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Allosteric Enhancement of Adenosine A_1 Receptor Binding and Function by 2-Amino-3-benzoylthiophenes

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SUMMARY

Several 2-amino-3-benzoylthiophenes were found to increase the binding of [^{3}H]N^6-cyclohexyladenosine to A_1 adenosine receptors in rat brain membranes. Concentration-response curves were bell-shaped, with up to 45% stimulation of binding at 10 μM followed by inhibition at higher concentrations. Because these compounds originated from a series of nonxanthine adenosine antagonists, the inhibition of binding was attributed to the presence of interfering adenosine antagonist activity. The compounds stimulated binding of several A_1 agonist ligands but only inhibited binding of the A_1 antagonist ligand [^{3}H]8-cyclopentyl-1,3-dipropylxanthine, indicating that enhancement was specific for the agonist conformation of the receptor. The enhancement was also specific for the A_1 receptor, because agonist binding to A_2 adenosine, M_2 muscarinic, α_2 adrenergic, and δ opiate receptors showed little or no enhancement. Uncoupling of the A_1 receptor from the inhibitory guanine nucleotide-binding protein did not prevent enhancement. The enhancers slowed the dissociation of [^{3}H]N^6-cyclohexyladenosine from the A_1 receptor, implying an allosteric mechanism of action. The inhibition of forskolin-stimulated cyclic AMP accumulation in FRTL-5 cells was employed as a functional index of A_1 receptor activation. The enhancers caused up to 19-fold leftward shifts in the concentration-response curve for N^6-cyclopentyladenosine and also caused up to 55% inhibition of cyclic AMP accumulation in the absence of agonist. The binding and functional results are consistent with a model in which the enhancers bind preferentially to the agonist conformation of the A_1 receptor, thereby shifting the receptor equilibrium in favor of agonist binding. Adenosine enhancers may be useful for ischemia and other conditions involving local energy deficits. More generally, allosteric enhancers may provide a means for strengthening physiological control circuits in a variety of receptor systems.

Adenosine acts as a local hormone in the cardiovascular, gastrointestinal, reproductive, and nervous systems. Produced when cellular energy charge becomes depressed, it protects tissues from damage by lowering oxygen demand and increasing oxygen supply (1, 2). The effects of adenosine are mediated by two subtypes of extracellular receptors, called A_1 and A_2 (3), which have opposite effects on adenylate cyclase activity (3, 4). The two receptor subtypes also appear to have complementary roles in regulating oxygen supply-demand balance (5, 6). A_1 responses usually decrease oxygen demand, for instance by inhibiting neuronal firing and depressing cardiac contractility, whereas A_2 responses tend to increase oxygen supply, for instance by dilating blood vessels, inhibiting platelet aggregation, and stimulating breathing.

Agents that augmented the response to adenosine would very likely be useful in conditions with a localized oxygen deficit, such as angina, myocardial infarction, and stroke. However, although attempts have been made to develop stable adenosine agonists for these conditions, direct-acting agonists have not proven successful,[2] because of their high potential for side effects. Undesirable effects of adenosine and stable adenosine agonists in humans include headache, nausea, retching, angina pain, and atrioventricular block (7–11). Such widespread side effects are seen because of the wide distribution of adenosine receptors and because direct-acting adenosine agonists activate all adenosine receptors to which they gain access, instead of only activating those adenosine receptors that are located in areas experiencing oxygen deficit.

Because of these problems with direct-acting adenosine agonists, it would be useful to consider alternative approaches that might activate adenosine receptors in a more selective manner. One potential strategy is illustrated by an example from the GABA receptor field. Diazepam and other benzodi-

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[2] Adenosine itself is used to terminate supraventricular tachycardia (7). Although it causes many side effects, these are tolerable because its actions are very short-lived when it is given by intravenous bolus.

ABBREVIATIONS: GABA, γ-aminobutyric acid; CGS 21680, 1-{6-amino-2-{[4-(2-carboxyethyl)phenyl]ethyl}amino}-9*H*-purine-9-yl}-1-deoxy-*N*-ethyl-β-D-ribofuranuronamide; CHA, N^6-cyclohexyladenosine; CMD, L-(+)-(*cis*)-2-methyl-4-trimethylammoniummethyl-1,3-dioxolane; CPA, N^6-cyclopentyladenosine; CPX, 8-cyclopentyl-1,3-dipropylxanthine; DSTLE, [D-Ser^2, Leu^5, Thr^6]enkephalin; G protein, guanine nucleotide-binding protein; G_i, inhibitory guanine nucleotide-binding protein; NECA, 1-(6-amino-9*H*-purin-9-yl)-1-deoxy-*N*-ethyl-β-D-ribofuranuronamide; NEM, *N*-ethylmaleimide; (*R*)-PIA, N^6-[(*R*)-1-methyl-2-phenylethyl]adenosine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DMSO, dimethyl sulfoxide.

azepines act as allosteric enhancers of the $GABA_A$ receptor. They potentiate the response to GABA by stabilizing a high affinity state of the GABA receptor, shifting the dose-response curve for GABA to the left (12). The advantages of allosteric enhancers as an approach to drug discovery are illustrated by the fact that benzodiazepines have an acceptable side effect profile and are used clinically; in contrast, direct-acting $GABA_A$ agonists have so far failed to show a satisfactory risk-benefit ratio for clinical use.

By analogy with the GABA receptor system, allosteric enhancers of adenosine receptor function might provide a more selective therapeutic effect than direct-acting adenosine agonists. Such agents might synergize with endogenous adenosine but have minimal effects in the absence of adenosine. Their actions would therefore be limited to times and locations at which significant release of adenosine occurred, so that systemic side effects would largely be avoided. However, compounds with allosteric effects at adenosine receptors have not been reported previously. The present study[3] describes the *in vitro* pharmacology of several 2-amino-3-benzoylthiophenes that enhance adenosine A_1 receptor binding and function. Like diazepam, these compounds appear to stabilize a high affinity, agonist-preferring receptor conformation. These agents may serve as prototypes for the discovery of drugs with clinical utility in conditions involving localized oxygen deficits.

Experimental Procedures

Materials. 2-Amino-3-benzoylthiophene derivatives were synthesized at Parke-Davis by the method of Tinney *et al.* (14). [^{3}H]CHA (specific activity, 34 Ci/mmol), [^{3}H](R)-PIA (52 Ci/mmol), [^{3}H]NECA (18 Ci/mmol), [^{3}H]CGS 21680 (48 Ci/mmol), [^{3}H]CMD (56 Ci/mmol), [^{3}H]UK-14,304 (88 Ci/mmol), and [^{3}H]DSTLE (30 Ci/mmol) were purchased from Du Pont NEN (Boston, MA), and [^{3}H]adenine and [^{32}P]cAMP from ICN (Irvine, CA). [^{3}H]CPX (117 Ci/mmol) was synthesized as described (15). Carbachol, GTP, NEM, HEPES, cyclic AMP, and adenosine deaminase (type VI) were from Sigma Chemical Co. (St. Louis, MO), [D-Ala^2,Met^5]enkephalinamide from Peninsula Laboratories (Belmont, CA), forskolin from Calbiochem (La Jolla, CA), neutral alumina, 80–200 mesh, Brockman activity 1, from Fisher Scientific (Fair Lawn, NJ), and AG50W ×4 200–400 (hydrogen form) from Bio-Rad (Richmond, CA). Rolipram was provided by Schering A. G. (Berlin, Germany). Adenosine agonists and antagonists were synthesized at Parke-Davis. Tissue culture reagents were from GIBCO (Grand Island, NY), and tissue culture flasks and plates were from Corning (Corning, NY). Dog and cow brains were from Pel-Freez (Rogers, AR). Human cerebral cortex was obtained from a 69-year-old man who died of a ruptured aorta. Approval of the human tissue use protocol was obtained from the University of Michigan Medical Center. The FRTL-5 (ATCC CRL 8305) and neuro-2a (ATCC CRL 131) cell lines were from the American Type Culture Collection (Rockville, MD). DMSO was spectrophotometric grade (Aldrich, Milwaukee, WI). Sources of other reagents were as previously described (16).

Adenosine receptor binding. Binding of 1 nM [^{3}H]CHA to A_1 receptors in rat forebrain membranes (20 mg original wet weight/tube) was carried out in triplicate at 25° for 1 hr in 50 mM Tris·HCl, pH 7.7, containing 0.1 units/ml adenosine deaminase, as described (16). Membranes were preincubated for 30 min at 37° with 10 units/ml adenosine deaminase (17). Nonspecific binding was defined as binding in the presence of 10 μM (*R*)-PIA. Test agents were dissolved in DMSO and added to the assay from a 100-fold concentrated solution in DMSO. Control incubations also contained 1% DMSO. Binding of 4 nM [^{3}H]NECA to A_2 receptors in rat striatal membranes was carried out at 25° for 1 hr in 50 mM Tris·HCl, pH 7.7, containing 10 mM $MgCl_2$, 50 nM CPA, and 10 units/ml adenosine deaminase, as described (16). [^{3}H]CHA binding to brain membranes from other species was carried out as for rat brain. Assays measuring [^{3}H](R)-PIA binding to A_1 receptors in rat forebrain membranes and [^{3}H]NECA binding to A_1 receptors in rat cerebellar membranes were also carried out as for [^{3}H]CHA, with the exception that [^{3}H]NECA was used at a concentration of 4 nM. Binding of 0.2 nM [^{3}H]CPX to A_1 receptors in rat forebrain membranes (15) was performed in the same way as [^{3}H]CHA binding, except that the amount of tissue was reduced to 5 mg wet weight.

Measurement of nonspecific binding. A 10 μM concentration of (*R*)-PIA was routinely used to measure nonspecific binding of [^{3}H]CHA, because of the observation that nonspecific binding defined by 1 mM theophylline (17) is increased by the adenosine enhancers. The increase in binding is explained by the fact that this concentration of theophylline is only about 40-fold higher than its IC_{50}, thus sparing a small amount of specific binding, which can be enhanced markedly by PD 117,975. True nonspecific binding, defined by addition of 10 μM (*R*)-PIA or CPX, is completely unaffected by 100 μM PD 117,975.

A common artifact that at first glance can be mistaken for binding enhancement is seen with insoluble compounds. When diluted from DMSO to assay buffer, an insoluble compound may precipitate. If the radioligand co-precipitates with the test agent, it will be trapped during the filtration step, causing an illusory increase in total binding. However, this artifact is easy to detect because nonspecific binding is elevated. The compounds in the present paper only increased nonspecific binding at 300 μM or higher.

Dissociation of [^{3}H]CGS 21680 from adenosine A_2 receptors. Rat striatal membranes (20 mg original wet weight) were incubated for 1 hr at 25° with 15 nM [^{3}H]CGS 21680 in 250 μl of 50 mM Tris·HCl, pH 7.7, containing 1 mM $MgCl_2$, 10 nM CPA, and 0.4 units/ml adenosine deaminase. A 10 μM concentration of NECA was used to define nonspecific binding. Dissociation was then begun by addition of 0.75 ml of Tris buffer containing 13.33 μM NECA, 1.333 mM EDTA, and 40 μM PD 81,723 or DMSO vehicle. Samples were filtered after incubation at 25° for the times indicated.

[^{3}H]CMD dissociation assay. This assay was modified from Closse *et al.* (18). Rat hearts (male Sprague-Dawley) were perfused with saline, disrupted in a Polytron (Brinkmann, Westbury, NY) for 30 sec at a setting of 6 in 20 volumes of ice-cold 10 mM Na-HEPES buffer, pH 7.4, washed by two rounds of centrifugation for 15 min at 40,000 × *g*, and stored as a suspension at −80°. For assays, membranes were thawed and resuspended by disruption for 5 sec in a Polytron. Membranes (7.2 mg tissue wet weight/tube) were preincubated with 2 nM [^{3}H]CMD in 1 ml of HEPES buffer for 1 hr at 25°, after which dissociation was initiated by addition of 1 ml of HEPES buffer containing 20 μM carbachol plus test agent or vehicle. After an additional period of time, incubations were terminated by filtration on a Brandel 48R cell harvester through GF/B filters presoaked in 0.3% polyethylenimine free base. Nonspecific binding was defined by addition of 10 μM carbachol during the preincubation.

[^{3}H]UK-14,304 dissociation assay. Rat forebrain membranes were prepared as for [^{3}H]CHA binding, except that the preincubation with adenosine deaminase was omitted. Other details of the assay were the same as for [^{3}H]CMD, except that the buffer was 50 mM Tris·HCl, pH 7.7, the ligand was 1 nM [^{3}H]UK-14,304, and 10 μM clonidine was used to initiate dissociation and define nonspecific binding.

[^{3}H]DSTLE dissociation assay. Details of the assay were the same as for [^{3}H]CMD, except that the tissue was rat forebrain membranes prepared in the same way as for [^{3}H]CHA binding, the buffer was 50 mM Tris·HCl, pH 7.7, the ligand was 1 nM [^{3}H]DSTLE, and 10 μM [D-Ala^2,Met^5]enkephalinamide was used to initiate dissociation and define nonspecific binding.

Cyclic AMP accumulation in FRTL-5 cells. FRTL-5 cells were grown in Coon's modified F12 medium with 5% calf serum, glutamine, and nonessential amino acids supplemented with six hormones, as described (19). No antibiotics were used. Atmosphere was 5% CO_2/95%

[3] A preliminary account of this work has been published (13).

humidified air at 37°. Stock cultures were grown in 75-cm^2 flasks with 30 ml of medium and subcultured weekly. Experimental cultures were grown as monolayers in six-well tissue culture plates. Cells were plated at 1:15 dilution from a near-confluent culture flask (96 35-mm wells from one 75-cm^2 flask) and grown for 5–7 days before the experiment. One or 2 days before the experiment, the medium was replaced with medium without the six hormones.

Cells were prelabeled for 3–5 hr with 2.5 μCi of [^{3}H]adenine (carrier-free) in 1 ml of medium without the six hormones, containing 1 unit/ml adenosine deaminase. Incorporation of [^{3}H]adenine into labeled nucleotides was typically about 950,000 cpm.

Incubations were for 10 min at 29°. The order of the incubations was randomized. Incubation medium was Tyrode-HEPES buffer (Tyrode's balanced salt solution with 50 mM glucose, 10 units/ml adenosine deaminase, and 15 mM HEPES, adjusted to pH 7.4 with NaOH). The phosphodiesterase inhibitor rolipram (100 μM) was added to the incubation medium from a 100 mM solution in DMSO. In some experiments, 10 μM forskolin (diluted from a 10 mM solution in DMSO) and 100 nM CPX (diluted from a 1 mM solution in DMSO) were also added to the stock medium. Test agents were dissolved at 1 mM or higher in DMSO and stored at −70°. Tubes containing incubation solutions were set up the same day as the experiment. Test agents were diluted in DMSO and added in a 5-μl volume to 1 ml of incubation medium. All tubes were adjusted to the same final concentration of DMSO (1.21% in most experiments). Each incubation was initiated by removing of the prelabeling medium from the well by aspiration, washing once with 0.9% NaCl, and adding the test medium. Incubations were terminated by removal of the test medium and addition of 0.6 ml of ice-cold stopping reagent (0.1 N HCl with 1 mM cyclic AMP and about 2000 cpm of [^{32}P]cAMP as marker). The plates were stored at −20° for later isolation of [^{3}H]cAMP by column chromatography by a modification of the method of Salomon *et al.* (20, 21).

Conversion of labeled adenine nucleotides to [^{3}H]cAMP was expressed as cpm of [^{3}H]cAMP/1,000,000 cpm of [^{3}H]adenine nucleotides (including [^{3}H]cAMP). Total [^{3}H]adenine nucleotides and recovery of [^{32}P]cAMP were measured for each sample. [^{3}H]cAMP was calculated as (cpm of [^{3}H]cAMP − blank)/recovery. The blank was measured from three 0.6-ml aliquots of stopping solution subjected to Dowex-alumina chromatography in parallel with the experimental samples.

Carbachol-inhibited cyclic AMP accumulation in neuro-2a cells was studied using the same protocol as for FRTL-5 cells, except that adenosine deaminase and CPX were omitted from the incubations.

Data analysis. Data from association and dissociation experiments were fit to one- and two-component exponential decay curves by nonlinear least-squares curve-fitting. Monotonic concentration-response curves were fit to a Langmuir isotherm or a logistic equation, depending on whether the latter model gave an improved fit compared with the former. Bell-shaped concentration-response curves were fit to a special model (UPDOWN), based on Eq. 1.

$$Y = N + \frac{S \cdot [1 + E \cdot X/(X + EC_{50})]}{(1 + X/IC_{50})} \quad (1)$$

In this equation, Y is observed binding, X is the concentration of enhancer, N is nonspecific binding, S is specific binding, E is the maximum enhancement possible in the absence of interference from competitive antagonism, EC_{50} is the concentration for half-maximal enhancement, and IC_{50} is the concentration for 50% inhibition. This model was only used to provide smooth curves for graphics and was not used for estimation of binding parameters.

Results

History of the chemical series. The chemical series described in the present study was first brought to our attention as the result of the screening of about 300 structures from the Parke-Davis compound bank in adenosine A_1 binding.[4] Among the 19 compounds with IC_{50} values below 10 μM was PD 78,416 (Fig. 1), which inhibited [^{3}H]CHA binding to rat brain membranes with a K_i of 970 nM. This compound came from a series of 2-amino-3-benzoylthiophenes originally made as chemical intermediates in the synthesis of benzodiazepine-like compounds (14). Following the discovery of its adenosine antagonist activity, PD 78,416 was tested in a variety of animal screens and was found to have some activity in a model of cognitive improvement (method described in Ref. 22). Because of this activity, several additional analogs were synthesized,[5] including PD 117,975 (Fig. 1). PD 117,975 was unexpectedly found to increase specific [^{3}H]CHA binding (see below). The experiments described in the present article were carried out to determine the mechanism of this unusual effect.

Later structure-activity studies (23) on the original series of Tinney *et al.* (14) identified PD 71,605 and PD 81,723 (Fig. 1) as analogs with somewhat improved profiles compared with PD 117,975. Most of the experiments in the present study were first carried out with PD 117,975 and later repeated with PD 71,605 or PD 81,723.

Effects of adenosine binding enhancers on binding of [^{3}H]CHA to rat brain membranes. The initial chemical lead, PD 117,975, increased specific [^{3}H]CHA binding by about 20% at 10 μM (Fig. 2). A bell-shaped concentration-response curve was seen, with about 40% inhibition of binding occurring at 100 μM. Because PD 117,975 originated from a series of adenosine antagonists, we attributed the inhibitory phase of the concentration-response curve to competitive antagonism (see below). PD 71,605 also inhibited [^{3}H]CHA binding at 30–100 μM. PD 81,723 appeared to show the least self-inhibition, increasing binding by 45% at 20 μM and returning binding to control levels at 100 μM (Fig. 2). The degree of self-inhibition by PD 81,723 was quite variable, with negligible inhibition seen in some experiments (6) and strong inhibition in others (see Ref. 24 and Fig. 3). This variability is probably attributable to different degrees of precipitation occurring at the 100 μM concentration. None of the compounds had any effect on appropriately defined nonspecific binding (see Experimental Procedures).

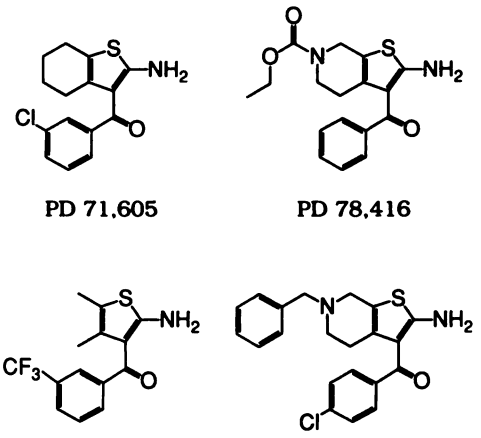

Fig. 1. Structures.

[4] L. Coughenour, G. Courtland, and T. Pugsley, personal communication.
[5] J. Dodd and J. McKee, personal communication.

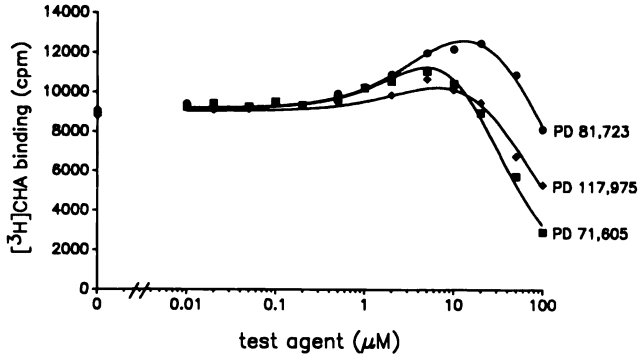

Fig. 2. Enhancement of [^{3}H]CHA binding by 2-amino-3-benzoylthiophenes. Binding of 1 nM [^{3}H]CHA to rat brain membranes was carried out for 1 hr at 25°. Values shown are specific binding and are means of triplicate determinations; standard errors were generally smaller than the symbols. The curves were generated using the best-fit parameters for the UPDOWN model (see Experimental Procedures). Taken with permission from Ref. 13.

Effects of enhancers on other adenosine receptor binding assays. PD 81,723 enhanced the binding of [^{3}H](R)-PIA and [^{3}H]NECA to A_1 receptors (Fig. 3), showing that the enhancement of [^{3}H]CHA binding extended to other A_1 agonists. [^{3}H]NECA binding was carried out in cerebellum, a tissue that has no A_2 component of [^{3}H]NECA binding (16). The concentration-response curves for PD 81,723 in [^{3}H](R)-PIA and [^{3}H]NECA binding were essentially identical to the curve in [^{3}H]CHA binding in the same experiment (not shown). In this experiment, there was a precipitous drop in binding at 100 μM PD 81,723, suggesting a nonspecific effect such as protein denaturation at this high concentration.

In contrast to its enhancement of adenosine agonist binding, PD 81,723 showed only inhibition when tested versus the A_1 antagonist ligand [^{3}H]CPX (Fig. 3). The inhibition of [^{3}H]CPX binding confirms that the enhancers retain significant competitive antagonist activity. In addition, the lack of enhancement of [^{3}H]CPX binding implies that the enhancing activity is limited to agonists and is not seen with antagonists. PD 117,975 did not enhance [^{3}H]NECA binding to striatal A_2 receptors (Fig. 3) but rather showed a weak inhibition, indicating that the enhancement of binding was specific for the A_1 receptor subtype.

Species differences in concentration-response curves. A_1 receptors contain a phenyl-binding pocket that increases the affinity of adenosine antagonists containing an appropriately placed phenyl ring. The strength of the "phenyl effect" varies markedly with species, with the order bovine > rat > human > dog (17, 25). Like many other adenosine antagonists, PD 117,975 possesses an exocyclic phenyl ring. We speculated that the phenyl effect might also contribute to the antagonist affinity of PD 117,975. If so, it might be possible to circumvent the interference from competitive antagonism by carrying out binding in a species with a weaker phenyl effect. The actual results bear out this prediction (Fig. 4); PD 117,975 showed a large monotonic enhancement of [^{3}H]CHA binding in human and dog, with an increase of more that 200% being seen in the latter species. In contrast, only inhibition was seen at the bovine A_1 receptor, which has a stronger phenyl effect than its rat counterpart. These results show that binding enhancement can be separated from competitive antagonism and that a large (over

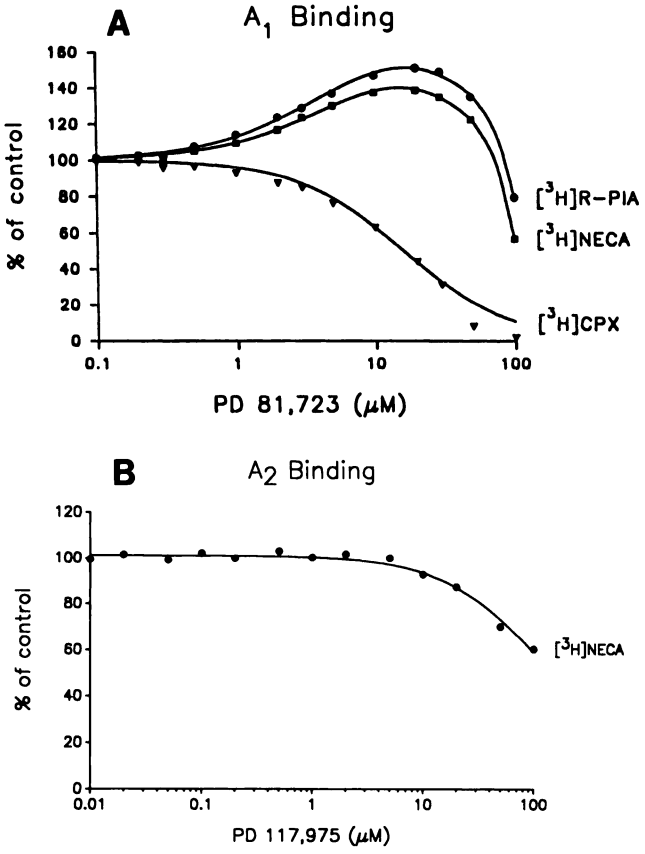

Fig. 3. Effects of enhancers on binding of A_1 and A_2 ligands. A, Effect of PD 81,723 on A_1 receptor binding. Experimental procedures for binding of 1 nM [^{3}H](R)-PIA and 0.2 nM [^{3}H]CPX to rat forebrain membranes and of 4 nM [^{3}H]NECA to rat cerebellar membranes were the same as in Fig. 2. Values are specific binding. Control values were [^{3}H](R)-PIA: total, 22,300 dpm; nonspecific, 620 dpm; [^{3}H]NECA: total, 10,000 dpm; nonspecific, 2,180 dpm; [^{3}H]CPX: total, 15,500 dpm; nonspecific, 460 dpm. [^{3}H](R)-PIA and [^{3}H]NECA curves are the best-fit solution to the UPDOWN model, except that the 100 μM concentration was omitted from the fitting and the portion of the curve from 50 to 100 μM was drawn by hand. Results for [^{3}H]CHA in the same experiment (not shown) could be superimposed on the [^{3}H](R)-PIA curve. The [^{3}H]CPX results (without the 50 and 100 μM data points) were fitted to a logistic model. B, Effect of PD 117,975 on A_2 receptor binding. Binding of 4 nM [^{3}H]NECA to rat striatal membranes was carried out in the presence of 50 nM CPA, as described (16). Control values were total, 1,540 cpm; nonspecific, 275 cpm.

200%) enhancement can be revealed when interference from competitive antagonism is minimized.

Unmasking by CPX of a robust binding enhancement by PD 117,975 in rat brain membranes. The large enhancement of human and dog A_1 receptor binding by PD 117,975 lent support to the idea that the rat A_1 receptor also might show a large degree of enhancement if the interfering effects of competitive blockade by PD 117,975 could somehow be nullified. To test this conjecture, concentration-response curves for PD 117,975 were carried out in the presence of various concentrations of the A_1 antagonist CPX (Fig. 5). The rationale for this experiment was as follows. Although absolute binding would be very low in the presence of CPX, competitive inhibition by PD 117,975 would add little to the already high degree of inhibition caused by CPX. Because PD 117,975 would still be free to exert its enhancing effects, the predicted result would be a large stimulation of binding, when expressed as a

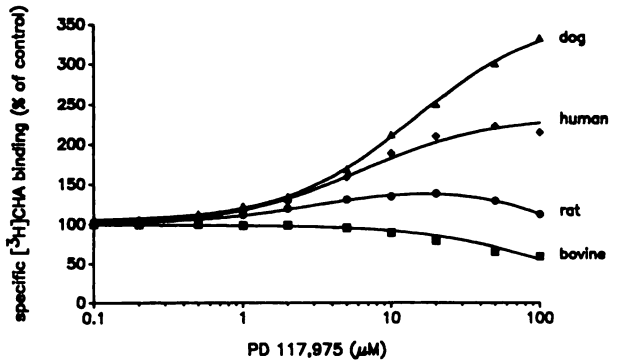

Fig. 4. Enhancement of [^{3}H]CHA binding by PD 117,975 in brain membranes from different species. Values are specific binding. Control binding values were dog frontal cortex: total, 682 cpm; nonspecific, 204 cpm; human frontal cortex: total, 1,970 cpm; nonspecific, 210 cpm; rat forebrain: total, 4,780 cpm; nonspecific, 209 cpm; bovine forebrain: total, 6,940 cpm; nonspecific, 217 cpm.

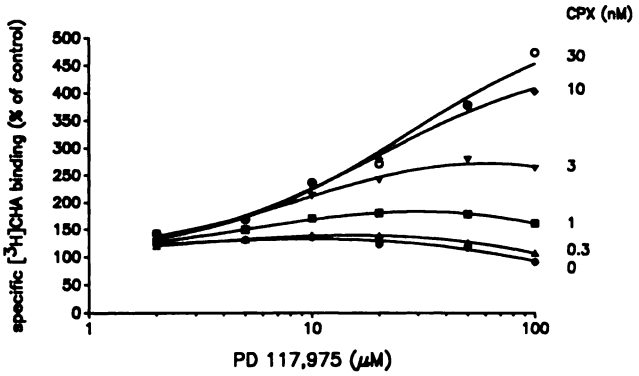

Fig. 5. Effect of the A_1 antagonist CPX on enhancement of [^{3}H]CHA binding by PD 117,975. Values are specific binding. Control specific binding values were no CPX, 5,170 cpm; 0.3 nM CPX, 4,360 cpm; 1 nM CPX, 2,630 cpm; 3 nM CPX, 1,190 cpm; 10 nM CPX, 398 cpm; 30 nM CPX, 145 cpm. Nonspecific binding averaged 177 cpm.

percentage of binding seen with CPX alone. The actual results agree with the prediction; progressively higher concentrations of CPX unmask progressively higher percentages of stimulation by PD 117,975, with up to 350% enhancement seen in the presence of 30 nM CPX. These results confirm that PD 117,975 can generate a robust severalfold stimulation of A_1 agonist binding when interference from its own competitive blockade is absent. The pharmacological stratagem described here (dilution of competitive effects of the test agent by addition of a second competitive antagonist) is also used later in this study to unmask functional enhancement by this series of compounds.

Interactions between PD 117,975 and agents that affect the inhibitory G protein. The A_1 receptor is coupled to adenylate cyclase through the inhibitory G protein G_i (4, 26, 27). G_i increases the affinity of the A_1 receptor for agonists by forming a complex with a receptor conformation that has high affinity for agonists (26). The enhancement of A_1 agonist affinity by PD 117,975 could therefore be due to a direct effect on the receptor or an indirect interaction via G_i. To distinguish these possibilities, we investigated the effects of GTP, NEM, and Mg^{2+} ions on the enhancement of [^{3}H]CHA binding by PD 117,975. The formation of the high affinity complex is strengthened by divalent cations and weakened by GTP and the sulfhydryl-alkylating reagent NEM (26, 27). GTP and NEM both inhibited binding of [^{3}H]CHA (see legend to Fig. 6). However, the percent enhancement by PD 117,975 was greater in the presence of GTP or NEM (Fig. 6), indicating that uncoupling of the receptor from G_i did not prevent the enhancing effect of PD 117,975. These results provide some tentative evidence that PD 117,975 may act directly on the A_1 receptor. This conclusion is strengthened by the observation (see below) that the adenosine enhancers do not enhance agonist binding to other G_i-linked receptors.

Conversely, addition of $MgCl_2$ increased [^{3}H]CHA binding but eliminated the enhancing effect of PD 117,975 (Fig. 6). The lack of additivity between PD 117,975 and magnesium ions suggests that both may promote the formation of the same high affinity conformation of the A_1 receptor. The enhancement by PD 117,975 was not due to heavy metal contamination, because it was not prevented by 1 mM EDTA.

Slowing of the dissociation of [^{3}H]CHA by PD 117,975. An agent that increased the binding of [^{3}H]CHA could do so either by accelerating the association of [^{3}H]CHA or by retarding its dissociation. Increases in affinity due to slowing of dissociation are common. For instance, magnesium ions slow the dissociation of [^{3}H]CHA (27), GABA slows the dissociation of benzodiazepines (12), and the allosteric enhancer ropizine slows the dissociation of [^{3}H]dextromethorphan (28). As anticipated based on these previous examples, PD 117,975 caused a marked slowing of the dissociation of [^{3}H]

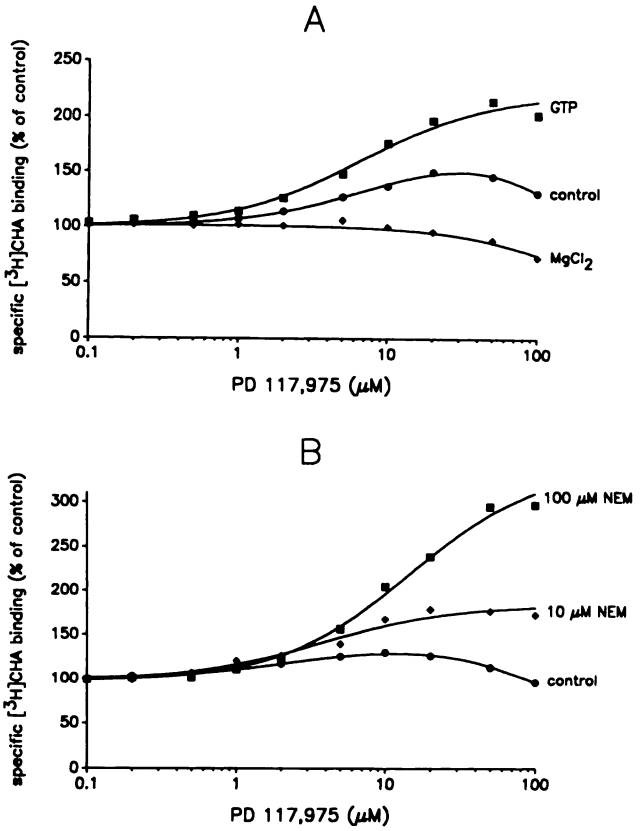

Fig. 6. Effects of GTP, $MgCl_2$, and NEM on the enhancement of [^{3}H]CHA binding by PD 117,975. GTP (100 μM), $MgCl_2$ (10 mM), or NEM (10 or 100 μM) was added at the same time as PD 117,975 and [^{3}H]CHA. Values are specific binding. Control specific binding values were as follows. A, No addition, 4,540 cpm; GTP, 1,890 cpm; $MgCl_2$, 8,700 cpm. B, No addition, 5,410 cpm; 10 μM NEM, 1,480 cpm; 100 μM NEM, 463 cpm.

CHA (Fig. 7). In the absence of PD 117,975, the dissociation of [^{3}H]CHA occurred in two phases, a rapid phase ($t_{1/2}$, 5 min) comprising about 60% of specific binding and a slow phase ($t_{1/2}$, 70 min) accounting for the remaining 40% of specific binding. In the presence of 10 μM PD 117,975, the slow component increased to about 62% of specific binding and the time constants for the fast and slow components were increased by factors of 1.8 and 1.6, respectively.

The dissociation of the antagonist [^{3}H]CPX was unaffected (Fig. 7) or slightly accelerated (data not shown), implying that the slowing of dissociation was specific for the agonist state of the A_1 receptor.

Concentration-response relationship for the slowing of [^{3}H]CHA dissociation by allosteric enhancers. As previously mentioned, the conflicting effects of allosteric enhancement and competitive inhibition produced bell-shaped concentration-response curves for PD 117,975 and other enhancers in the standard [^{3}H]CHA binding assay. However, the enhancement occurs largely via a slowing of the dissociation rate for [^{3}H]CHA, whereas competitive inhibition operates by preventing association. Therefore, it should be possible to obtain a concentration-response relationship for enhancement alone by adding the enhancer under conditions where only dissociation occurs. Such an experiment is shown in Fig. 8. [^{3}H]CHA was preassociated with the receptor for 1 hr, after which various

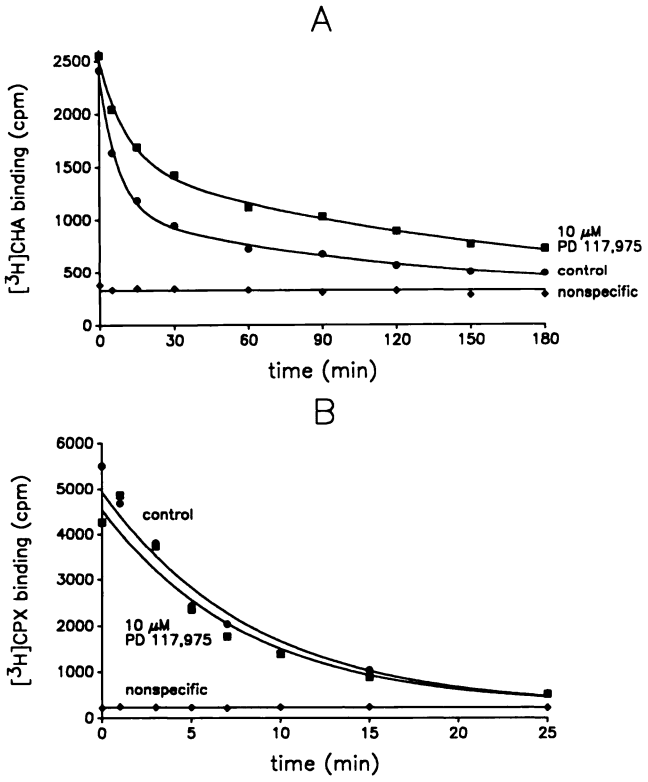

Fig. 7. Effect of PD 117,975 on the dissociation of the agonist [^{3}H]CHA and the antagonist [^{3}H]CPX from A_1 adenosine receptors in rat brain membranes. A, [^{3}H]CHA (1 nM) was preassociated with 20 mg of rat forebrain membranes for 1 hr at 25° without additions (total binding) or in the presence of 10 μM (*R*)-PIA (nonspecific binding). At the end of the preincubation period, 100 μM CPA was added simultaneously with vehicle or 10 μM PD 117,975. The incubations were terminated after the additional times indicated on the *x*-axis. B, The experiment was carried out in the same way as in A, except that the ligand was 0.2 nM [^{3}H]CPX and the amount of tissue was 5 mg.

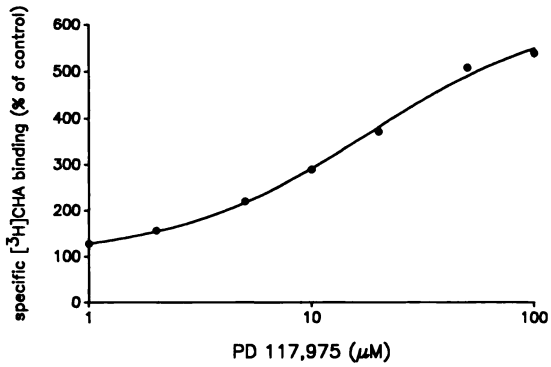

Fig. 8. Concentration-response curve for the slowing of the dissociation of [^{3}H]CHA by PD 117,975. [^{3}H]CHA (1 nM) was preassociated with 20 mg of rat forebrain membranes for 1 hr at 25° without additions (total binding) or in the presence of 10 μM (*R*)-PIA (nonspecific binding). At the end of the preincubation period, 100 μM CPA was added simultaneously with vehicle or various concentrations of PD 117,975. The incubations were terminated after an additional 2 hr. Control specific binding at the end of the 2-hr dissociation period was 400 cpm. Taken with permission from Ref. 13.

concentrations of the enhancer were added along with sufficient unlabeled CPA (100 μM) to halt instantly any further association of [^{3}H]CHA with the receptor. Samples were filtered after an additional 2 hr, during which control binding decayed to about 15% of initial binding. Up to 439% enhancement of residual binding could be seen with this protocol (Fig. 8). Interestingly, although the initial chemical lead PD 78,416 showed only inhibition in the standard [^{3}H]CHA binding assay, it showed significant enhancement (112% at 100 μM) in the dissociation protocol. The lack of enhancement by PD 78,416 in the standard [^{3}H]CHA assay was undoubtedly due to interference from its high affinity as a competitive antagonist (K_i 530 nM in [^{3}H]CPX binding). In a companion study (23) we have used the dissociation assay described here to assess structure-activity relationships for A_1 enhancement in the absence of interference from competitive inhibition.

Specificity of effects of A_1 enhancers on dissociation of agonists. An important issue is whether the enhancement by PD 117,975 and analogs is specific for the A_1 adenosine receptor. Because [^{3}H]CGS 21680 selectively labels A_2 adenosine receptors in rat striatal membranes (29), we used this ligand to investigate whether agonist dissociation from the A_2 receptor was altered by the enhancers. PD 81,723 had no effect on the dissociation of [^{3}H]CGS 21680 (Fig. 9). The enhancers also had little or no effect on the time-courses for dissociation of [^{3}H]CMD from M_2 muscarinic receptors, [^{3}H]UK-14,304 from α_2 adrenergic receptors, or [^{3}H]DSTLE from δ opiate receptors (Fig. 9). Because M_2, α_2, and δ receptors are linked to inhibitory G proteins (30, 31), these results imply that analogs of PD 117,975 are not general enhancers of G_i-linked receptors.

Inhibition of cyclic AMP accumulation in FRTL-5 cells: a model system for A_1 functional responses. The A_1-mediated inhibition of cyclic AMP accumulation in the FRTL-5 rat thyroid cell line (32) was characterized as a model for the A_1 functional response. Forskolin (10 μM) increased cyclic AMP by 80-fold in the presence of the A_1 antagonist CPX (Table 1). Omission of CPX resulted in a 50% inhibition of forskolin-stimulated cyclic AMP accumulation, even though a very high concentration of adenosine deaminase (10 units/

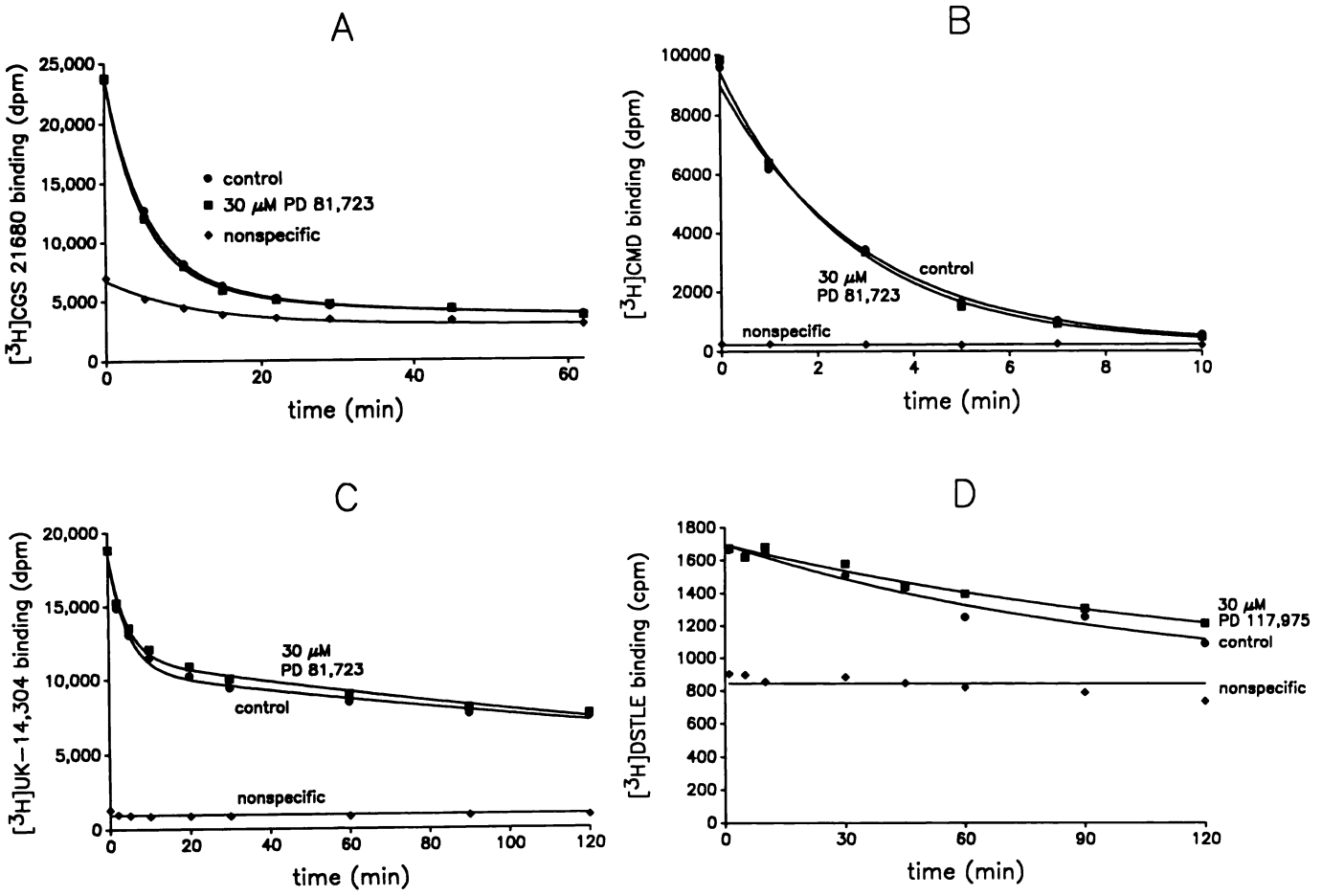

Fig. 9. Lack of effect of enhancers on the dissociation of agonists from A_2 adenosine, M_2 muscarinic, α_2 adrenergic, and δ opiate receptors (A–D, respectively). Membranes were preincubated for 1 hr at 25° with labeled agonist, after which an unlabeled agonist was added simultaneously with enhancer (or vehicle), and the incubations were terminated after the additional times indicated on the *x*-axis. The A_2 and M_2 assays were carried out in rat striatal membranes and rat heart membranes, respectively, and the other two binding assays were carried out in rat forebrain membranes. In order to achieve a sufficient level of specific binding, the preassociation step for the A_2 assay was carried out in the presence of 1 mM $MgCl_2$, and possible interfering effects of magnesium ions during the dissociation were eliminated by addition of a 4-fold excess of EDTA at the beginning of the dissociation step. Additional details of the assays are provided in Experimental Procedures. The curves in A and C are based on best-fit parameter values for a two-site dissociation model (16), which gave a significantly better fit (*F* test, $p < 0.01$) than a one-site model. In A, the model also incorporated an extra component consisting of dissociable nonspecific binding (*F* test, $p < 0.01$). The curves in B and D are for a one-site model, because a two-site model did not give an improved fit.

TABLE 1

A_1 receptor-mediated inhibition of cyclic AMP accumulation in FRTL-5 cells

Incubations with test agents were carried out for 10 min at 29° in 1 ml of Tyrode-HEPES containing 100 μM rolipram and 10 units/ml adenosine deaminase, as described in Experimental Procedures. Values are means ± standard errors of triplicate determinations and are expressed as ppm, cpm of [^{3}H]cAMP/1,000,000 cpm of total [^{3}H]adenine nucleotides. Tritiated adenine nucleotides averaged 745,000 cpm. Δ represents the increase in cyclic AMP caused by forskolin, expressed as a percentage of the increase seen in the absence of receptor activation by endogenous adenosine.

	[^{3}H]cAMP	
	Accumulation	Δ
	ppm	%
Basal	440 ± 110	0
10 μM Forskolin	43,000 ± 3,000	52
+100 nM CPX	82,000 ± 2,000	100
+30 nM CPA	920 ± 40	0.5

ml) was included in the incubation.[6] Most dramatically, the A_1 agonist CPA inhibited forskolin-stimulated cyclic AMP accumulation by 97% or more (for instance, 99.5% in Table 1). To our knowledge, this is the most profound receptor-mediated inhibition of cyclic AMP accumulation yet reported. Although Berman *et al.* (32) did not report an exact magnitude for maximum inhibition of cyclic AMP accumulation, their Fig. 2 appears to show a 98% inhibition of forskolin-stimulated cyclic AMP accumulation by 100 nM (*R*)-PIA. The IC_{50} for CPA was 0.5 nM under control conditions (data not shown) and 230 nM

[6] The CPX-sensitive inhibition of cyclic AMP accumulation in the presence of 10 units/ml adenosine deaminase could be accounted for by any of three different mechanisms. First, there could be an intracellular pool of A_1 receptors (for instance, on endocytotic vesicles) accessible to endogenous adenosine and CPX (both of which cross cell membranes) but not to adenosine deaminase. Second, a very high turnover of adenosine could overwhelm the deaminase. Third, the unoccupied A_1 receptor could have intrinsic activity due to a small population of receptors in the active conformation in equilibrium with a much larger population in the ground state. However, the latter interpretation would probably require an unusually high density of A_1 receptors or an unusually strong coupling between the A_1 receptor and adenylate cyclase.

in the presence of 100 nM CPX (see Fig. 10, below). The concentration-response curve for CPA was steep, with a Hill coefficient of 1.9.

Effects of A_1 enhancers on the concentration-response relationship for CPA in FRTL-5 cells. A true allosteric enhancer should cause a leftward shift in the dose-response curve for agonist. However, in the 2-amino-3-benzoylthiophene series, this effect might be obscured by competitive inhibition, which should produce a shift to the right. To avoid this confounding influence, the effect of PD 71,605 on the concentration-response curve for CPA was studied in the presence of a 100 nM concentration of the competitive antagonist CPX. The addition of CPX at approximately 200 times its K_d was designed to overwhelm any smaller competitive effect by PD 71,605. Under these conditions, 100 μM PD 71,605 caused a 19-fold shift to the left in the concentration-response relationship for CPA (Fig. 10). A smaller 7-fold shift was seen with 10 μM PD 71,605. These results demonstrate that PD 71,605 can cause functional enhancement when tested under the appropriate conditions. Similar results were seen with PD 117,975 (24) and PD 81,723 (data not shown).

Besides shifting the CPA curve to the left, PD 71,605 also lowered cyclic AMP by 44–55% in the absence of CPA (Fig. 10). PD 117,975 (24) and PD 81,723 lowered cyclic AMP to a similar degree. Because the experiment was carried out in the presence of high concentrations of both adenosine deaminase and CPX, it is unlikely that this effect was due to potentiation of endogenous adenosine. Furthermore, the lowering of cyclic AMP was probably not a nonspecific toxic effect of PD 71,605, because it was not seen in the neuro-2a cell line (see below). Unlike FRTL-5 cells, neuro-2a cells do not show an A_1 inhibition of cyclic AMP accumulation (data not shown). In the absence of an alternative explanation, it seems possible that the A_1 enhancers may have significant intrinsic activity; this would be predicted from the proposed mechanism of enhancement (see Discussion).

Concentration dependence of functional enhancement. To determine the concentration-response properties of PD 71,605, the compound was tested in FRTL-5 cells under three conditions: with 100 nM CPX and 100 nM CPA (to measure enhancement in the absence of any significant competitive inhibition), with 0.3 nM CPA alone (to measure the combined effects of enhancement and blockade by PD 71,605), and with 100 nM CPX alone (to measure intrinsic activity of PD 71,605). In the presence of 100 nM CPX, 100 nM CPA caused a 44% inhibition of cyclic AMP accumulation, which was increased to 94% inhibition by 30 μM PD 71,605. Half-maximal potentiation by PD 71,605 was seen at 580 nM, and significant potentiation was seen at concentrations as low as 100 nM (Fig. 11).

Without CPX, PD 71,605 potentiated the response to 0.3 nM CPA, with half-maximal inhibition being seen around 200 nM (Fig. 11). Maximal potentiation was seen at 3 μM PD 71,605. The concentration-response curve for PD 71,605 turned upward at higher concentrations, presumably because of competitive inhibition.

PD 71,605 also showed apparent intrinsic activity in the presence of 100 nM CPX (Fig. 11). Maximum inhibition of 57% was seen at 30 μM, and the affinity of the effect (EC_{50}, 1.7 μM) was about 3-fold less than for potentiation of CPA.

Specificity of the functional enhancement by PD 71,605. PD 71,605 did not potentiate the inhibition of forskolin-stimulated cyclic AMP accumulation by carbachol in the neuro-2a cell line (Fig. 12). The inhibition of cyclic AMP accumulation by carbachol in this cell line is probably mediated by an m4 muscarinic receptor (33).

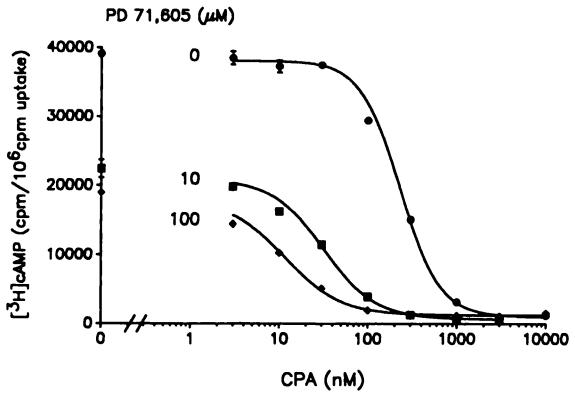

Fig. 10. Effect of PD 71,605 on the inhibition of forskolin-stimulated cyclic AMP accumulation in FRTL-5 cells by the A_1 agonist CPA. FRTL-5 cells were incubated for 10 min with test agents plus (except for basal) 100 nM CPX and 10 μM forskolin, as described in Experimental Procedures. *Uptake*, incorporation of [^{3}H]adenine into total adenine nucleotides, which averaged 1,078,000 cpm. IC_{50} values for CPA were control, 230 nM; with 10 μM PD 71,605, 32 nM; with 100 μM PD 71,605, 11.7 nM. Taken with permission from Ref. 13.

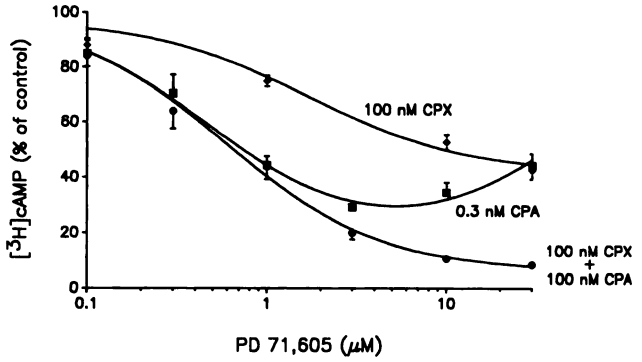

Fig. 11. Concentration-response curves for inhibition of forskolin-stimulated cyclic AMP accumulation in FRTL-5 cells by PD 71,605 under various conditions. Experimental procedures were the same as in Fig. 10. Values for basal and forskolin-stimulated [^{3}H]cAMP accumulation in this experiment are given in Table 1.

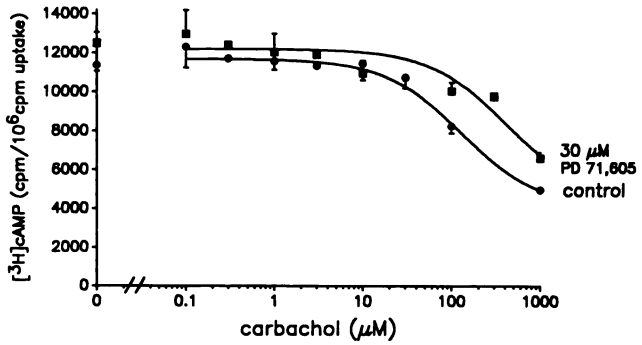

Fig. 12. Effect of 30 μM PD 71,605 on the inhibition of forskolin-stimulated cyclic AMP accumulation in neuro-2a cells by carbachol. Experimental conditions were the same as in Fig. 10, except that CPX and adenosine deaminase were omitted from the incubation. Total [^{3}H]adenine nucleotides averaged 617,000 cpm.

Discussion

This study presents evidence that 2-amino-3-benzoylthiophenes act as allosteric enhancers at the adenosine A_1 receptor, both in binding assays and in a functional assay *in vitro*. The allosteric nature of the interaction is shown by the slowing of the dissociation of the agonist [^{3}H]CHA. The enhancing effect is specific for the A_1 receptor, because no enhancement is seen at the A_2 receptor or at several other receptors that (like the A_1 receptor) couple to inhibitory G proteins. Furthermore, the effect is specific for the agonist state of the receptor, because A_1 antagonist binding is not enhanced.

Although allosteric enhancers are known for receptors from other superfamilies (12, 28, 36), the A_1 enhancers described in the present study may be the first such compounds to be reported for the large superfamily of G protein-linked receptors.[7] As such, their mechanism of action deserves detailed analysis, because they may serve as prototypes for allosteric modulators of other receptors in this family. Fortunately, much of the experimental and theoretical knowledge gained from other allosteric enzyme and receptor systems appears to be applicable to the present system. In particular, many of the effects of the adenosine enhancers seen in the present study are in fact similar to phenomena that have been reported before in other receptor systems. These include the slowing of the dissociation of agonist (12, 28), the leftward shift of the concentration-response curve for agonist in functional models (12, 36), and the ability of the enhancer to cause a functional response in the absence of agonist (36).

A simple model of receptor allosterism (Fig. 13) appears to explain the behavior of the adenosine enhancers. This model, based on the theory of linked equilibria (37–39), presumes a single-subunit receptor with two different binding sites, one for agonist and one for enhancer. The receptor exists in two conformations, a ground state, *R*, and an activated state, *R**, the latter being responsible for the response that is controlled by the receptor (for example, inhibition of adenylate cyclase). Under basal conditions *R* may outnumber *R** by a factor of ~1000. Agonists activate the receptor by binding selectively to the *R** state of the receptor, driving the receptor equilibrium in the direction of *AR** (illustrated by the front face of the cube in Fig. 13).

In this model, the role of enhancer is simple; it behaves in exactly the same way as agonist, except that it binds to a different site. Thus, the enhancer, like agonist, drives the receptor from *R* to *R** by binding selectively to *R**. At high concentrations, if its affinity preference for *R** over *R* is high enough, the enhancer can elicit a response without agonist being present. At lower concentrations, the enhancer potentiates the response to agonist. For instance, under basal conditions a cell may have 1 receptor in the *R** conformation and 999 receptors in the *R* conformation. With the addition of a low concentration of enhancer the equilibrium may shift to 1 *R**, 9 *R*E*, and 990 *R*. Because there are now 10 receptors in the activated state, compared with only 1 without enhancer, it will be 10 times easier for agonist to drive the equilibrium further toward the activated state. Hence, in this example the enhancer should cause a 10-fold leftward shift in the concentration-response curve for agonist.

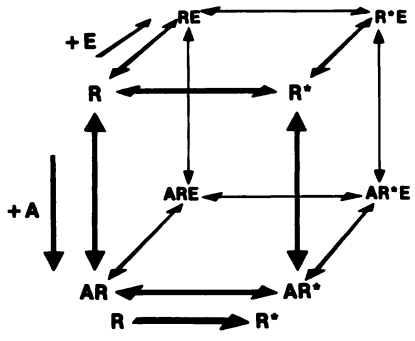

Fig. 13. Equilibrium diagram for receptor, agonist, and allosteric enhancer. *R*, Ground state of receptor; *R**, activated state of receptor; *A*, agonist; *E*, enhancer.

The model described above is a special case of model III of Monod *et al.* (39), which explains allosterism on the basis of interactions between separate sites. Models I and II deserve mention as possible alternative explanations for the behavior of the enhancers. Model I assumes that the enhancers bind to the same site as agonist. This model could account for the biphasic curves seen in Fig. 2 if the receptor is assumed to be a dimer with the two subunits allosterically coupled.[8] At a low concentration of enhancer, one of the two subunits would be occupied by enhancer, forcing the other into the agonist state and increasing its affinity for agonist, thereby increasing labeled agonist binding. At higher enhancer concentrations, both sites would be occupied by enhancer and binding of labeled agonist would be inhibited. Although this model provides a unitary explanation for the observed biphasic dose-response curves, several other predictions of the model are inconsistent with actual observations. In particular, this model predicts that any unlabeled agonist should stimulate labeled agonist binding in a manner similar to the enhancers. Contrary to this prediction, stimulation of [^{3}H]CHA binding by unlabeled agonists has never been observed. In addition, the ability of the enhancers to slow the dissociation of [^{3}H]CHA in the presence of a 200,000-fold excess of unlabeled agonist (Fig. 7) cannot be explained by this model.

Model II involves a direct chemical interaction between agonist and enhancer, bound to adjacent sites on the receptor. This model generally predicts behavior very similar to that predicted by model III, but two considerations tend to argue against this model. First, there is no obvious means by which the enhancer would bind to receptor-bound adenosine. Most of the functional groups of adenosine (for example, 2′- and 3′-hydroxyls, 6-amino group, N^7, etc.) are already bound by the receptor, and there is little free space available in the area surrounding adenosine except near N^6 and (to a lesser extent) 5′ (40, 41). The latter two spaces can be fully occupied (for instance, in (*R*)-PIA and NECA, respectively) without disruption of the enhancement. In addition, model II assumes that the receptor activation by the enhancer is indirect, via enhancement of the affinity of adenosine, rather than by a direct shifting of the receptor conformation by enhancer alone. The apparent intrinsic activity of the enhancers in the absence of adenosine is therefore inconsistent with model II.

The chemical mechanisms of enhancement appears to in-

[7] Gallamine acts as an allosteric inhibitor at the M_2 muscarinic receptor (34). The kinetics of this effect are complex (35).

[8] Technically, Model I assumes a single site for which agonist and enhancer compete; allosteric coupling between two such sites is described as part of Model III (37, 39).

volve a reversible noncovalent binding between enhancer and receptor. Washing experiments using slide-mounted sections indicate that enhancement of binding by PD 117,975 is reversible.[9] From their structures, the enhancers would appear to have little chemical reactivity except for a susceptibility to oxidation (seen as darkening on storage in solution) due to the aromatic amino group. Furthermore, any covalent reaction with the receptor from an oxidized enhancer molecule would be expected to be slow (due to gradual accumulation of reactant with progressive oxidation) and irreversible, whereas the actual kinetics are rapid (see the early time points in Fig. 7A) and reversible. The sulfhydryl reagents, 5,5′-dithio-bis-(2-nitrobenzoic acid) and dithiothreitol did not enhance binding, implying that the effects of the enhancers were not attributable to oxidation or reduction of sulfhydryl groups.

The 2-amino-3-benzoylthiophenes described in the present study represent the first generation of adenosine enhancers, and as such their use as pharmacological tools may have important limitations. An obvious factor limiting the use of these agents is their adenosine antagonist activity, which will tend to reverse any enhancing actions of these compounds. The antagonism by these compounds is highly species dependent, being strong in rat and cow and weak in dog and human. Fortunately, the structure-activity relationships for enhancement are separable from the structure-activity relationships for competitive antagonism (23), implying that it should be possible to discover improved enhancers that lack antagonist activity. Other limitations of these compounds include their low solubilities (about 50 μM) and their moderate potencies (IC_{50} values about 0.5 μM in intact cells). The resultant ratio of about 100 between solubility and pharmacological activity may not be adequate for good activity *in vivo* (42). Furthermore, the specificity of these compounds for the A_1 receptor compared with other receptors has been investigated to only a limited extent, so the possibility of other interfering activities cannot be excluded. Because of these uncertainties, we urge that caution be exercised in the interpretation of results obtained using these compounds as pharmacological tools. Nevertheless, we do believe that the presently available enhancers are worthy of careful testing in relevant models. Because PD 81,723 appears to have the best overall combination of potency, solubility, and lack of antagonist activity, we recommend that this compound be employed in such studies.

Improved A_1 enhancers may be useful as therapeutic agents. In light of the role of adenosine in protection against damage due to inadequate tissue oxygenation, ischemia would appear to be a particularly promising area for application of adenosine enhancers. Neuroprotective actions of adenosine that are mediated by A_1 receptors include inhibition of neuronal firing (43), inhibition of the release of excitatory amino acids (44), and inhibition of pre- and postsynaptic calcium uptake (45). Cardioprotective A_1 effects include reductions in heart rate (46), prevention of the positive inotropic effects of β-adrenergic agonists (47), and inhibition of the release of catecholamines (48). Because adenosine production is much higher in ischemic than in normal tissue, the actions of an adenosine enhancer should be naturally selective for the ischemic zone and a small penumbra surrounding this zone.

Several other possible therapeutic applications for A_1 enhancers deserve consideration. Adenosine is known to serve an important role in reducing metabolic work in the kidney (49). An A_1 enhancer would therefore be predicted to protect the kidney from damage due to metabolic overload, slowing the progression of chronic renal failure. In the nonischemic central nervous system, A_1 enhancers might be useful for conditions involving neuronal overexcitation, such as epilepsy, mania, paranoia, and rage or anger. A_1 enhancers should selectively inhibit neurons that are metabolically overactive, because these neurons should produce adenosine under nonischemic conditions. A_1 enhancers also might protect against brain damage from hypoxia during birth.

Although the adenosine enhancers in the present study were discovered by accident, we see no reason why other enhancers could not be found by screening. Because enhancers already exist for several receptor superfamilies, it would appear that almost any receptor could be a target for such an approach (50). The dissociation protocol illustrated in Fig. 8 might furnish a particularly sensitive and selective means to screen for allosteric enhancers. Such enhancers would represent an attractive strategy for strengthening already-existing control mechanisms.

Acknowledgments

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