0026-895X/03/6406-1557–1564$7.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 64:1557–1564, 2003

Vol. 64, No. 6
2762/1114434
Printed in U.S.A.

Allosteric Enhancers of A_1 Adenosine Receptors Increase Receptor-G Protein Coupling and Counteract Guanine Nucleotide Effects on Agonist Binding

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Received June 25, 2003; accepted September 11, 2003

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Endogenous ligands of G protein-coupled receptors bind to *orthosteric* sites that are topologically distinct from *allosteric* sites. Certain aminothiophenes such as (2-amino-4,5-dimethyl-3-thienyl)-[3-(trifluromethyl)-phenyl]-methanone (PD81,723) and 2-amino-4,5,6,7-tetrahydro-benzo[b]thiophen-3-yl)-biphenyl-4-yl-methanone (ATL525) are positive allosteric regulators, or *enhancers*, of the human A_1 adenosine receptor (A_1AR). In equilibrium binding assays, ^{125}I-N^6-aminobenzyladenosine (^{125}I-ABA) binds to two affinity states of A_1AR with K_D-high (0.33 μM) and K_D-low (~10 nM). Enhancers have little effect on K_D-high but convert all A_1AR binding sites to the high-affinity state. Enhancers decrease the potency of guanosine 5′-*O*-(3-thio)triphosphate (GTPγS) as an inhibitor of agonist binding by 100-fold and increase agonist-stimulated guanine nucleotide exchange. The association of ^{125}I-ABA to high-affinity receptors on Chinese hamster ovary (CHO)-hA_1 membranes does not follow theoretical single-site association kinetics but is approximated by a bi-exponential equation with $t_{1/2}$ values of 1.85 and 12.8 min. Allosteric enhancers selectively increase the number of slow binding sites, possibly by stabilizing newly formed receptor-G protein complexes. A new rapid assay method *scores* enhancer activity on a scale from 0 to 100 based on their ability to prevent the rapid dissociation of ^{125}I-ABA from A_1AR in response to GTPγS. Compared with PD81,723, ATL525 (100 μM) scores higher (27 versus 79) and has less antagonist activity. ATL525 functionally enhances A_1 signaling to inhibit cAMP accumulation in CHO-hA1 cells. These data suggest that simultaneously binding orthosteric and allosteric enhancer ligands convert the A_1AR from partly to fully coupled to G proteins and prevents rapid uncoupling upon binding of GTPγS.

Ligands that target G protein-coupled receptors generally bind to the same site as the endogenous ligand, defined as the *orthosteric* site. Receptors can also be influenced by compounds that bind to topologically distinct *allosteric* sites, as reviewed recently (Christopoulos, 2002; Christopoulos and Kenakin, 2002). Various orthosteric ligands do not necessarily have exactly overlapping binding domains but overlap in their binding sites enough that two orthosteric ligands cannot bind at the same time. As a functional consequence, orthosteric ligands are competitive in equilibrium binding assays, and the binding of one orthosteric ligand does not affect the dissociation kinetics of another. Allosteric ligands have little or no overlap with the orthosteric site. Hence allosteric and orthosteric ligands can both bind simultaneously. As a functional consequence of binding, positive allosteric regulators decrease the dissociation kinetics of orthosteric ligands, and negative allosteric regulators increase the dissociation kinetics of orthosteric ligands. PD81,723 (Fig. 1) is a 2-aminothiophene extensively characterized as an allosteric activator or *enhancer* of A_1ARs (Bruns and Fergus, 1989; Amoah-Apraku et al., 1993; Musser et al., 1993, 1999; Bhattacharya and Linden, 1995; Kollias-Baker et al., 1997; Van der Klein et al., 1999; Kourounakis et al., 2001). Several groups have recently prepared new 2-aminothiophenes that are more potent and effective than PD81,723 as A_1AR allosteric enhancers (Baraldi et al., 2000; Tranberg et al., 2002; Lutjens et al., 2003). These compounds act to selectively increase the binding affinity of agonists to the A_1AR and have little or no activity at other G protein-coupled receptors or on the other adenosine receptor subtypes A_{2A},

This work was supported by grant number RO1-HL36555 from the National Institutes of Health.

ABBREVIATIONS: PD81,723, (2-amino-4,5-dimethyl-3-thienyl)-[3-(trifluromethyl)-phenyl]-methanone; A_1AR, A_1 adenosine receptor; ATL525, (2-Amino-4,5,6,7-tetrahydro-benzo[b]thiophen-3-yl)-biphenyl-4-yl-methanone; DMSO, dimethyl sulfoxide; ABA, N^6-4-amino-3-benzyladenosine; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; CPX, 8-cyclopentyl-1,3-dipropylxanthine; CPA, N^6-cyclopentyladenosine; CPT, 8-cyclopentyltheophylline; CHO-hA1, recombinant human A_1AR stably expressed in Chinese hamster ovary-K1 cells; R-G, receptor-G protein; LRG, ligand-receptor-G protein.

A_{2B}, or A_3 (Mizumura et al., 1996). At high concentrations, some 2-aminothiophenes, including PD81,723, also act as competitive antagonists of the orthosteric site.

PD81,723 increases equilibrium agonist radioligand binding to the A_1AR, slows agonist dissociation kinetics, and increases the functional sensitivity of tissues to A_1AR-mediated responses in the heart and central nervous system (Janusz et al., 1991; Amoah-Apraku et al., 1993). We have shown that it is possible to improve on the moderate enhancing efficacy of PD81,723 by modifying its structure such that the C-4 and C-5 atoms of the thiophene ring are incorporated into a cycloalkyl bridge or by adding bulky 3-aroyl-substituents (Tranberg et al., 2002). ATL525 (Fig. 1) incorporates both of these features by including the C-4 and C-5 methyl groups in a cyclohexyl ring and by containing a 3-(*p*-phenylbenzoyl) substituent. In this study, we used PD81,723 and ATL525 as tools to investigate the molecular mechanisms by which A_1 allosteric enhancers influence the interactions between A_1ARs, G proteins, and guanine nucleotides. ATL525 is more potent than PD81,723 in radioligand binding and functional assays. Both promote a slow ($t_{1/2}$ >10 min) conversion of the A_1AR from a mixture of receptors in the low- and high-affinity states to one consisting only of high-affinity receptors coupled to G proteins. The enhancers also reduce sensitivity of agonist radioligand binding to inhibition by GTPγS. We conclude that allosteric enhancers increase agonist binding by stabilizing the receptor-G protein complex.

Materials and Methods

Materials. PD81723 and ATL525 were synthesized as described previously (Tranberg et al., 2002). Stock 10 mM solutions were prepared in DMSO and stored frozen in small aliquots to avoid oxidation upon repeated freeze-thawing. ABA was a gift of Dr. Susan Daluge (GlaxoSmithKline, Research Triangle Park, NC); $Na^{125}I$ was purchased from Amersham Biosciences (Piscataway, NJ); [^{35}S]GTPγS and [^{3}H]CPX from PerkinElmer Life and Analytical Sciences (Boston, MA); adenosine deaminase was from Roche Diagnostics (Indianapolis, IN), and GTPγS, rolipram, forskolin, and 8-CPT were from Sigma (St. Louis, MO).

Equilibrium Binding Assays. ^{125}I-ABA was prepared by the chloramine-T catalyzed radioiodination of ABA with carrier-free $Na^{125}I$ followed by purification over a 25-cm C18 column using a System Gold high-performance liquid chromatograph (Beckman Coulter, Fullerton, CA) with UV detection at 268 nM and a Beckman in-line γ-counter to detect ^{125}I. Chromatography was initiated with methanol/5 mM NaH_2PO_4, pH 6.0 (1:1) for 5 min, followed by a linear gradient of methanol to 100% over 15 min. The elution times for iodine, ABA, and ^{125}I-ABA are 1.5, 8, and 13 min, respectively. Membranes were prepared in buffer with protease inhibitors as described previously (Robeva et al., 1996) from CHO-K1 cells stably transfected with human A_1 adenosine receptors (CHO-hA_1). Membranes derived from these cells express approximately 2 pmol/mg of protein of A_1 receptors. For equilibrium binding assays membranes were prepared in buffer (10 mM HEPES, pH 7.4) and preincubated with 2 U/ml adenosine deaminase for 10 min. Aliquots of membranes (15 μg of protein in 50 μl) were added to the wells of 96-well Millipore (Bedford, MA) multiscreen filtration plates (GF/B filter) with 50 μl/well of ^{125}I-ABA or [^{3}H]CPX with or without allosteric enhancers in HEPES buffer, usually with 1 mM $MgCl_2$ (final concentration, 0.5 mM $MgCl_2$ in 0.1 ml) at 21°C for 3 h. For competition binding experiments, inhibitors were added with radioligand. All of the wells were washed simultaneously using a Brandel Microdispenser with 5 × 200 μl of ice-cold 10 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.4. To determine nonspecific binding, 100 μM 8-CPT was added to the membranes before the radioligand, and allosteric enhancers were omitted. We found the addition of PD81,723 or ATL525 produced an apparent increase in nonspecific binding only in membranes expressing A_1 receptors. This probably results from the very high affinity and slow dissociation of the radioligand ^{125}I-ABA in the presence of allosteric enhancers. For this reason, true nonspecific binding was measured in the absence of allosteric enhancers and represented <20% of total binding in all experiments. For binding isotherms, nonspecific binding was fit by least-squares regression to a straight line. Nonspecific binding calculated for each concentration of radioligand was subtracted from total binding to determine specific binding. Specific equilibrium binding was fit to a 1- or 2-site hyperbolic equation using Prism (version 3.0; GraphPad Software, San Diego, CA) For curvilinear Scatchard analyses (2-site plots of Bound versus B/F) B/F was calculated using the quadratic equation: $Y = -B + [(B^2 + 4AC)^{1/2}/2A]$ where Y = bound/free, X = specific binding, $A = K_{D1} \times K_{D2}$, $B = X \times (K_{D1} + K_{D2}) - B_{max1} \times K_{D2} - B_{max2} \times K_{D1}$, $C = X \times (X - B_{max1} - B_{max2})$. Optimal parameter values were determined by nonlinear least-squares interpolation (Marquardt, 1963). All results shown in the figures are typical of 3 to 5 experiments. Each point represents the mean ± S.E.M. of 3 determinations, unless otherwise indicated.

Effects of Allosteric Enhancers on GTPγS Inhibition of ^{125}I-ABA Binding. Membranes from CHO-hA_1 cells were diluted to 0.3 mg protein/ml in HEPES/EDTA buffer (10 mM HEPES and 1 mM EDTA, pH 7.4) and preincubated with 2 U/ml adenosine deaminase for 15 min at 21°C. ^{125}I-ABA in HEPES/EDTA buffer containing $MgCl_2$ was added for 90 min, followed by ATL525 (25 μM) or vehicle (0.4% DMSO) for 5 min. Finally, various concentrations of GTPγS were added for 90 min. The final composition of the assay mixture was: 15 μg of membrane protein, 25 μM enhancer, 1 U/ml adenosine deaminase, 1.25 mM $MgCl_2$, and 0.5 nM radioligand.

Association Kinetics. The rate of ^{125}I-ABA binding to CHO-hA_1 membranes at 30°C was measured by adding, at 90-s intervals over a 30-min period, radioligand with $MgCl_2$ (final concentration, 0.5 mM) ± 25 μM allosteric enhancer to sets of wells containing membranes pretreated with adenosine deaminase (2 U/ml for 15 min in $MgCl_2$-free HEPES buffer) in a final volume of 200 μl. Incubations continued until the last set of wells had incubated for 90 s, then the entire ensemble of membranes was filtered and washed simultaneously. Equilibrium (2.5 h) specific binding (B_{eq}) also was determined in parallel. Binding theory predicts that association to a single binding site is described by the relationship $\ln[B_{eq}/(B_{eq} - B)] = K_{obs} \times t$ where $K_{obs} = K_{-1} + K_1 \times$ [radioligand], where K_{-1} is the dissociation rate constant and K_1 is the association rate constant. Theoretically, a plot of $\ln[B_{eq}/(B_{eq} - B)]$ versus time yields a straight line with a slope = K_{obs}. Because such a plot did not result in a straight line, association data were also fit by nonlinear least-squares to the bi-exponential equation $B = B1(1 - \exp(-k_1 t) +$

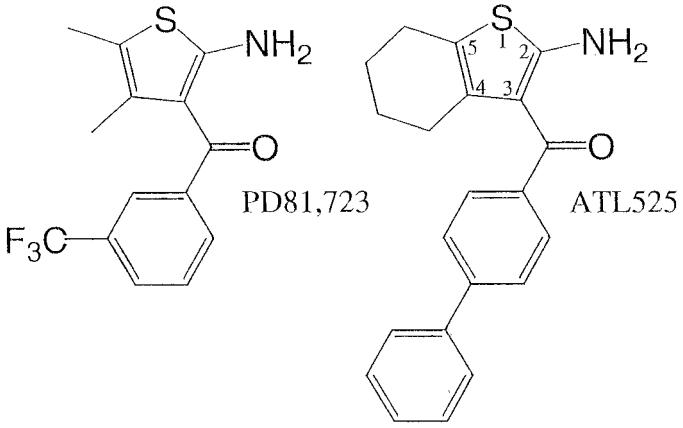

Fig. 1. The structures of 2-amino-3-aroyl-tiophene allosteric enhancers of A_1 adenosine receptors.

B2(1 − exp($-k_2t$); where $B1$ and $B2$ approximate the number of rapid and slow binding sites, respectively.

Dissociation Kinetics. CHO-hA_1 membranes (15 μg of protein in 100 μl) at 21°C were preincubated with adenosine deaminase (2 U/ml) in $MgCl_2$-free buffer. ^{125}I-ABA (final concentration, 0.7 nM with 0.5 mM $MgCl_2$) was added, and incubation continued for 90 min to achieve binding equilibrium. Either vehicle or allosteric enhancer (1–100 μM in 50 μl) was added, followed 5 min later by 50 μl of GTPγS and 8-cyclopentyltheophylline (final concentrations, 50 and 100 μM, respectively). Data were fit to bi-exponential decay curves. Some experiments measured the dissociation kinetics of the antagonist [^{3}H]CPX (2–4 nM) using a similar protocol, except that $MgCl_2$ and GTPγS were omitted and data were fit to a monoexponential equation. The inhibition of ^{125}I-ABA dissociation by allosteric enhancers was scored between 0 and 100%, as illustrated in Fig. 5.

Guanine Nucleotide Exchange Assays. CHO-hA_1 membranes (15 μg of membrane protein in 50 μl) in $MgCl_2$-free buffer (10 mM HEPES, pH 7.4) were preincubated with adenosine deaminase (2 U/ml) for 10 min at 21°C. Either 50 μl of vehicle (0.5% DMSO) or allosteric enhancer (25 μM final) was added, followed by various concentrations of CPA in (final concetration) 10 mM HEPES, pH 7.4, 10 μM GDP, 5 mM $MgCl_2$, 5 μg/ml saponin, 100 mM NaCl, 1 μM 8-CPT, and 2 mM dithiothreitol. CPT counteracted the effects of low levels of endogenous adenosine that escaped deamination. Tubes were incubated for 30 min at 30°C. [^{35}S]GTPγS (final concentration, 0.5 nM in 10 μl) was added 10 min before filtration.

Cyclic AMP Assays. CHO-hA_1 cells cultured in Ham's F12 medium with 10% fetal calf serum, 1% penicillin/streptomycin, and 250 μg/ml G418 were removed from plates by incubation for 5 to 10 min with EDTA in phosphate-buffered saline. Cells were washed twice and resuspended in phosphate-buffered saline containing 10 mM HEPES and 2 U/ml adenosine deaminase, pH 7.2. Aliquots of cells (200 μl, 0.75–1 × 10^5 cells/tube) were transferred to polypropylene test tubes and preincubated for 30 min at 21°C. Stock concentrations of compounds in 50 μl of phosphate-buffered saline/HEPES buffer containing (final concentration) 5 μM forskolin and 10 μM rolipram with or without allosteric enhancers or CPA were added to cells and the tubes were transferred to a 37°C shaking water bath for 10 min. The addition of 0.5 ml of 0.15 N HCl lysed the cells. A 0.5-ml aliquot of the supernatant after centrifugation at 1700g for 10 min was removed for acetylation and radioimmunoassay of cyclic AMP (Brooker et al., 1979).

Results

Equilibrium Binding Studies. We investigated the effects of two allosteric enhancer compounds, PD81,723, which has been extensively characterized as a moderately effective allosteric enhancer of A_1AR in prior studies, and ATL525, recently described as a new high efficacy enhancer (Tranberg et al., 2002). In the absence of allosteric enhancers, ^{125}I-ABA binds to two affinity states. The high-affinity K_D is 0.33 nM. The low-affinity K_D cannot be measured accurately because of high nonspecific binding at radioligand concentrations above 5 nM, but it is estimated to be ~10 nM (Fig. 2). In the presence of either allosteric enhancer, binding was to a single high-affinity site. This is readily apparent in Fig. 2 as a shift in Scatchard plots from curvilinear in the absence of enhancers to linear in the presence of enhancers. The addition of either enhancer produced a >2-fold increase in the number of high-affinity binding sites. Because our previous studies have established that the high- and low-affinity sites correspond to G protein-coupled and -uncoupled receptors, respectively (Figler et al., 1996, 1997), such a result suggests that the allosteric enhancers increase equilibrium binding primarily by stabilizing receptor-G protein complexes, resulting in a population of receptors fully coupled to G protein.

Interaction between Allosteric Enhancers and Guanine Nucleotides. The binding of guanine nucleotides such as GTPγS rapidly uncouples receptor-G protein complexes, reducing high-affinity agonist binding. Fig. 3A shows that the addition of ATL525 opposes that effect of GTPγS. It is noteworthy that this result does not represent an effect on

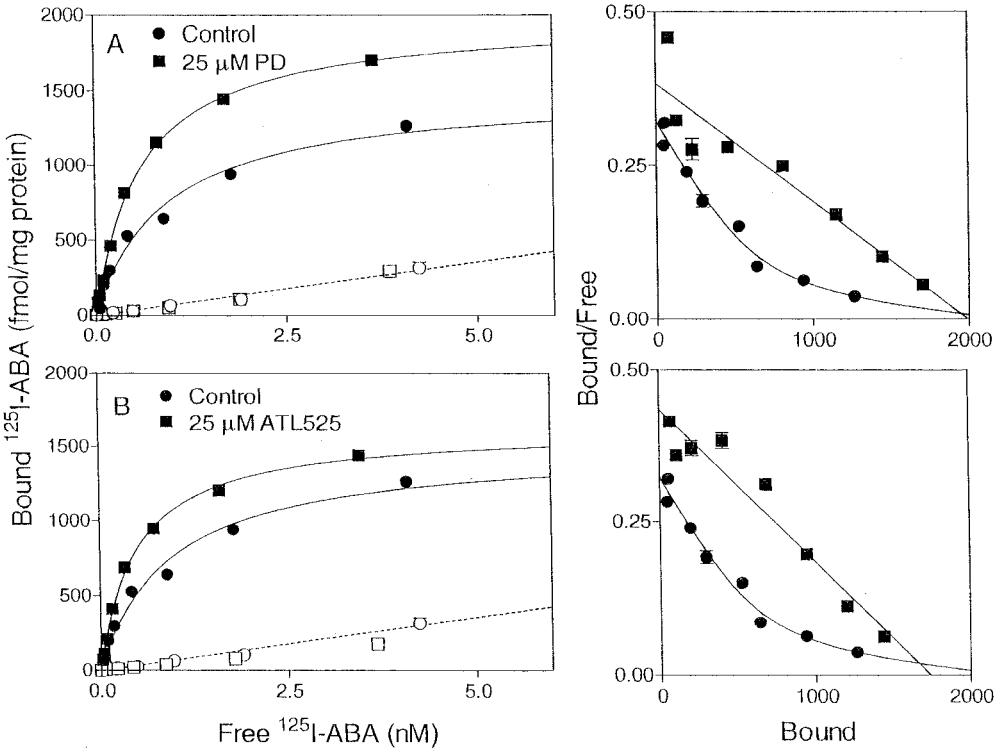

Fig. 2. Effect of allosteric enhancers, either 25 μM PD81725 (A) or ATL525 (B), on equilibrium binding of ^{125}I-ABA to A_1ARs in CHO-hA_1 membranes. Untransformed (left) and Scatchard transformed (right) equilibrium binding is shown. The open symbols show nonspecific binding. Binding in the absence of enhancers (control) was optimally fit to two sites: B_1 = 716 ± 330 fmol/mg protein, K_{D1} = 0.33 ± 0.16 nM, B_2 = 2119 ± 2775 fmol/mg, K_{D2} = 10 ± 25 nM. In the presence of enhancers, a single binding site was detected. For PD81723, B_{max} = 1992 ± 32, K_D = 0.62 nM ± 0.03; for ATL525, B_{max} = 1623 ± 26.2, K_D = 0.50 ± 0.02 nM. Protein, 0.015 mg; volume, 0.1 ml.

binding equilibrium, because the uncoupling effect of GTPγS is both concentration- and time-dependent. The data indicate, however, that enhancers substantially slow the reduction of high-affinity binding by GTPγS. Two mechanisms could account for such a result. First, bound allosteric enhancer might prevent GTPγS from binding to the G protein, either by steric hindrance, which denies access to the GTP-binding site, or by allosterically lowering affinity for the site. Alternatively, GTPγS may bind normally, and the enhancer might retard the rapid dissociation of receptor-G complexes initiated by guanine nucleotide binding. The results shown in Fig. 3B support the latter explanation: ATL525 increases the guanine nucleotide exchange stimulated by the agonist CPA. These experiments suggest that allosteric enhancers offset the reduction in high-affinity agonist binding caused by guanine nucleotide binding.

Effects of Allosteric Enhancers on the Kinetics of ^{125}I-ABA Binding. We next examined the effects of the enhancers on the kinetics of ^{125}I-ABA binding to the A_1AR. In Fig. 4A, binding has been transformed to plot $\ln[B_{eq}/(B_{eq} - B)]$ versus time. If there is a single binding site, binding theory predicts that this transformation results in a straight line with a slope of K_{obs}. The transformed data were not linear beyond 8 min, suggesting the existence of more than one kinetically distinct binding site. A bi-exponential equation incorporating two rate constants was found to fit the association kinetics well (Fig. 4B). The $t_{1/2}$ values for the fast and slow components of binding are 1.85 and 12.8 min, respectively, a difference of 6.9-fold. The effects of the allosteric enhancers PD81,723 and ATL425 on bi-exponential binding parameters are summarized in Table 1. The enhancers have no effect on the number of fast binding sites, B_{max1}. The most striking effect of the enhancers is the increase, by >60%, in the number of slowly occupied binding sites, B_{max2}. Thus, there is little effect of the enhancer on rapid radioligand binding during the first 10 min. Beyond 10 min, binding in the presence of the enhancers increases above control binding. One interpretation of this result is that the rapid binding phase reflects ^{125}I-ABA binding to preformed receptor-G protein (R-G) complexes, whereas the slow phase represents the binding to new R-G complexes recruited from the pool of previously uncoupled receptors. This is consistent with the observation that the enhancers act to stabilize R-G complexes.

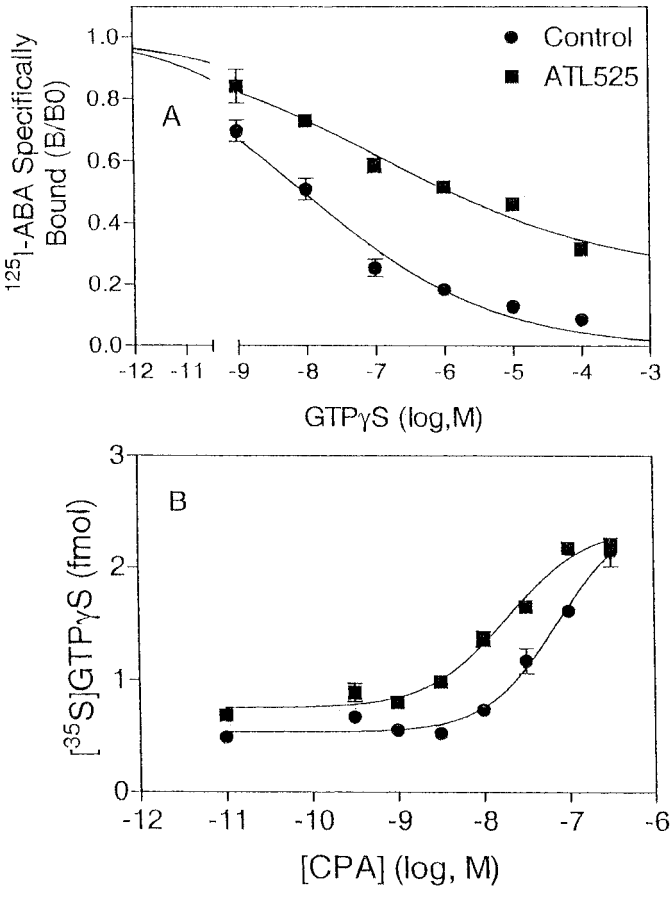

Fig. 3. Effects of ATL525 and GTPγS on ^{125}I-ABA binding to A_1AR-G protein complexes. A, effect of 25 μM AT525 on the dose dependence of GTPγS (added last, see *Materials and Methods*) to inhibit ^{125}I-ABA binding. Without enhancer, GTPγS log IC_{50} = −8.02 ± 0.013 M; with enhancer, GTPγS log IC_{50} = −6.92 ± 0.51 M. ^{125}I-ABA, 0.6 nM; *B*0, 3.22 fmol; nonspecific binding, 0.19 fmol; protein, 0.015 mg; volume, 0.1 ml. B, effects of ATL525 (25 μM) on CPA-stimulated guanine nucleotide exchange to CHO-hA1 membranes. Without enhancer, the CPA logEC_{50} = −7.13 ± 0.17 M; with enhancer, the CPA logEC_{50} = −7.96 ± 0.09 M; [^{35}S]GTPγS, 0.45 nM; protein, 0.015 mg; final volume, 0.11 ml. Each point is the mean ± S.E.M. of triplicate determinations.

Assessment of Allosteric Enhancer Score. PD81,725 was found to have two opposing effects on equilibrium binding: it has a positive allosteric effect to increase orthosteric agonist binding, but it also has activity as a competitive antagonist and can thereby decrease equilibrium radioligand binding. An allosteric action to increase the binding of any adenosine contaminating membrane preparations may also contribute to a decrease in equilibrium radioligand binding. For this reason, determining the kinetics of agonist radioligand dissociation from the orthosteric site has been used as an unambiguous measure of allosteric activity (Bruns and Fergus, 1990). High-affinity agonists such as ^{125}I-ABA dissociate slowly ($t_{1/2}$ = 34 min) from R-G complexes in the absence of guanine nucleotides (Fig. 5A). In the presence of the allosteric enhancer PD81,724 (25 μM), the $t_{1/2}$ for ^{125}I-ABA dissociation increases by 8.1-fold to 276 min. To measure kinetics accurately, it is necessary to conduct binding assays lasting several hours. To simplify measuring enhancer activity, we examined the effect of allosteric enhancers on the kinetics of agonist dissociation promoted by 50 μM GTPγS. In the absence of an allosteric enhancer, the addition of the guanine nucleotide caused very rapid dissociation of bound ^{125}I-ABA, the $t_{1/2}$ being less than 1 min (Fig. 5B). The addition of the high efficacy enhancer ATL525 (25 μM) resulted in the complete disappearance of the rapid phase of dissociation. ^{125}I-ABA dissociated mono-exponentially with a $t_{1/2}$ of 28 min. This GTPγS-resistant state might reflect receptors occupied by both the orthosteric and allosteric ligands. By contrast, in the presence of PD81,725 (25 μM), GTPγS evoked a rapid and a slow phase of ^{125}I-ABA dissociation that could correspond to population of receptors without and with PD81,723 bound to the allosteric site, respectively. As a means of quantifying the ability of the allosteric regulators to prevent rapid dissociation of the orthosteric radioligand, we *scored* the enhancers on a scale of 0 to 100, as illustrated in Fig. 5B. In this system, a compound that has no effect on GTPγS action (measured at 10 min after initiating dissociation) has a score of 0, whereas a compound completely preventing dissociation at 10 min would have the

maximal score of 100. To conduct this assay, the orthosteric radioligand is bound to equilibrium (2 h) and then the allosteric enhancer is added 5 min before initiating dissociation with a combination of an antagonist, CPT, and GTPγS. Five minutes is enough time for the allosteric enhancer effect to be fully evident but short enough to cause little or no change in the equilibrium binding of the radioligand.

Allosteric enhancer score was dose-dependent (Fig. 5C). At the highest dose used, 100 μM, ATL525 and PD81,723 had scores of 79 and 28, respectively. The curve relating the concentration of ATL525 to its score as an allosteric enhancer had a Hill coefficient approaching unity (0.87). This is consistent with the possibility the enhancer score is proportional to occupancy of a single saturable allosteric site.

Effect of Allosteric Enhancers on Antagonist Binding. PD81,723 and other allosteric enhancers inhibit binding of antagonist radioligands such as [^{3}H]CPX (Bruns and Fergus, 1989). An example of this action of PD81,723 is illustrated in Fig. 6A. ATL525 is not only more efficacious than PD81,723 as an enhancer of agonist binding but also inhibits antagonist binding only slightly. The effect of PD81,723 to reduce equilibrium [^{3}H]CPX binding could be attributed to either a competitive or an allosteric inhibitory action. Fig. 6B shows that neither allosteric compound has any effect on the kinetics of antagonist dissociation from A_1ARs. These data suggest that the binding of enhancers has little or no allosteric effect on antagonist binding and confirm previous reports that there is no correlation between the enhancer and antagonist activities of 2-aminothiophenes.

Functional Effects of ATL525. Although ATL525 is more potent and effective than PD81,723 as an allosteric enhancer of ^{125}I-ABA binding, it has not yet been shown to have functional enhancer activity. In CHO-hA1 cells, ATL525 produced a dose-dependent inhibition in cyclic AMP levels, even in the absence of CPA (Fig. 7A). Others have noted such partial agonist activity by enhancers (Bruns et al., 1990). In the presence of 300 nM CPX, added to reverse the partial agonist effect of ATL525, the effect of the ATL525 was manifested as a leftward shift in the dose-response curve to CPA. As shown in Fig. 7B, 3 μM ATL525 enhanced the potency of CPA by 10-fold.

Discussion

Allosteric modulators of G protein-coupled receptors have been thought of as compounds that bind to receptors and either increase or decrease the binding of orthosteric ligands. The binding sites of allosteric enhancers of the A_1AR are not known, but PD81,723 and ATL525 are both positive allosteric regulators because they decrease the dissociation kinetics of the orthosteric agonist ligand ^{125}I-ABA. The mathematical modeling of allosterism has been characterized by a cooperativity factor, α, that characterizes the strength of the interaction between the allosteric and orthosteric sites (Christopoulos, 2002). In the present study, we have examined two positive allosteric regulators of A_1ARs, a well characterized, moderately effective enhancer, PD81,723, and a recently synthesized, more efficacious enhancer, ATL525. This study focused on how these compounds influence the coupling of A_1ARs to G proteins. The results indicate that both compounds act more to increase the fractional coupling of A_1ARs to G proteins than to increase the affinity of the receptors for

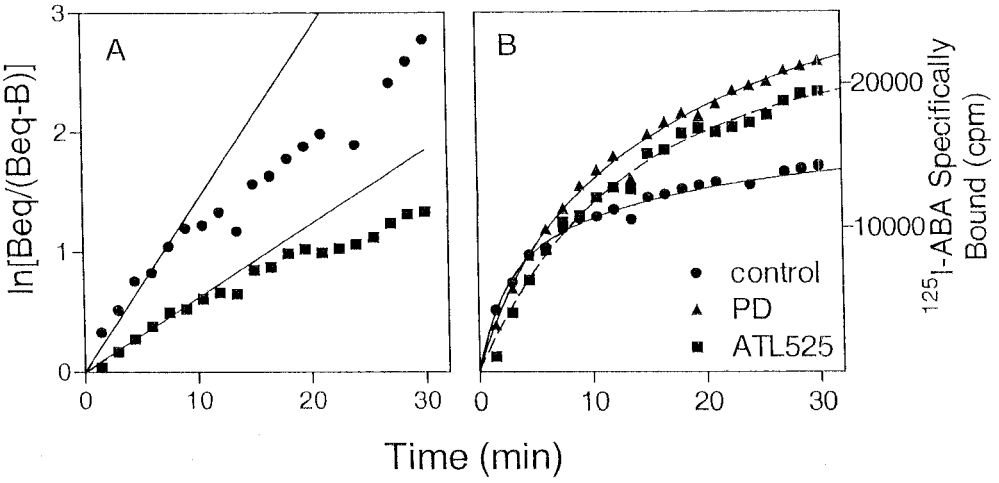

Fig. 4. Kinetics of association of ^{125}I-ABA to CHO-hA_1 receptors. A, data were transformed and results from the first 9 min only were fit to a straight line. B, specific binding was fit to bi-exponential association curves. Binding of ^{125}I-ABA was measured without enhancer (control) or with concurrent addition of 25 μM PD81723 (PD) or ATL525. Binding parameters are summarized in Table 1. Nonspecific binding was 0.16 fmol. The total ^{125}I-ABA added to each tube was 83 fmol. Equilibrium binding parameters are: control, 4.3 fmol; PD81723, 7.6 fmol; ATL525, 8.1 fmol; protein, 0.015 mg; final volume, 0.2 ml.

TABLE 1

Parameters for ^{125}I-ABA binding to CHO-hA_1 membranes with or without 25 μM concentrations of allosteric enhancers, PD81723, or ATL525. Association kinetics from experiments conducted on the same day using the same pool of membranes and radioligand were optimally fit to a bi-exponential equation as described under *Materials and Methods*. The parameter values and S.E. are derived from least squares nonlinear regression fits.

	B_{max1}	B_{max2}	k_1	k_2	B_{max2}
	fmol	*fmol*	min^{-1}	min^{-1}	*% of control*
Control	1.49 ± 0.29	2.48 ± 0.16	0.375 ± 0.046	0.054 ± 0.002	100
$t_{1/2}$ (min)			1.85	12.8	
PD81,723	1.39 ± 0.21	4.76 ± 0.61	0.24 ± 0.014	0.038 ± 0.005	193
$t_{1/2}$ (min)			2.9	18.2	
ATL525	1.64 ± 0.08	3.97 ± 0.6	0.125 ± 0.018	0.015 ± 0.001	160
$t_{1/2}$ (min)			5.5	46	

agonists. Thus, in equilibrium binding assays employing agonist radioligands, both PD81,723 (Kollias-Baker et al., 1994; Bhattacharya and Linden, 1995) and ATL525 increase the fraction of receptors found in the high-affinity (G protein-coupled) state, but have little or no effect on the radioligand K_D. These findings suggest the enhancers stabilize the conformational state of receptors that bind agonists with high affinity. It is notable that enhancers fail to increase agonist binding to A_1ARs on adipocyte membranes very highly coupled to G proteins even in the absence of enhancers (Jarvis et al., 1999). In view of these findings, we conclude that a cooperativity factor, α, is not adequate to model allosteric regulation of G protein-coupled receptors and that an effect on G protein coupling also should be included.

The action of enhancers to increase R-G coupling is opposite that of guanine nucleotides such as GTPγ S, which uncouple ligand-receptor-G protein (LRG) complexes. Previous work showed that PD81,723 produced a small (2.2-fold) rightward shift in the potency of GTPγS to decrease agonist binding (Bhattacharya and Linden, 1995). In that study, PD81,723 and GTPγS were added to membranes at the same time. To the extent that GTPγS binds to G proteins before the enhancer binds to receptors, the guanine nucleotide may act to uncouple R-G complexes before the enhancer can bind. In the current study, we show that if ATL525 and ^{125}I-ABA are added to membranes before GTPγS, there is a very large decrease (>100-fold) in the potency of the guanine nucleotide to decrease agonist binding measured at 90 min (Fig. 3A). Fig. 3B shows that ATL525 enhances rather than inhibits the ability of CPA to stimulate guanine nucleotide exchange on the G protein. Together, the results suggest that the

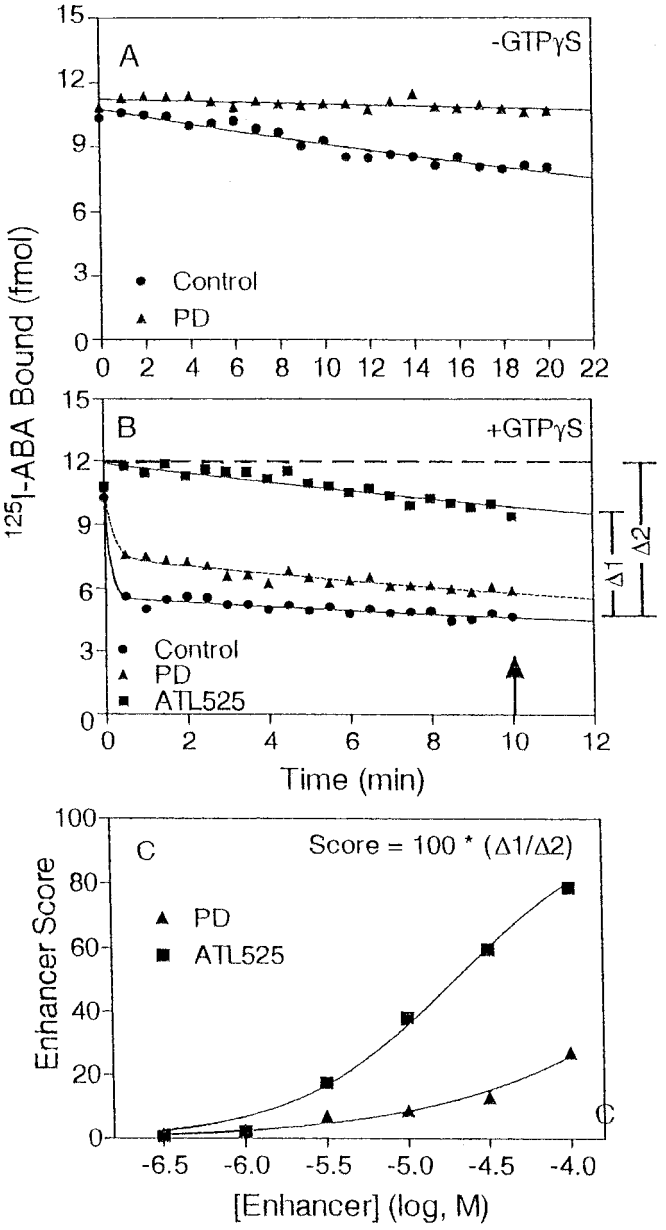

Fig. 5. Kinetics of dissociation of ^{125}I-ABA from CHO-hA_1 receptors ± GTPγ S. A, dissociation kinetics measured in the absence of GTPγS ± 100 μM PD81725. B, dissociation kinetics measured in the presence of GTPγS (50 μM) ± 100 μM PD81725 or ATL525. Enhancer *scores* were calculated as 100 × (Δ1/Δ2) as illustrated on the graph, where Δ1 is the difference is residual binding between the enhancer and control curves measured at 10 min after the initiation of dissociation and Δ2 is the difference between binding at equilibrium (dashed line) and residual binding without enhancer at 10 min. For A and B, total ^{125}I-ABA was 78 fmol, nonspecific binding was 2.16 fmol; protein, 0.015 mg; final volume, 0.2 ml. Each point represents a single determination. C, dose dependence of PD81725 and ATL525 to increase enhancer score. The logED_{50} of ATL525 was −4.71 ± 0.04 M. Total ^{125}I-ABA, 105 fmol; total binding, 4.46 fmol; nonspecific binding, 0.27 fmol; protein, 0.01 mg; final volume, 0.1 ml.

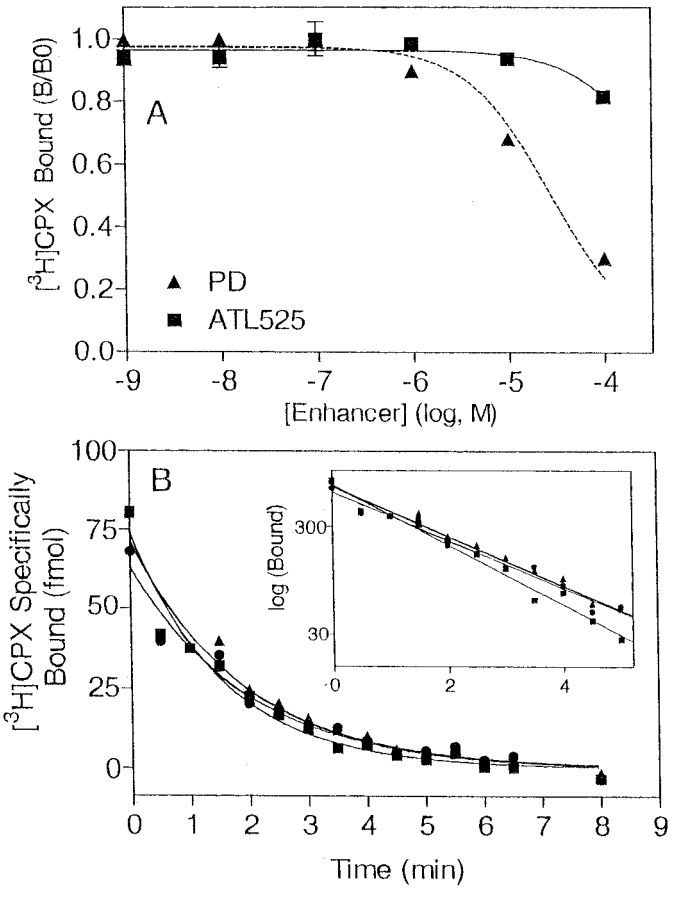

Fig. 6. The effects of allosteric enhancers on the binding of the antagonist, [^{3}H]CPX. A, dose dependence of PD81723 and ATL525 to inhibit equilibrium binding. B, kinetics of [^{3}H]CPX dissociation ± 25 μM PD81723 or ATL525. In triplicate experiments, dissociation $t_{1/2}$ values for [^{3}H]CPX are 1.35 ± 0.20 min (control), 1.30 ± 0.23 min (PD81,723), and 1.07 ± 0.14 min (ATL525). Total [^{3}H]CPX, 275 fmol; nonspecific binding, 3.1 fmol; *B*0, 90 fmol; protein, 0.01 mg; volume, 0.2 ml.

simultaneous binding of an enhancer and an orthosteric agonist to the R-G complex does not prevent GTPγS from binding, but does slow uncoupling of the R-G complex.

The actions of enhancers, orthosteric agonist ligands, and GTP on the stability of the LRG complex can be conceptualized in an expansion of the classic ternary complex model of receptor-G protein interactions (De Lean et al., 1980). Binding of the orthosteric agonist facilitates receptor-G protein coupling and the formation of the high-affinity conformational state, R*. We propose, as an expansion of the ternary complex model, that the simultaneous binding of both an allosteric enhancer, E, and ligand causes enhanced stability of R*G complex and resistance to uncoupling upon the binding of GTP. In the context of this model, the effects of enhancers on the kinetics of ^{125}I-ABA binding are interesting. Careful analysis of binding kinetics reveals two phases of orthosteric agonist binding. This is consistent with the possibility that a fraction of A_1ARs in CHO-hA1 membranes are precoupled to G proteins and consequently bind agonists more quickly than receptors initially uncoupled from G proteins. Other evidence that some GPCRs are precoupled is based on: coimmunoprecipitation of R-G complexes, the kinetics of G protein activation, and the kinetics of GPCR-regulated K^+ channel activation (Shea and Linderman, 1997; Roka et al., 1999; Martin, 2002; Zhang et al., 2002). Our experiments show allosteric enhancers selectively increase the population of receptors that bind agonists slowly (Fig. 4, Table 1), consistent with the concept that this slow phase of binding reflects the gradual formation of R-G complexes and that the R-G complexes that do form are stabilized by enhancers.

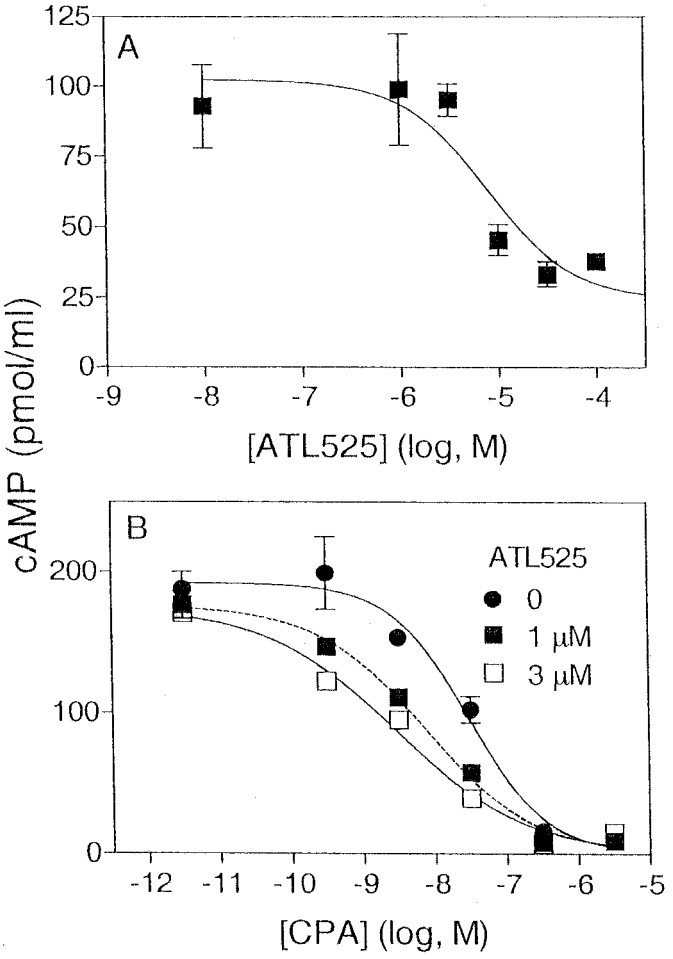

Fig. 7. Effects of ATL525 and CPA on cyclic AMP accumulation in CHO-hA_1 cells. A, cells were incubated with 5 μM forskolin, 10 μM rolipram, and ATL525 as indicated. Basal cyclic AMP in the absence of forskolin was 2.5 pmol/ml. ATL525 inhibited forskolin-stimulated cyclic AMP accumulation with a logIC_{50} of −5.1 ± 0.35 M. B, cells were incubated in the presence of 300 nM CPX with forskolin, rolipram, and CPA ± ATL525 as indicated. logIC_{50} values of CPA were: control, −7.52 ± 0.14 M; 1 μM ATL525, −8.12 ± 0.10; and 3 μM ATL525, −8.53 ± 0.15. Each point is the mean of duplicate determinations assayed in duplicate.

The effects of allosteric enhancers on equilibrium binding of orthosteric agonists are variable because these effects depend on the coupling state of receptors and are influenced by the competitive antagonist properties of some of these compounds. Hence, the use of agonist dissociation kinetics has been used as a pure measure of allosteric enhancer activity (Bruns and Fergus, 1989). One limitation of this assay stems from the fact that in the presence of enhancers, agonist dissociation can be very slow (see Fig. 5A). In this study, we have developed a new procedure for rapidly assessing enhancer activity that measures resistance to the effect of GTPγS to uncouple R-G complexes as a robust means of quantifying enhancement. The assay procedure, illustrated in Fig. 5, exploits the fact that the GTPγS minimally uncouples ELRG complexes over a period of 10 min. We normalize GTPγS-resistant high-affinity agonist binding by relating it to that observed in the absence of any enhancer (0%) and no dissociation over 10 min (100%), thereby deriving an enhancer *score*. Note in Fig. 5B that ^{125}I-ABA dissociation in the presence of PD81,723 occurs in two clearly discernible kinetic phases that probably correspond to LRG complexes without (fast dissociation) and with (slow dissociation) bound enhancer. In the presence of 100 μM ATL525, the LRG complexes are nearly saturated with enhancer, only slow dissociation is observed, and the score is nearly 100%. Lower doses of ATL525 generating lower scores result in biphasic dissociation kinetics (data not shown), which is consistent with the hypothesis that enhancer score is related to LRG occupancy by enhancer. In principle, it is likely that different enhancers might stabilize the ELRG complex to different extents. This would manifest itself as different rates in the slow phase of agonist dissociation.

Assessing functional responses in intact cells is an important way to evaluate the effect of enhancers to increase the potency of orthosteric agonists. In assays based on A_1-agonist induced lowering of cyclic AMP, 30 μM PD31723 was found to produce a 3- to 4-fold increase in agonist potency (Bhattacharya and Linden, 1995, 1996). By contrast, as shown in Fig. 7, 3 μM ATL525 produced a 10-fold shift in agonist potency, consistent with its greater enhancer score than PD81,723.

In conclusion, the results of this study indicate the primary effect of two allosteric enhancers of A_1ARs is to stabilize the high-affinity agonist binding conformational state of receptor-G protein complexes. This is manifested in equilibrium binding as an increase in steady-state coupling and in kinetic assays as an increase in the fraction of slow binding as receptors convert from uncoupled to coupled over time. The potency and efficacy of enhancers can be accurately and quickly *scored* based on their ability to acutely counteract the action of GTPγS to uncoupled receptor-G protein complexes.

Acknowledgments

We gratefully acknowledge Susan Daluge of Glaxo SmithKline for her gift of aminobenzyladenosine and Robert Pursley for his assistance in the preparation of this manuscript.

References

Amoah-Apraku B, Xu J, Lu JY, Pelleg A, and Belardinelli L (1993) 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.

Baraldi PG, Zaid AN, Lampronti I, Fruttarolo F, Pavani MG, Tabrizi MA, Shryock JC, Leung E, and Romagnoli R (2000) Synthesis and biological effects of a new series of 2-amino-3-benzoylthiophenes as allosteric enhancers of A1-adenosine receptor. *Bioorg Med Chem Lett* **10:**1953–1957.

Bhattacharya S and Linden J (1995) The allosteric enhancer, PD 81,723, stabilizes human A_1 adenosine receptor coupling to G proteins. *Biochim Biophys Acta* **1265:** 15–21.

Bhattacharya S and Linden J (1996) Effects of long-term treatment with the allosteric enhancer, PD81,723, on Chinese hamster ovary cells expressing recombinant human A_1 adenosine receptors. *Mol Pharmacol* **50:**104–111.

Brooker G, Terasaki WL, and Price MG (1979) Gammaflow: a completely automated radioimmunoassay system. *Science (Wash DC)* **194:**270–276.

Bruns RF and Fergus JH (1989) Allosteric enhancers of adenosine A_1 receptor binding and function, in *Adenosine Receptors in the Nervous System* (Ribeiro JA ed) pp 53–60, Taylor & Francis, London.

Bruns RF and Fergus JH (1990) Allosteric enhancement of adenosine A1 receptor binding and function by 2-amino-3-benzoylthiophenes. *Mol Pharmacol* **38:**939–949.

Bruns RF, Fergus JH, Coughenour LL, Courtland GG, Puggsley TA, Dodd JH, and Tinney FJ (1990) Structure-activity relationships for enhancement of adenosine A_1 receptor binding by 2-amino-3-benzoylthiophenes. *Mol Pharmacol* **38:**950–958.

Christopoulos A (2002) Allosteric binding sites on cell-surface receptors: novel targets for drug discovery. *Nat Rev Drug Discov* **1:**198–210.

Christopoulos A and Kenakin T (2002) G protein-coupled receptor allosterism and complexing. *Pharmacol Rev* **54:**323–374.

De Lean A, Stadel JM, and Lefkowitz RJ (1980) A ternary complex model explains the agonist-specific binding properties of the adenylate cyclase-coupled β-adrenergic receptor. *J Biol Chem* **255:**7108–7117.

Figler RA, Graber SG, Lindorfer MA, Yasuda H, Linden J, and Garrison JC (1996) Reconstitution of recombinant bovine A1 adenosine receptors in Sf9 cell membranes with recombinant G proteins of defined composition. *Mol Pharmacol* **50:** 1587–1595.

Figler RA, Lindorfer MA, Graber SG, Garrison JC, and Linden J (1997) Reconstitution of bovine A_1 adenosine receptors and G proteins in phospholipid vesicles: $\beta\gamma$-subunit composition influences guanine nucleotide exchange and agonist binding. *Biochemistry* **36:**16288–16299.

Janusz CA, Bruns RF, and Berman RF (1991) Functional activity of the adenosine binding enhancer, PD 81,723, in the in vitro hippocampal slice. *Brain Res* **567:** 181–187.

Jarvis MF, Gessner G, Shapiro G, Merkel L, Myers M, Cox BF, and Martin GE (1999) Differential effects of the adenosine A_1 receptor allosteric enhancer PD 81,723 on agonist binding to brain and adipocyte membranes. *Brain Res* **840:**75–83.

Kollias-Baker C, Ruble J, Dennis D, Bruns RF, Linden J, and Belardinelli L (1994) Allosteric enhancer PD 81,723 acts by novel mechanism to potentiate cardiac actions of adenosine. *Circ Res* **75:**961–971.

Kollias-Baker CA, Ruble J, Jacobson M, Harrison JK, Ozeck M, Shryock JC, and Belardinelli L (1997) Agonist-independent effect of an allosteric enhancer of the A1 adenosine receptor in CHO cells stably expressing the recombinant human A1 receptor. *J Pharmacol Exp Ther* **281:**761–768.

Kourounakis A, Visser C, de Groote M and IJzerman AP (2001) differential effects of the allosteric enhancer (2-amino-4,5- dimethyl-trienyl)[3-(trifluoromethyl) phenyl-]methanone (PD81,723) on agonist and antagonist binding and function at the human wild-type and a mutant (T277A) adenosine A(1) receptor. *Biochem Pharmacol* **61:**137–144.

Lutjens H, Zickgraf A, Figler H, Linden J, Olsson RA, and Scammells PJ (2003) 2-Amino-3-benzoylthiophene allosteric enhancers of A(1) adenosine agonist binding: new 3,4- and 5-modifications. *J Med Chem* **46:**1870–1877.

Marquardt DM (1963) An algorithm for least-squares estimation of nonlinear parameters. *J Soc Indust Appl Math* **11:**431–441.

Martin SS (2002) Identification of G-proteins coupling to the vasoactive intestinal peptide receptor VPAC(1) using immunoaffinity chromatography: evidence for precoupling. *Biochem Biophys Res Commun* **290:**1300–1307.

Mizumura T, Auchampach JA, Linden J, Bruns RF, and Gross GJ (1996) PD 81,723, an allosteric enhancer of the A1 adenosine receptor, lowers the threshold for ischemic preconditioning in dogs. *Circ Res* **79:**415–423.

Musser B, Morgan ME, Leid M, Murray TF, Linden J, and Vestal RE (1993) Species comparison of adenosine and beta-adrenoceptors in mammalian atrial and ventricular myocardium. *Eur J Pharmacol* **246:**105–111.

Musser B, Mudumbi RV, Liu J, Olson RD, and Vestal RE (1999) Adenosine A1 receptor-dependent and -independent effects of the allosteric enhancer PD 81,723. *J Pharmacol Exp Ther* **288:**446–454.

Robeva AS, Woodard R, Luthin DR, Taylor HE, and Linden J (1996) Double tagging recombinant A1- and A2A-adenosine receptors with hexahistidine and the FLAG epitope. Development of an efficient generic protein purification procedure. *Biochem Pharmacol* **51:**545–555.

Roka F, Brydon L, Waldhoer M, Strosberg AD, Freissmuth M, Jockers R, and Nanoff C (1999) Tight association of the human Mel_{1a}-melatonin receptor and G_i: precoupling and constitutive activity. *Mol Pharmacol* **56:**1014–1024.

Shea L and Linderman JJ (1997) Mechanistic model of G-protein signal transduction. Determinants of efficacy and effect of precoupled receptors. *Biochem Pharmacol* **53:**519–530.

Tranberg CE, Zickgraf A, Giunta BN, Luetjens H, Figler H, Murphree LJ, Falke R, Fleischer H, Linden J, Scammells PJ, et al. (2002) 2-Amino-3-aroyl-4,5-alkylthiophenes: agonist allosteric enhancers at human A(1) adenosine receptors. *J Med Chem* **45:**382–389.

Van der Klein PAM, Kourounakis AP, and IJzerman AP (1999) allosteric modulation of the adenosine A(1) receptor. Synthesis and biological evaluation of novel 2-amino-3-benzoylthiophenes as allosteric enhancers of agonist binding. *J Med Chem* **42:**3629–3635.

Zhang Q, Pacheco MA, and Doupnik CA (2002) Gating properties of GIRK channels activated by $g\alpha_o$- and $g\alpha_i$-coupled muscarinic m2 receptors in *Xenopus* oocytes: the role of receptor precoupling in RGS modulation. *J Physiol* **545:**355–373.

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