounds (50 μl) were added at 5× the final concentration, and the cells were incubated for 10 min at 37° and then lysed by the addition of 500 μl of 0.15 M HCl. Each tube contained (final) 5 μM forskolin and 1 μM CPX. The CPX was added to attenuate constitutive inhibition of adenylyl cyclase found in cells overexpressing recombinant A_1AR. CPA was added acutely at concentrations sufficient to overcome competitive blockade by CPX.

Data analysis. Binding parameters (K_d, B_{max}, K_i, and IC_{50}) were fit by Marquardt's (23, 24) nonlinear least-squares interpolation to equations for one or multiple binding sites. Dissociation rates were fit to a two-component exponential binding model. Statistical analyses were performed by using the one-tailed paired Student's *t* test. The analysis of cAMP experiments was complicated by the fact that pretreatment of cells with PD or CPA changed both control cAMP levels and the potency of CPA to reduce cellular cAMP. To determine if these shifts were statistically significant, data sets from three sets of experiments were pooled, fit to a four-parameter logistic equation, and the resulting curves compared using sum of squares of fits to separate and pooled data sets as described previously (25). The *F* statistic evaluated at $p < 0.05$ was used to determine whether data sets were fit significantly better by two curves than by one.

Results

Desensitization of cAMP inhibition. Activation of A_1ARs inhibits adenylyl cyclase activity (1). The functional effects of pretreating cells with the allosteric compound, PD, and/or the orthostatic agonist CPA on cAMP responses were measured in intact CHO-K1 cells stably expressing recombinant human A_1AR. The concentration of PD used was 20 μM, which in previous studies with human A_1AR produced maximal enhancement (6). After a 24-hr pretreatment period, cells were washed and acutely exposed to forskolin and various concentrations of CPA. The A_1AR antagonist CPX (1 μM) also was added acutely to attenuate constitutive activity of the overexpressed recombinant receptors, a strategy similar to the one used by Bruns and Fergus (26) to study PD responses in FRTL-5 thyroid cells. In the concentration range of 10–1000 nM, CPA overcomes the competitive blockade of CPX (Fig. 1). On desensitization, there was an increase in control (i.e., the response of cells stimulated with forskolin and no CPA) cAMP levels (Fig. 1, *inset*, Table 1). After pretreatment with PD, the concentration-response curve to CPA was slightly (1.5-fold) shifted to the right (Fig. 1 and Table 1). Pretreatment of cells with CPA caused a much larger (4.0-fold) desensitization than did pretreatment of cells with PD. Pretreatment of cells with PD and CPA produced a 1.5-fold shift compared with CPA alone (p = NS).

In some experiments, cells were acutely treated with PD after 24 hr of pretreatment with PD and/or CPA. The functional effects of PD to acutely enhance the potency of CPA were not diminished after such pretreatment. In all cases, the acute addition of PD shifted the CPA dose-response curve by 4–5-fold (Fig. 2 and Table 1). These data suggest that the allosteric effect of PD to enhance the action of the orthostatic agonist CPA is not subject to desensitization.

Acute treatment of cells expressing recombinant A_1ARs with PD alone lowers control cAMP levels (Fig. 2). For example, PD alone (without CPA) lowers cAMP by 40% (Fig. 2A). In addition to altering the potency of CPA and elevating control cAMP levels, desensitization has a third effect: to diminish the ability of PD alone to lower control cAMP levels. In cells pretreated for 24 hr with a combination of PD and CPA, the acute addition of PD alone no longer lowers control

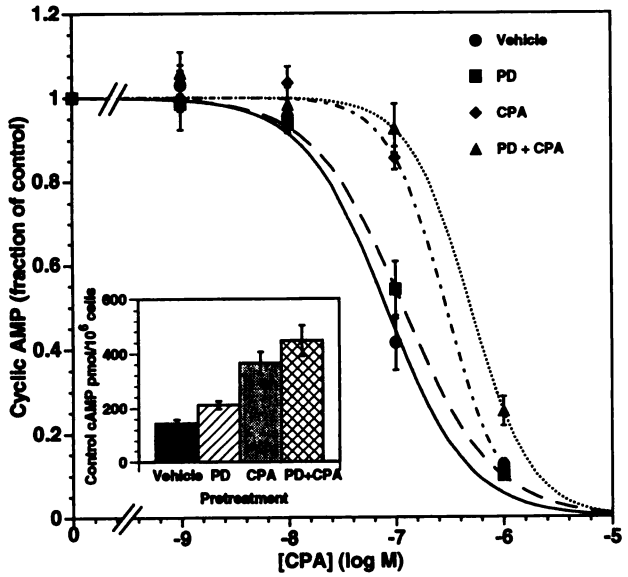

Fig. 1. Effects of pretreatment conditions on the acute dose-response curve of CPA to lower cAMP accumulation in CHO cells transfected with human A_1AR. Cells were pretreated for 24 hr with vehicle (0.3% DMSO), PD (20 μM), CPA (10 μM), or PD + CPA in the presence of 1 unit/ml adenosine deaminase. The pretreated cells were washed with PBS (three times) and incubated for 10 min with 1 μM CPX, 10 units/ml adenosine deaminase, 5 μM forskolin, and various concentrations of CPA. Data are expressed as fraction of control (forskolin without CPA) for each pretreatment condition. *Inset*, absolute cAMP accumulation corresponding to four control responses. *Points*, mean ± standard error of three experiments, each assayed in triplicate.

cAMP levels, but PD still acts to acutely increase the potency of CPA (Fig. 2D).

Membrane binding studies. We examined the effects of pretreating cells with PD and/or CPA on the binding of radioligands to cell membranes. For these experiments, the number of A_1 receptors coupled to G proteins was estimated from the binding of the agonist radioligand ^{125}I-ABA, and the total number of receptors was estimated based on the binding of the antagonist [^{3}H]CPX (27). Cells were preincubated under conditions identical to those used to produce desensitization, i.e., 20 μM PD and/or 10 μM CPA for 24 hr. In three experiments, pretreatment with PD was found to have no effect on the number of ^{125}I-ABA binding sites or on the radioligand K_D (Fig. 3A and Table 1). Pretreatment of cells with 10 μM CPA reduced the number of ^{125}I-ABA binding sites by >40%. The magnitude of receptor down-regulation caused by pretreatment with 10 μM CPA was unaffected by the simultaneous addition of 20 μM PD (Fig. 3A and Table 1). As shown in Fig. 3B, the total number of [^{3}H]CPX binding sites is not altered by pretreatment with 20 μM PD alone (Table 1). Pretreatment with CPA reduced the number of [^{3}H]CPX binding sites by 29% (Fig. 3B and Table 1). The magnitude of this CPA-induced down-regulation was unaffected by the simultaneous addition of PD. The down-regulation of [^{3}H]CPX binding sites was not accompanied by any change in the radioligand K_D, suggesting that it is not caused by failure to completely remove CPA used during the pretreatment protocol. Furthermore, the down-regulation persisted when binding assays were conducted in the presence of 50 μM GTPγS, which would reduce the affinity of receptors for any residual CPA (data not shown). These data suggest that pretreatment of cells with CPA acted to decrease the number of receptors and not to mask some receptors as a result of persistent high affinity binding.

Although PD had no effect on the magnitude of receptor down-regulation produced by a high concentration of CPA (10 μM), it seemed possible that PD might influence the concentration dependence of CPA to cause receptor down-regulation. To test this possibility, we wanted to determine the effects of PD pretreatment on receptor down-regulation caused by various concentrations of CPA. The addition of PD for 24 hr with CPA failed to modify the CPA concentration dependence of receptor down-regulation as evaluated through agonist (Fig. 4A) or antagonist (Fig. 4B) radioligand binding.

In previous studies, it has been shown that PD acts to selectively decrease the rate of dissociation of agonists from A_1ARs (5, 6, 26). We next sought to determine whether pretreating cells with CPA and/or PD causes any change in agonist dissociation kinetics or influences the acute action of PD to slow agonist dissociation. The results are shown in Fig. 5 and summarized in Table 1. ^{125}I-ABA dissociation occurs in two kinetically distinct components. The majority of binding dissociates slowly, probably reflecting binding to high affinity receptor/G protein complexes. In three experiments, the acute addition of PD to membranes slowed the slow component of ^{125}I-ABA binding by 2–2.5-fold. This acute effect of PD was not changed by pretreatment of the cells with CPA and/or PD for 24 hr.

Intact cell binding studies. In the studies described above, it is notable that pretreatment of the cells with PD produces a small functional desensitization (Figs. 1 and 2 and Table 1) that is not accompanied by a detectable change in the parameters of radioligand binding to membranes (Figs. 3–5 and Table 1). Pretreatment of cells with CPA produces a large (4-fold) desensitization response (Fig. 1) associated with relatively smaller changes in radioligand binding parameters (Fig. 3). These data suggest that some factor or factors that contribute to desensitization responses in whole cells is lost or reversed on cell lysis. This prompted us to conduct a series of experiments designed to determine whether pretreatment of cells with PD and/or CPA influences the binding of radioligands to intact cells more than to broken cell membranes. Whole-cell binding assays were conducted on ice to prevent possible reversal of the effects of pretreatment during the course of the radioligand binding assays. As shown in Fig. 6A and Table 1, the total number of A_1 receptors measured on intact cells as [^{3}H]CPX binding sites was reduced by 20% after pretreatment of the cells for 24 hr with PD. Pretreating cells with PD also significantly reduced the number of high affinity ^{125}I-ABA binding sites measured in intact cells by 37% (Fig. 6B and Table 1). These high affinity ^{125}I-ABA binding sites represent only a small proportion of the total receptor pool (<1%), but it is striking that the number of such sites is strongly influenced by PD pretreatment. Pretreating cells with CPA produced a large reduction (>75%) in the number of high affinity ^{125}I-ABA binding sites measured on intact cells (Fig. 6C and Table 1).

We next sought to determine whether pretreatment of cells with PD and/or CPA modifies the distribution of receptors between the cell surface and an internalized compartment. We reasoned that due to its lipophilic character, [^{3}H]CPX will label the cell surface and internalized receptors, whereas the charged compound 8-SPT will be selectively accessible to

TABLE 1

Effects of pretreatment of CHO-K1 cells stably transfected with recombinant human A_1 adenosine receptors on cellular cAMP accumulation and radioligand binding parameters

Acute response	Not pretreated	PD (20 μM) pretreatment	CPA (10 μM) pretreatment	PD + CPA pretreatment
Whole-cell functional studies: cAMP accumulation, response to CPA[a]				
IC_{50} without PD (nM)	82 ± 9	127 ± 13[c]	331 ± 56[d]	486 ± 86[d]
IC_{50} with PD (nM)	18.6 ± 4.8	33.8 ± 15.4[c]	55 ± 12[d]	113 ± 13[c,d]
Control cAMP (pmol/10^6 cells)	109 ± 10	159 ± 11.3[c]	275 ± 31	335 ± 42
[^{3}H]CPX binding to membranes				
B_{max}	6,699 ± 603	6,595 ± 569	4,749 ± 505[d]	4,559 ± 491[d]
K_d	1.97 ± 0.387	1.93 ± 0.28	1.59 ± 0.36	1.8 ± 0.266
^{125}I-ABA binding to membranes				
B_{max}	992 ± 237	1,084 ± 239	567 ± 193[d]	596 ± 184[d]
K_d	1.1 ± 0.27	0.85 ± 0.12	0.776 ± 0.1	1.1 ± 0.247
T½ of ^{125}I-ABA dissociation (min)				
Without PD	13 ± 3.9	13 ± 0.64	14 ± 0.8	14 ± 3.3
With PD[a]	30 ± 4.8	30.7 ± 11	37.4 ± 6.4	34.5 ± 4.3
[^{3}H]CPX binding to whole cells				
B_{max}	10,253 ± 1,498	8,228 ± 808[c]		
K_d	2.42 ± 0.67	1.65 ± 0.29		
^{125}I-ABA binding to whole cells				
B_{max}	60 ± 29	38 ± 19[c]	14.6 ± 0.7[d]	11.7 ± 1.3[d]
K_d	1.3 ± 0.5	1.1 ± 0.23	0.94 ± 0.52	0.45 ± 0.21
Competition [^{3}H]CPX with 8-SPT				
% Internalized[b]	12.3 ± 3.5	14 ± 5	17.6 ± 6[d]	19 ± 5[d]

[a] After a 24-hr pretreatment period, cells were washed and acutely exposed to forskolin, CPA, and/or PD as described in Materials and Methods. The acutely treated cells also were treated with 1 μM CPX to minimize differences between groups in control cAMP levels. The CPA added was sufficient to overcome the competitive blockade by 1 μM CPX. Concentration-response curves with fit to a four-parameter logistic equation to data pooled from triplicate experiments. Standard errors of parameter values are listed.

[b] Internalization was estimated as the percentage of specific whole cell [^{3}H]CPX binding that is insensitive to blockade by 8-SPT.

[c] Significantly different than control ($p < 0.05$) for the effect of PD by the F test or the paired one-tailed Student's t test.

[d] Significantly different than not pretreated ($p < 0.05$).

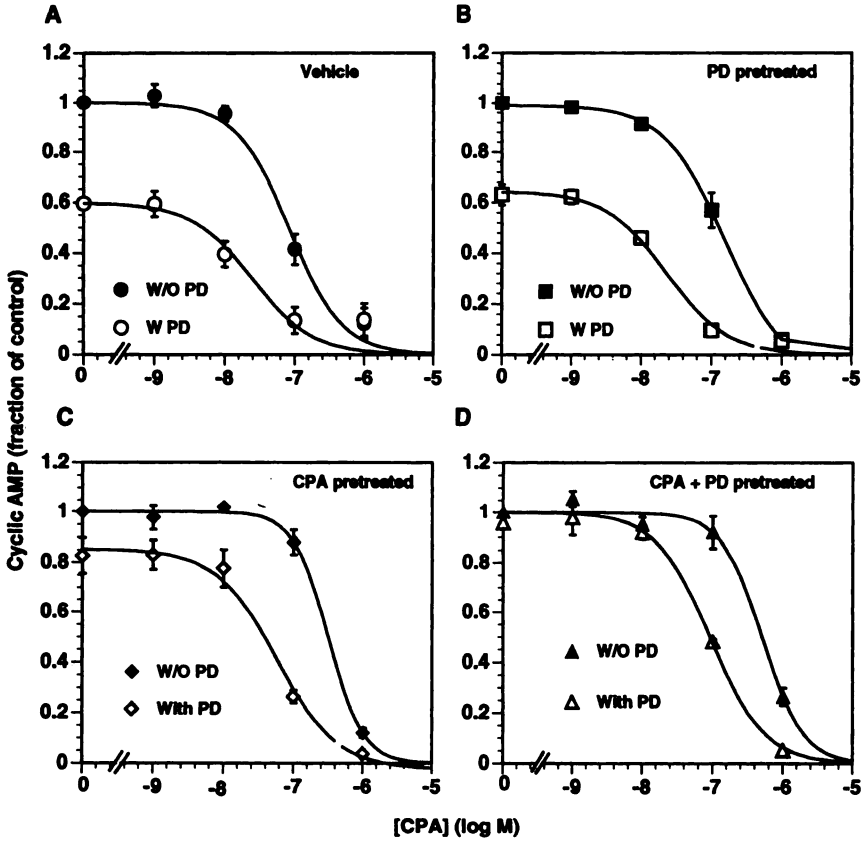

Fig. 2. Effect of PD on the dose-dependence of CPA to modify cAMP accumulation in CHO cells after various pretreatments. Cells pretreated as described in legend to Fig. 1 were washed and incubated without (*W/O*) or with (*W*) PD (20 μM) for 10 min with 10 units/ml adenosine deaminase, 1 μM CPX, 5 μM forskolin, and various concentrations of CPA. Data are expressed as fraction of control (forskolin without CPA or PD) for each pretreatment condition. *Points*, the mean ± standard error of three experiments, each assayed in triplicate.

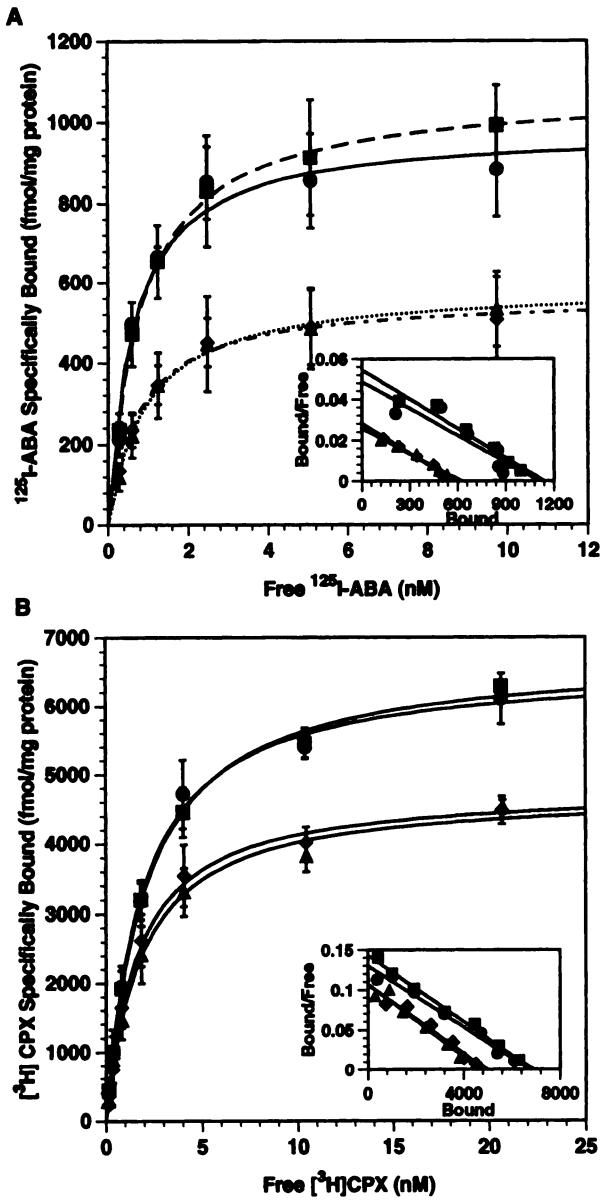

Fig. 3. Effects of pretreatment conditions of the equilibrium binding of ^{125}I-ABA (A) and [^{3}H]CPX (B) to membranes of CHO cells expressing A_1AR. The cells were pretreated for 24 hr with vehicle (0.3% DMSO, ●), PD (20 μM, ■), CPA (10 μM, ♦), or PD + CPA (▲) in the presence of 1 unit/ml adenosine deaminase and washed, and membranes were isolated for radioligand binding. Untransformed specific binding and Scatchard transformed (*inset*) binding isotherms are shown. Values are mean ± standard error of specific binding pooled from three independent experiments, each assayed in triplicate. Binding parameters are summarized in Table 1.

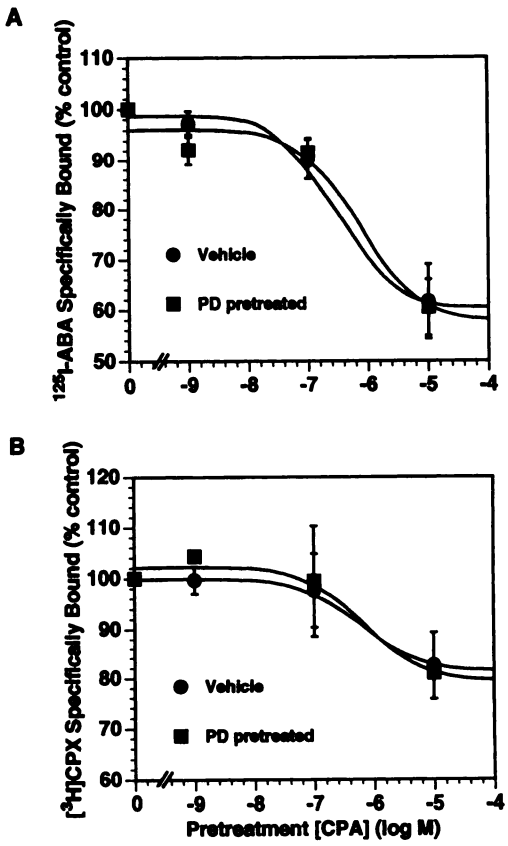

Fig. 4. Effect of PD (20 μM) on CPA-induced down-regulation of A_1AR. CHO cells expressing A_1AR were pretreated for 24 hr with different concentrations of CPA without (●) or with (■) PD (20 μM) and washed, and membranes were isolated for ^{125}I-ABA (A) and [^{3}H]CPX (B) radioligand binding. Data are expressed as percentage of specific binding obtained from cells not pretreated with CPA and are mean ± standard error of specific binding pooled from three independent experiments, each assayed in triplicate.

receptors on the cell surface. Therefore, we defined specific [^{3}H]CPX binding to intact cells that is insensitive to displacement by a saturating concentration (1 mM) of 8-SPT as an internalized pool. As shown in Fig. 7 and Table 1, this pool represents 12% of the total number of receptors in cells not pretreated with CPA (Fig. 7). Pretreatment of cells with 10 μM CPA for 24 hr increased the proportion of the internalized receptor pool. Pretreatment with PD did not significantly increase the proportion of internalized receptors in the absence or presence of CPA.

Discussion

Allosteric enhancers of receptors are defined as compounds that potentiate responses to orthostatic agonists. An example of a well characterized class of compounds that is thought to act allosterically is the benzodiazepines, including diazepam. These compounds are enhancers of $GABA_A$ receptors. They potentiate the response to GABA by stabilizing a high affinity state of the receptor and shifting the dose-response curve of GABA to the left (28). The clinical advantages of allosteric enhancers over agonists is illustrated by the fact that benzodiazepines have relatively few side effects and are used clinically. In contrast, direct-acting $GABA_A$ agonists produce numerous side effects and are not used clinically.

The 2-amino-3-benzoylthiophene compounds such as PD may be useful for enhancing the protective effects of adenosine mediated by A_1 adenosine receptors. This approach is particularly attractive as a therapeutic strategy because adenosine is elevated selectively in hypoxic areas of ischemic tissues. Allosteric enhancers might be used chronically to treat ischemic diseases if the effects of these compounds are not subject to desensitization. In this study, we demonstrated that long term exposure of cells to PD does not produce desensitization to its allosteric action. Thus, the acute effect of PD to shift the CPA dose-response curve to the left by 4–5-fold was not significantly influenced by pretreatment of

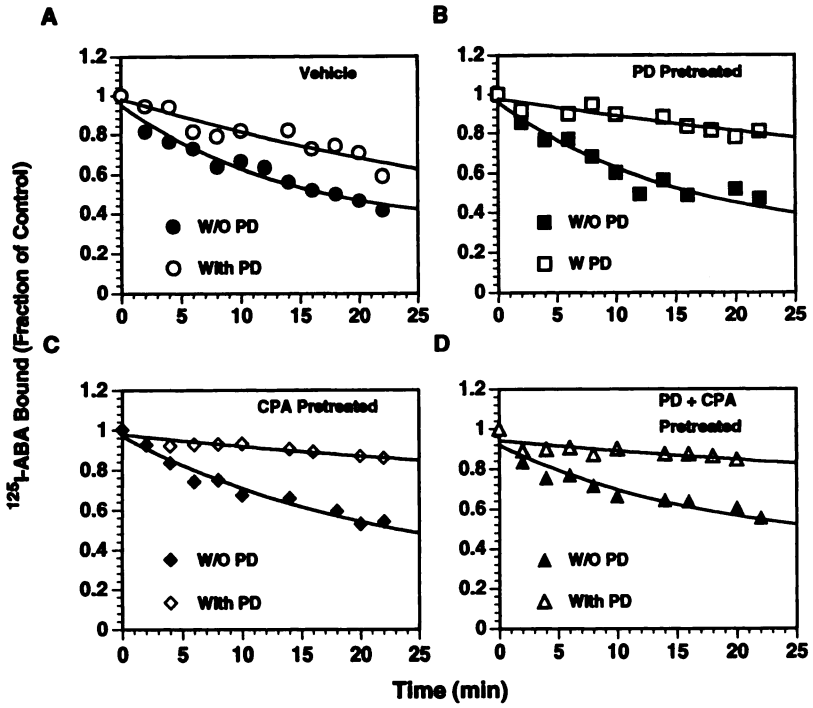

Fig. 5. Effect of PD on the kinetics of dissociation of ^{125}I-ABA from membranes of CHO cells after various pretreatments. CHO cell membranes pretreated for 24 hr with vehicle (0.3% DMSO), PD (20 μM), CPA (10 μM), or PD + CPA in the presence of 1 unit/ml adenosine deaminase were incubated with ^{125}I-ABA (0.5 nM) for 1 hr at 37°. *R*-PIA (20 μM) without (*W/O*) or with (*W*) PD (20 μM) was added for various times before filtration. Specific binding is normalized to binding at equilibrium. Data are from a single experiment that was repeated three times with similar results. The dissociation rates are summarized in Table 1.

cells with PD and/or CPA. Also, the effect of PD to slow the kinetics of agonist dissociation from receptors, a sensitive index of the allosteric response (2), was not affected by the various pretreatment conditions. Pretreatment of cells with PD also produced minimal desensitization to an orthostatic agonist, CPA.

A disadvantage of using recombinant cells to investigate desensitization and down-regulation responses is that recombinant receptors may not be normally regulated. However, in this study, we showed that the orthostatic agonist CPA produces desensitization and down-regulation that are typical of native adenosine receptors of tissues or cultured cells (16–19). The advantages of using recombinant receptors is that overexpressed receptors are homogeneous, human receptors can be characterized, and receptors can be quantified with great precision because of their high abundance.

As an initial functional assay for allosteric enhancers of A_1 adenosine receptors, Bruns and Fergus (4, 26) demonstrated that these compounds cause a leftward shift in the dose-response curve of CPA to inhibit forskolin-stimulated cAMP accumulation in FRTL-5 rat thyroid cells. In addition to shifting the CPA curve to the left, the enhancers lower cAMP in the absence of CPA. In accordance with one of the models of allosterism reviewed by Monod *et al.* (29), Bruns and Fergus (4) proposed that the 2-amino-3-benzoylthiophenes may behave as agonists except that they bind to an allosteric site distinct from the orthostatic adenosine binding site. According to this model, allosteric enhancers potentiate the response to orthostatic agonists. The fact that PD produces little desensitization by itself is consistent with the possibility that it acts as a weak agonist.

It is notable that despite potentiating the action of CPA to lower cAMP, PD did not potentiate CPA-induced desensitization. PD increases agonist binding to human A_1AR by increasing agonist binding affinity and by enhancing coupling of receptors to G proteins (6). This is manifested as an increase in the proportion of receptors found in a high affinity state and apparently results in a more efficient activation of G proteins than is produced in the absence of the enhancer. The fact that the enhancer produces little effect by itself and does not substantially increase down-regulation or desensitization induced by CPA suggests that the conformation state of the receptor that activates G proteins is not selectively targeted for down-regulation. This is consistent with the notion that it is receptors uncoupled from G proteins that are subject to phosphorylation by G protein-coupled receptor kinases and become internalized (30). PD did produce a modest desensitization associated with minor changes in radioligand binding parameters that could be detected in intact cells but not in cell membranes. Because PD enhances both the constitutive activity of recombinant receptors and the actions of the orthostatic agonist CPA, PD apparently is capable of triggering some G protein activation, even in the absence of CPA. This will result in the generation of some free βγ subunits, which in turn may produce some receptor down-regulation and desensitization by causing a βγ-mediated increase in G protein-coupled receptor kinase activity (31).

In this study, PD lowered cAMP in the absence of an orthostatic agonist. A similar effect of PD was noted previously in cultured thyroid cells (4). This could occur because PD is a partial agonist, because PD enhances the intrinsic activity of A_1AR, or because PD augments the action of a low level of adenosine present in cells that is not completely removed by the addition of adenosine deaminase. The fact that competitive antagonists such as CPX antagonize this direct action of PD does not distinguish among these possibilities. It is notable, however, that PD alone has little functional effect on tissues, possibly because the density of A_1AR is relatively low (5). A small degree of intrinsic activity by unliganded receptors could take on increased significance in recombinant as opposed to wild-type systems due to the expression of very large numbers of receptors. We show here that this intrinsic effect of PD is diminished after CPA-induced desensitization.

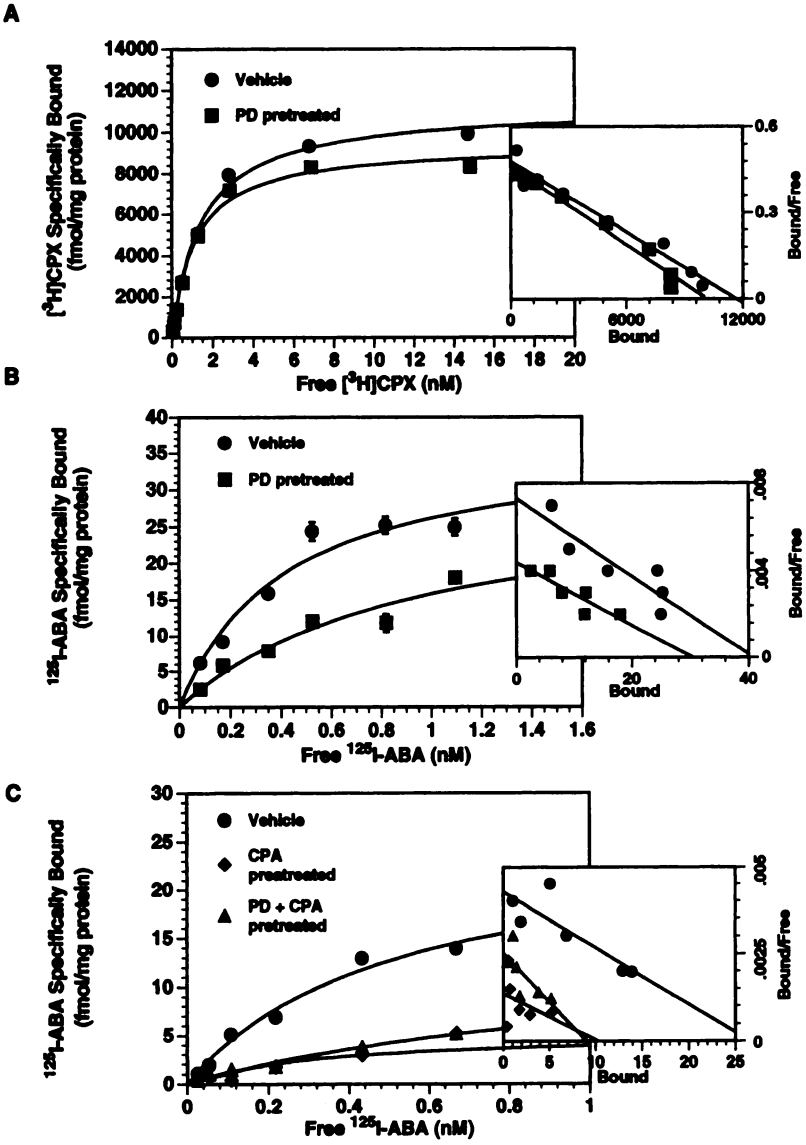

Fig. 6. Effects of pretreatment conditions on the equilibrium binding of [^{3}H]CPX (A) or ^{125}I-ABA (B and C) to intact CHO cells. Cells were pretreated for 24 hr with vehicle (0.3% DMSO), PD (20 μM), CPA (10 μM) or PD + CPA in presence of 1 unit/ml adenosine deaminase; washed; and used for radioligand binding on ice. Untransformed specific binding and Scatchard transformed (*insets*) binding isotherms are shown. Data are mean ± standard error from a single experiment assayed in triplicate. Similar results were obtained in three experiments. Binding parameters are summarized in Table 1.

Pretreatment of cells with PD produced a small degree of desensitization and some changes in the binding of radioligands to intact cells. It is striking, however, that pretreatment with PD failed to influence radioligand binding to broken cell membranes. These data suggest that PD produces some modification of receptors that is not preserved on cell lysis. Another possibility is that PD has a selective effect on an internalized compartment of receptors that is disrupted when cells are lysed. Prolonged treatment of cells with CPA produced changes in radioligand binding characteristics to intact cells and membranes. Reduced radioligand binding to membranes most probably reflects down-regulation of receptor number. Such changes in receptor number likely contribute to the substantial functional desensitization produced by long term exposure of cells to CPA. The interpretation of changes in radioligand binding to intact cells is more complex. When considering the effect of pretreating cells with CPA on radioligand binding to intact cells, it seems possible that some of the changes might be due to a slowly reversible uptake of CPA into an internalized compartment of receptors that serves to mask radioligand binding to these sites. It is significant in this regard that whole-cell, but not membrane

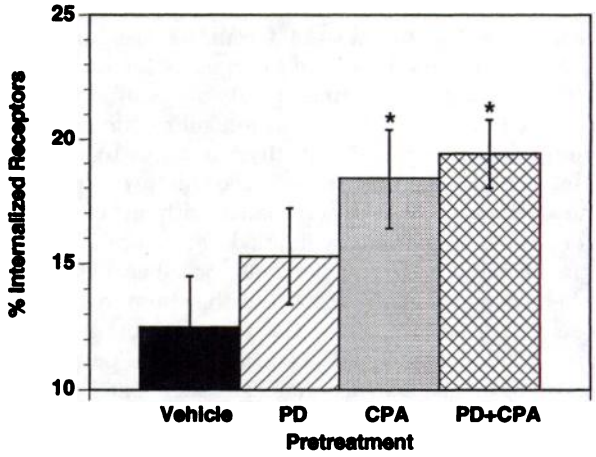

Fig. 7. Effects of pretreatment conditions on the competition by 8-SPT for [^{3}H]CPX binding to intact cells. Percent internalization is defined as percentage of specific [^{3}H]CPX binding sites not displaced by 1 mM 8-SPT in intact CHO cells expressing A_1AR. Cells were preincubated for 24 hr with vehicle (0.3% DMSO), PD (20 μM), CPA (10 μM), and PD + CPA. *Bars*, mean ± standard error of three experiments, each assayed in triplicate. Data are summarized in Table 1.

binding assays, were conducted on ice to prevent possible reversal of down-regulation responses during the incubation of intact cells with radioligands. The cold temperature may have retarded the washout of compounds from internalized receptor compartments and/or reduced the accessibility of radioligands to internalized receptors. On the other hand, there apparently is a CPA-induced receptor internalization manifested as an increase in the proportion of specific [^{3}H]CPX binding to intact cells that is insensitive to blockade by the charged compound 8-SPT. PD and endogenous adenosine may also gain access to internalized binding sites during long term incubations. This might account for the curious observation that long term pretreatment with PD reduces the number of [^{3}H]CPX binding sites on intact cells but not on cell membranes. It is likely that the small amount of desensitization caused by long term exposure of cells to PD is associated with some redistribution of receptors into a pool that, for some reason, has limited access to radioligands.

In summary, in this study, we demonstrated that long term exposure of intact cells to the allosteric enhancer PD produces a small desensitization response that is also manifested by some subtle changes in radioligand binding to intact cells but not to cell membranes. In contrast, long term pretreatment with the orthostatic agonist CPA produces a much larger desensitization and down-regulation responses. The minimal desensitization caused by PD is viewed as encouraging in terms of the therapeutic potential of A_1 adenosine receptor enhancer compounds.

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References

1. Tucker, A. L., and J. Linden. Cloned receptors and cardiovascular responses to adenosine. *Cardiovasc. Res.* **27**:62–67 (1993).
2. Linden, J., M. A. Jacobson, C. Hutchins, and M. Williams. Adenosine receptors, in *Handbook of Receptors and Channels: G Protein Coupled Receptors* (S. J. Peroutka, ed.). CRC Press, Boca Raton, FL, 29–44 (1994).
3. Linden, J. Cloned adenosine A3 receptors: pharmacological properties, species differences and receptor functions (Review). *Trends Pharmacol. Sci.* **15**:298–306 (1994).
4. Bruns, R. F., and J. H. Fergus. Allosteric enhancement of adenosine A_1 receptor binding and function by 2-amino-3-benzoylthiophenes. *Mol. Pharmacol.* **38**:939–949 (1990).
5. Kollias-Baker, C., J. Ruble, D. Dennis, R. F. Bruns, J. Linden, and L. Belardinelli. Allosteric enhancer PD 81,723 acts by novel mechanism to potentiate cardiac actions of adenosine. *Circ. Res.* **75**:961–971 (1994).
6. Bhattacharya, S., and J. Linden. The allosteric enhancer, PD 81,723, stabilizes human A_1 adenosine receptor coupling to G proteins. *Biochim. Biophys. Acta Mol. Cell Res.* **1265**:15–21 (1995).
7. Janusz, C. A., and R. F. Berman. The adenosine binding enhancer, PD 81,723, inhibits epileptiform bursting in the hippocampal brain slice. *Brain Res.* **619**:131–136 (1993).
8. Mudumbi, R. V., S. C. Montamat, R. F. Bruns, and R. E. Vestal. Cardiac functional responses to adenosine by PD 81:723, an allosteric enhancer of the adenosine A1 receptor. *Am. J. Physiol.* **264**:H1017–H1022 (1993).
9. Amoah-Apraku, B., J. Xu, J. Y. Lu, A. Pelleg, and L. Belardinelli. Selective potentiation by an A_1 adenosine receptor enhancer of the negative dromotropic action of adenosine in the guinea pig heart. *J. Pharmacol. Exp. Ther.* **266**:611–617 (1993).
10. Mizumura, T., J. A. Auchampach, J. Linden, R. F. Bruns, and G. J. Gross. PD 81:723, an allosteric enhancer of the A1 adenosine receptor, lowers the threshold for ischemic preconditioning in dogs. *Circ. Res.*, in press.
11. Auchampach, J. A., and G. J. Gross. Adenosine A_1 receptors, K_{ATP} channels, and ischemic preconditioning in dogs. *Am. J. Physiol.* **264**:H1327–H1336 (1993).
12. Parrratt, J. R. Protection of the heart by ischaemic preconditioning: mechanisms and possibilities for pharmacological exploitation. *Trends Pharmacol. Sci.* **15**:19–25 (1994).
13. Linden, J. Structure and function of the A_1 adenosine receptor. *FASEB J.* **5**:2668–2676 (1991).
14. Daval, J.-L., D. K. J. E. Von Lubitz, J. Deckert, D. J. Redmond, and P. J. Marangos. Protective effect of cyclohexyladenosine on adenosine A_1-receptors, guanine nucleotide and forskolin binding sites following transient brain ischemia: a quantitative autoradiographic study. *Brain Res.* **491**:212–226 (1989).
15. Mitchell, H. L., W. A. Frisella, R. W. Brooker, and K.-W. Yoon. Attenuation of traumatic cell death by an adenosine A_1 agonist in rat hippocampal cells. *Neurosurgery* **36**:1003–1008 (1995).
16. Liang, B. T., and L. A. Donovan. Differential desensitization of A_1 adenosine receptor-mediated inhibition of cardiac myocyte contractility and adenylate cyclase activity: relation to the regulation of receptor affinity and density. *Circ. Res.* **67**:406–414 (1990).
17. Ramkumar, V., M. E. Olah, K. A. Jacobson, and G. L. Stiles. Distinct pathways of desensitization of A_1- and A_2-adenosine receptors in DDT_1 MF-2 cells. *Mol. Pharmacol.* **40**:639–647 (1991).
18. Shryock, J., A. Patel, L. Belardinelli, and J. Linden. Downregulation and desensitization of A1-adenosine receptors in embryonic chicken heart. *Am. J. Physiol.* **256**:H321–H327 (1989).
19. Lee, H. T., C. I. Thompson, A. Hernandez, J. L. Lewy, and F. L. Belloni. Cardiac desensitization to adenosine analogues after prolonged R-PIA infusion *in vivo*. *Am. J. Physiol.* **265**:H1916–H1927 (1993).
20. Linden, J., A. Patel, and S. Sadek. [^{125}I]Aminobenzyladenosine, a new radioligand with improved specific binding to adenosine receptors in heart. *Circ. Res.* **56**:279–284 (1985).
21. Robeva, A. S., R. L. Woodard, D. R. Luthin, H. E. Taylor, and J. Linden. Double tagging recombinant A_1 and A_{2A} adenosine receptors with hexahistidine and the FLAG epitope: development of an efficient generic protein purification procedure. *Biochem. Pharmacol.*, in press.
22. Stowell, C. P., T. G. Kuhlenschmidt, and C. A. Hoppe. A fluorescamine assay for submicrogram quantities of protein in the presence of Triton X-100. *Anal. Biochem.* **85**:572–580 (1978).
23. Marquardt, D. M. An algorithm for least-squares estimation of nonlinear parameters. *J. Soc. Ind. Appl. Math.* **11**:431–441 (1963).
24. Tabata, T., and R. Ito. Effective treatment of the interpolation factor in Marquardt's nonlinear least-squares fit algorithm. *Comput. J.* **18**:250–251 (1975).
25. Motulsky, H. J., and L. A. Ransnas. Fitting curves to data using nonlinear regression: a practical and nonmathematical review (Review). *FASEB J.* **1**:365–374 (1987).
26. Bruns, R. F., and J. H. Fergus. Allosteric enhancers of adenosine A_1 receptor binding and function, in *Adenosine Receptors in the Nervous System* (J. A. Ribeiro, ed.). Taylor & Francis, London, 53–60 (1989).
27. Yeung, S. H., and R. D. Green. Agonist and antagonist affinities for inhibitory adenosine receptors are reciprocally affected by 5'-guanylylimidodiphosphate or *N*-ethylmaleimide. *J. Biol. Chem.* **258**:2334–2339 (1983).
28. Barker, J. L., N. L. Harrison, and A. P. Mariani. Benzodiazepine pharmacology of cultured mammalian CNS neurons. *Life Sci.* **39**:1959–1968 (1986).
29. Monod, J., J.-P. Changeux, and F. Jacob. Allosteric proteins and cellular control systems. *J. Mol. Biol.* **6**:306–329 (1963).
30. Lefkowitz, R. J. G protein-coupled receptor kinases (Review). *Cell* **74**:409–412 (1993).
31. Kameyama, K., K. Haga, T. Haga, K. Kontani, T. Katada, and Y. Fukada. Activation by G protein *beta gamma* subunits of *beta*-adrenergic and muscarinic receptor kinase. *J. Biol. Chem.* **268**:7753–7758 (1993).

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