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Adenosine receptors in brain membranes: Binding of N^6-cyclohexyl[^{3}H]adenosine and 1,3-diethyl-8-[^{3}H]phenylxanthine

(methylxanthines/adenylate cyclase)

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ABSTRACT **N^6-Cyclohexyl[^{3}H]adenosine ([^{3}H]CHA) and 1,3-diethyl-8-[^{3}H]phenylxanthine ([^{3}H]DPX) bind to adenosine receptors in brain membranes. The agonist [^{3}H]CHA has high affinity in both bovine and guinea pig brain (K_d, 0.7 nM and 6 nM, respectively). [^{3}H]CHA binding kinetics are slow (dissociation $t_{1/2}$, 60 min); binding is much higher at 25°C than at 0°C and is inhibited by guanine nucleotides. Potencies of nucleosides and xanthines in competing for [^{3}H]CHA sites indicate that specific binding is entirely to A_1 adenosine receptors. In bovine brain, the antagonist [^{3}H]DPX exhibits high-affinity binding (K_d, 5 nM) to the same A_1 receptors that bind [^{3}H]CHA. Binding kinetics are rapid (dissociation $t_{1/2}$, 1 min), and binding is moderately higher at 0°C than at 25°C. In guinea pig brain, [^{3}H]DPX binding has only moderate affinity (K_d, 50 nM), and about 60% of specific binding is to sites that resemble A_2 adenosine receptors.**

In addition to its role in intermediary metabolism, adenosine displays a number of receptor-mediated physiological actions, including dilation of coronary vessels, inhibition of platelet aggregation, and inhibition of lipolysis. Adenosine also behaves as a neuromodulator or neurotransmitter, inhibiting neuronal firing (1) and synaptic transmission (2) and altering cyclic AMP concentrations in brain tissue (3). In tissue preparations, adenosine can decrease or increase cyclic AMP levels. The structural specificity for decreasing adenylate cyclase activity differs from that for increasing adenylate cyclase activity, suggesting the existence of two distinct adenosine receptors: A_1 sites mediating decreases in adenylate cyclase activity and A_2 sites mediating increases (4–6). In a preliminary communication (7) and in the present paper, we describe the direct identification of A_1 and A_2 receptors in brain membranes by ^{3}H-labeled ligand binding techniques. A_1 receptors were labeled with N^6-cyclohexyl[^{3}H]adenosine ([^{3}H]CHA); both A_1 and A_2 receptors appeared to be labeled with 1,3-diethyl-8-[^{3}H]-phenylxanthine ([^{3}H]DPX) (Fig. 1).

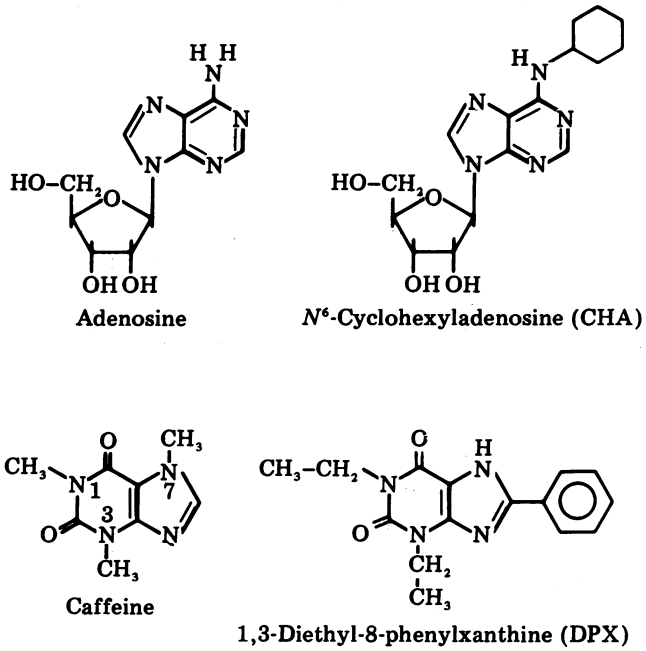

FIG. 1. Adenosine, caffeine, CHA, and DPX.

MATERIALS AND METHODS

Materials. Unless stated otherwise, materials were from standard sources. Adenosine-5′-cyclopropylcarboxamide was from Abbott; CHA, N^6-(D-phenylisopropyl)adenosine (D-PIA), and N^6-(L-phenylisopropyl)adenosine (L-PIA) were from Boehringer Mannheim; 2-(*p*-methoxyphenyl)adenosine (CV 1674) was from Takeda Chemical Industries (Osaka, Japan); and 1,3-dipropylxanthine was from G. D. Searle (Chicago, IL). D-PIA and L-PIA are diastereomers, differing only at the phenylisopropyl moiety. The sugar in both is D-ribose. DPX, *p*-bromo-DPX, and 8-(*p*-sulfophenyl)theophylline were synthesized by modifications of standard methods (8, 9). [^{3}H]DPX (13.6 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) was produced by catalytic dehalogenation of *p*-bromo-DPX with tritium gas (New England Nuclear), and [^{3}H]CHA (14.5 Ci/mmol) was produced from CHA by exchange (Amersham/Searle, Arlington Heights, IL). After purification by thin-layer chromatography (silica gel with acetonitrile as solvent for [^{3}H]DPX and 9:1 chloroform/methanol for [^{3}H]CHA), the compounds had radiochemical purities of >90% based on thin-layer chromatographic analysis. Solutions of methylxanthines and [^{3}H]DPX were stored at refrigerator temperature. [^{3}H]DPX was stable for at least 9 months under these conditions. Unlabeled DPX was dissolved at 1 mM in 0.1 M NaOH. At neutral pH, DPX is soluble up to 3 μM. Solutions of nucleosides were stored at −20°C. [^{3}H]CHA was stored at −20°C in Tris containing 1% ethanol and was stable for at least 4 months.

Preparation of Membranes. Crude washed guinea pig brain membranes were prepared as follows: A frozen guinea pig brain (Pel-Freez) was thawed and then disrupted for 30 sec in a Polytron (setting 5) in 20 ml of 50 mM Tris·HCl at pH 7.7. The homogenate was centrifuged at 50,000 × *g* for 10 min, and the

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Abbreviations: CHA, N^6-cyclohexyladenosine; DPX, 1,3-diethyl-8-phenylxanthine; L-PIA, N^6-(L-phenylisopropyl)adenosine; D-PIA, N^6-(D-phenylisopropyl)adenosine; GMP-PNP, guanylylimidodiphosphate.

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pellet was resuspended in Tris, centrifuged, and resuspended in Tris containing 2 international units of adenosine deaminase (Sigma, type III) per ml. After a 30-min incubation at 37°C, the membranes were recentrifuged, and the pellet was stored at −70°C. The procedure for bovine brain was similar: a thawed bovine forebrain was disrupted in 350 ml of Tris, centrifuged and resuspended three times, incubated at 37°C for 30 min with 400 international units of adenosine deaminase, centrifuged, and stored at −70°C. Because there is only a single centrifugation step after the adenosine deaminase incubation, a great deal of adenosine deaminase is probably carried over to the ^{3}H-labeled ligand incubation.

Incubation Conditions. Incubations were in 50 mM Tris·HCl at pH 7.7. The order of incubations was random. Standard conditions were, for [^{3}H]CHA in guinea pig brain, 1 nM [^{3}H]CHA and membranes from 15 mg (wet weight) of tissue in 1 ml at 25°C for 2 hr, resulting in about 1500 cpm of specific binding; for [^{3}H]DPX in guinea pig brain, 5 nM [^{3}H]DPX and 20 mg of tissue in 1 ml at 0°C for 1 hr (900 cpm); for [^{3}H]CHA in bovine brain, 0.2 nM, [^{3}H]CHA and 10 mg of tissue in 2 ml at 25°C for 4 hr (1500 cpm); and for [^{3}H]DPX in bovine brain, 1 nM [^{3}H]DPX and 10 mg of tissue in 2 ml at 25°C for 4 hr (2000 cpm).

Filtration Procedure. The sample was poured onto a 25-mm Whatman GF/B filter under reduced pressure; 4 ml of ice-cold buffer was added to the sample tube, and this was poured onto the filter; the filter was washed twice with 4 ml of ice-cold buffer. Due to very rapid dissociation, an abbreviated wash procedure for [^{3}H]DPX in guinea pig brain was used: The sample was poured onto the filter, and the filter was immediately washed once with ice-cold buffer.

RESULTS

Properties of [^{3}H]CHA Binding to Brain Membranes. In both guinea pig and bovine brain membranes, [^{3}H]CHA binding was linear with tissue concentration up to 15 mg (wet weight)/ml and 5 mg (wet weight)/ml, respectively. [^{3}H]CHA binding was also temperature dependent; specific binding was much lower at 0°C than at 25°C or 37°C. Specific binding of 1 nM [^{3}H]CHA at 0°C in guinea pig brain membranes was only 150 cpm even after 6 hr. In typical experiments at 25°C in guinea pig brain membranes at 1 nM [^{3}H]CHA, total binding was about 1700 cpm, compared with 150 cpm nonspecific binding in the presence of 10 μM L-PIA. Maximal displacement of [^{3}H]CHA binding was essentially the same with 10 μM L-PIA, 10 μM 2-chloroadenosine, 10 μM CHA, or 1 mM theophylline.

Specific [^{3}H]CHA binding was saturable (Fig. 2), but nonspecific binding increased linearly with [^{3}H]CHA concentration. Scatchard analysis showed a binding site in guinea pig brain membranes that had a dissociation constant (K_d) of 6 nM, a B_{max} of 37 pmol/g of wet weight, and a Hill coefficient of 0.9. In bovine brain, the Scatchard plot was curved and had a high-affinity component (K_d, of 0.26 nM; B_{max}, 34 pmol/g) and a low-affinity component (K_d, 1.8 nM; B_{max}, 20 pmol/g). Half-maximal binding occurred at 0.7 nM.

Kinetics of [^{3}H]CHA binding were slow; $t_{1/2}$ for dissociation (measured in the presence of 10 μM L-PIA) at 25°C was about 1 hr in both guinea pig and bovine brain. Association and dissociation were extremely slow at 0°C; $t_{1/2}$ for dissociation was about 24 hr.

Structural Specificity of [^{3}H]CHA Binding Sites in Guinea Pig. A_1 and A_2 adenosine receptors differ in specificity for

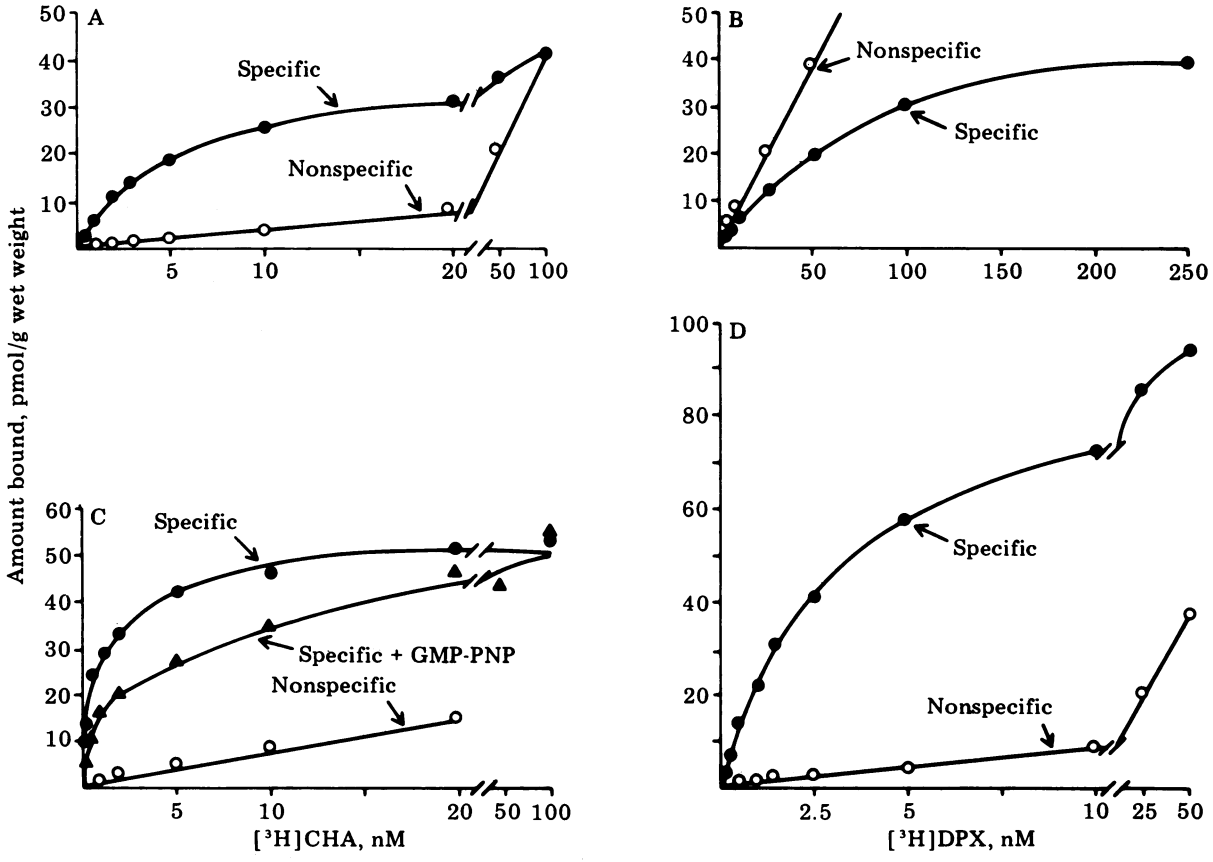

FIG. 2. Binding of [^{3}H]CHA (*A* and *C*) and [^{3}H]DPX (*B* and *D*) in guinea pig brain (*A* and *B*) and bovine brain (*C* and *D*). n = 6 for total binding and 3 for nonspecific binding and for total binding with 100 μM of GMP-PNP. For [^{3}H]DPX binding in guinea pig brain (*B*), n = 12 for total and 9 for nonspecific binding; in this experiment, the tissue was preincubated for 1 hr at 25°C with 100 nM unlabeled CHA to eliminate presumptive A_1 receptor binding.

Table 1. Adenosine receptor properties in guinea pig brain

	Inhibition*		Cyclic AMP generation†	
	[^{3}H]CHA: IC_{50}, nM	[^{3}H]DPX: IC_{60}, nM	EC_{50}, nM	K_i, nM
Nucleosides				
Agonists				
L-PIA	4	150,000	150,000	
CHA	6	80,000	160,000	
2-Chloroadenosine	10	2,000	24,000	
Adenosine-5′-cyclopropylcarboxamide	10	100,000	3,000	
D-PIA	150	100,000	750,000	
2-(*p*-Methoxyphenyl)adenosine	2,500	5,000	>1,000,000	
N^6-Dimethyladenosine	10,000	200,000	>1,000,000	
6-(2-Hydroxy-5-nitrobenzyl)thioinosine	200,000	50,000	>1,000,000	
Inosine	400,000	>1,000,000	>1,000,000	
8-Bromoadenosine	500,000	50,000	>1,000,000	
Antagonists				
5′-Deoxy-5′-iodoadenosine	90	3,000		8,000
5′-Deoxy-5′-methylthioadenosine	300	3,000		8,000
5′-Deoxy-5′-isobutylthioadenosine	2,000	25,000		>1,000,000
2′,5′-Dideoxyadenosine	100,000	30,000		280,000
Purine bases				
DPX	500	50		40
8-Phenyltheophylline	1,000	800		180
1,3-Dipropylxanthine	1,200	500		700
3-Isobutyl-1-methylxanthine	10,000	3,000		3,500
Theophylline	15,000	20,000		4,800
8-(*p*-Sulfophenyl)theophylline	20,000	100,000		1,200
Caffeine	100,000	200,000		13,000
Theobromine	300,000	300,000		130,000
Adenine	800,000	200,000		200,000
Isocaffeine	3,000,000	2,000,000		3,000,000

* Structural specificity of [^{3}H]CHA and [^{3}H]DPX binding in guinea pig brain. To determine IC_{50} values, 5–8 concentrations of displacer were examined, each in triplicate.

† EC_{50} or K_i values of compounds for A_2 receptor-mediated increases in cyclic AMP in human fibroblasts. These values are from ref. 6 or are unpublished data.

nucleosides as well as in their coupling to adenylate cyclase (4–6). Adenosine analogs have nanomolar 50% effective concentration (EC_{50}) values at A_1 receptors and micromolar EC_{50} values at A_2 receptors. At A_1 sites, L-PIA is more potent than adenosine or 2-chloroadenosine; at A_2 sites, 2-chloroadenosine is about 10 times more potent than L-PIA. The A_1-mediated actions of PIA are stereospecific; L-PIA is up to 100 times more potent than D-PIA (10). A_2-mediated effects, however, show much less stereoselection (10).

The potency levels of adenosine derivatives at [^{3}H]CHA binding sites in guinea pig brain indicate an association with A_1 receptors. Adenosine derivatives had affinities nanomolar. L-PIA was about 2 times more potent than 2-chloroadenosine and about 40 times more potent than D-PIA (Table 1; Fig. 3). By contrast, adenosine derivatives are much less potent in enhancing cyclic AMP formation in fibroblasts, an A_2 action (6). Among the methylxanthines, DPX was the most potent agent with an IC_{50} of 0.5 μM. Theophylline was about 5 times as potent as caffeine which, in turn, was about 3 times more potent than theobromine; isocaffeine was only about 3% as potent as caffeine. Displacement curves for adenosine analogs and methylxanthines at [^{3}H]CHA sites in guinea pig brain were monophasic with Hill coefficients of about 1. None of the [^{3}H]CHA binding was to nucleoside uptake sites, because the extremely potent uptake blocker 6-(2-hydroxy-5-nitrobenzyl)thioinosine (11) had no effect on binding, except at micromolar concentrations (Table 1). In preliminary experiments, the affinities of adenosine analogs were similar at [^{3}H]CHA sites in bovine and guinea pig brain, with the exceptions of CHA, L-PIA, and D-PIA, which were about 10-fold more potent in bovine brain (data not shown).

Effects of Guanine Nucleotides on [^{3}H]CHA Binding. Binding of ligands to receptors associated with adenylate cyclase is frequently regulated by guanine nucleotides, which selectively decrease affinities of agonists but not of antagonists for binding sites (12). The hydrolysis-resistant GTP analog guanyl-5′-yl-imidodiphosphate (GMP-PNP) reduced the specific binding of 1 nM [^{3}H]CHA in guinea pig brain membranes by more than 90%, with an IC_{50} of 300 nM. In bovine brain, the [^{3}H]CHA saturation curve was shifted 5-fold to the right by 100 μM GMP-PNP (Fig. 2). GTP and GDP were highly active, but GMP had little effect (R. Goodman, personal communication). This pattern of guanine nucleotide effects on CHA binding resembles the pattern of these substances at most neurotransmitter and hormone receptors that are linked to adenylate cyclase. In addition to the guanine nucleotide effects, divalent cations increased and chelators decreased [^{3}H]CHA binding (R. Goodman, personal communication).

Properties of [^{3}H]DPX Binding in Bovine Forebrain. In contrast to [^{3}H]CHA, [^{3}H]DPX binding to membranes from bovine forebrain was moderately higher at 0°C than at 25°C. At 1 nM [^{3}H]DPX and 25°C, total binding was about 2200 cpm, while nonspecific binding in the presence of 10 μM L-PIA is about 150 cpm. Theophylline (1 mM) plus 10 μM L-PIA gave the same nonspecific binding as L-PIA alone. Kinetics of [^{3}H]DPX binding were rapid; $t_{1/2}$ of dissociation (measured in the presence of 1 mM theophylline) was 1 min at 25°C. Specific binding of [^{3}H]DPX was saturable; K_d, 5 nM; B_{max}, 100 pmol/g; and Hill coefficient, 1.0 (Fig. 2).

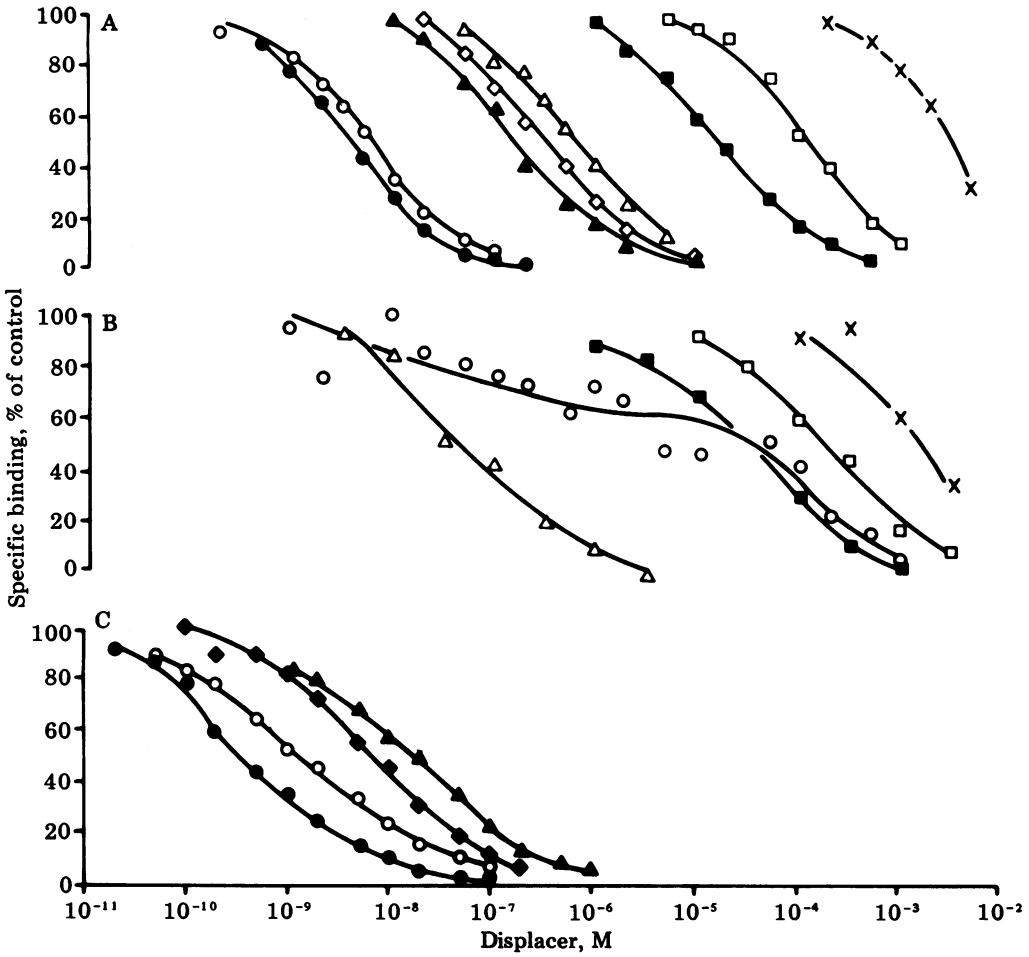

FIG. 3. Displacement curves for nucleosides and methylxanthines. (*A*) [^{3}H]CHA in guinea pig brain. (*B*) [^{3}H]DPX in guinea pig brain. (*C*) [^{3}H]DPX in bovine brain. ●, L-PIA; ○, CHA; ▲, D-PIA; ◇, 5′-deoxy-5′-methylthioadenosine; △, DPX; ■, theophylline; □, caffeine; X, isocaffeine; ◆, CHA + 100 μM GMP-PNP. n = 12 for control binding, 6 for nonspecific binding, and 3 for binding in the presence of displacers. For the unlabeled CHA displacement curve (*B*), samples were incubated for 2 hr at 25°C and then for 30 min at 0°C; n = 6 for CHA in this experiment.

The structure–activity profile for displacement of [^{3}H]DPX indicated that binding was to the same A_1 receptors that bind [^{3}H]CHA. Unlabeled DPX displaced [^{3}H]CHA with an IC_{50} of 4 nM. CHA displaced 1 nM [^{3}H]DPX with an IC_{50} of 1.3 nM (Fig. 3) and a Hill coefficient of 0.7, suggesting some heterogeneity of binding sites. L-PIA was 50-fold more potent than D-PIA. As expected for an antagonist, [^{3}H]DPX binding was not inhibited by GMP-PNP; indeed, binding of 1 nM [^{3}H]DPX was increased about 8% by GMP-PNP. The displacement curve for unlabeled CHA versus [^{3}H]DPX binding was shifted 5-fold to the right by GMP-PNP (Fig. 3) as was the saturation curve for specific binding of [^{3}H]CHA (Fig. 2).

Properties of [^{3}H]DPX Binding in Guinea Pig Brain. [^{3}H]DPX binding in guinea pig brain membranes differed markedly from binding in bovine forebrain. Specific binding dissociated within about 5 sec at 0°C, necessitating an abbreviated wash procedure. Centrifugation could not be used to separate bound from free ligand because nonspecific binding was very high when this technique was used. Nonspecific binding at 0°C in the presence of 1 mM theophylline at 5 nM [^{3}H]DPX was about 50% of total binding of about 1800 cpm. Specific binding was almost undetectable at 25°C. Maximal displacement by 1 mM 2-chloroadenosine, 300 μM 5′-deoxy-5′-iodoadenosine, and 3 μM DPX was about the same as with 1 mM theophylline. [^{3}H]DPX binding was linear with tissue concentration up to 20 mg/ml.

Specific [^{3}H]DPX binding to guinea pig membranes was saturable, but nonspecific binding increased linearly with [^{3}H]DPX concentration (Fig. 2). Scatchard analysis indicated a K_d value of 70 nM and a B_{max} value of 50 pmol/g.

Structural Specificity of [^{3}H]DPX Binding Sites in Guinea Pig. Displacement curves for xanthines were monophasic and had Hill coefficients near 1. Potencies of xanthines in displacing [^{3}H]DPX correlated well ($r \geq 0.9$) with their potencies at A_2 receptors (adenosine antagonism in human fibroblasts). By contrast, adenosine analogs displayed extremely shallow and multiphasic displacement curves at [^{3}H]DPX sites. For instance, CHA displaced about 40% of specific [^{3}H]DPX binding at nanomolar concentrations and the remaining 60% only at high micromolar concentrations of CHA (Fig. 3). This may indicate that 40% of specific binding is to A_1 receptors and the remaining 60% is to A_2 receptors. In agreement with this interpretation, the nucleosides having displacement at nanomolar levels (CHA, L-PIA, 2-chloroadenosine, and adenosine-5′-cyclopropylcarboxamide) had affinities at nanomolar levels at [^{3}H]CHA sites. In addition, the displacement at the nanomolar level was shifted to the right by GMP-PNP. However, the structure–activity profile of the displacement at micromolar level (presumptive A_2 receptors) was at variance with the known structure–activity relationships for nucleosides in causing increases in cyclic AMP via A_2 receptors in guinea pig cortical slices and human fibroblasts. To provide a better indication of affinity at the micromolar level sites, 60% inhibition concentration (IC_{60}, rather than IC_{50}) values for displacement of [^{3}H]DPX are given in Table

1. Compounds that are neither agonists nor antagonists in fibroblasts (6) and brain slices (3), such as 8-bromoadenosine, displaced [^{3}H]DPX, but the potent agonist adenosine-5′-cyclopropylcarboxamide was a weak displacer of [^{3}H]DPX. The A_1 agonist 2-(*p*-methoxyphenyl)adenosine (13), inactive in human fibroblasts, displaced [^{3}H]DPX binding potently, up to a maximum of 120%—i.e., it was able to displace a portion of nonspecific binding. GMP-PNP had no effect on the micromolar phase of nucleoside displacement curves.

Possible Influence of Endogenous Adenosine on Ligand Binding to Adenosine Receptors. In initial experiments, satisfactory binding of [^{3}H]CHA to guinea pig brain membranes could be obtained only in the presence of adenosine deaminase. Inosine, the product of this reaction, had no effect on binding of [^{3}H]CHA (Table 1). The very potent and irreversible adenosine deaminase inhibitor deoxycoformycin reduced [^{3}H]CHA binding 75% in guinea pig membranes. This suggests that, even from washed membranes, endogenous adenosine is generated in sufficient quantity to inhibit [^{3}H]CHA binding when the exogenous adenosine deaminase is inhibited by deoxycoformycin. This conclusion is supported by the finding that inhibition by deoxycoformycin was maximal at 10 nM, consonant with its potency as an inhibitor of adenosine deaminase (14), and remained constant at 75% up to 10 μM. In addition, the effect of deoxycoformycin was absent at very low tissue concentrations, which would be expected to generate less endogenous adenosine. Moreover, the adenosine deaminase inhibitor *erythro*-9-[3-(2-hydroxynonyl)]adenine (EHNA) had the same effects as deoxycoformycin. Repeated washing of the membranes, which removes adenosine deaminase, reduced [^{3}H]CHA binding, which could be restored by adding back adenosine deaminase. It is clear that [^{3}H]CHA and [^{3}H]DPX did not bind to adenosine deaminase, because many compounds that displace binding were neither substrates nor inhibitors of adenosine deaminase. Because of the apparent generation of endogenous adenosine, we could not determine the potency of adenosine itself at [^{3}H]CHA or [^{3}H]DPX sites.

DISCUSSION

The major finding of the present study is that the A_1 subtype of adenosine receptors can be labeled in bovine brain membranes with [^{3}H]CHA and [^{3}H]DPX and that [^{3}H]DPX may label A_2 sites in guinea pig brain. The failure of earlier attempts to label physiologically relevant adenosine receptors with [^{3}H]adenosine (15–17) may have been due to competition from endogenous adenosine slowly released from washed membranes and to the existence of large numbers of nonreceptor binding sites for [^{3}H]adenosine.

Conceivably, further subdivisions of adenosine receptors may exist. Thus, we found high- and low-affinity [^{3}H]CHA binding sites whose possible differential substrate specificity has not yet been adequately explored.

Interpretation of the [^{3}H]DPX binding data in guinea pigs is difficult. Although about 40% of specific binding is to presumptive A_1 receptors, it is difficult to characterize this component in detail due to high nonspecific binding and the resultant data scatter. The remaining 60% of [^{3}H]DPX binding resembles A_2 receptors in drug specificity, but several discrepancies suggest that this may be a different site or that there exist A_2 subtypes.

Adenosine decreases adenylate cyclase activity in some systems through intracellular sites designated P sites, whose drug specificity differs from A_1 and A_2 receptors (18). Neither [^{3}H]CHA nor [^{3}H]DPX appear to label P sites; binding of these ligands is displaced by methylxanthines and by N^6-modified nucleosides (Table 1), which are inactive at P sites (18).

The close correlation between methylxanthine affinities for A_1 and A_2 receptors indicates that these substances do not markedly differentiate the two receptor subtypes. Methylxanthines may exert their central stimulant effects by blocking adenosine receptors; their relative potencies in blocking adenosine effects parallel behavioral stimulant actions and they are substantially more potent in inhibiting effects of adenosine than in eliciting other biochemical actions (such as inhibition of phosphodiesterase) (19). It is unclear whether such actions of methylxanthines involve A_1 or A_2 receptors.

The adenosine analog 5′-deoxy-5′-methylthioadenosine is an antagonist of adenosine activation of adenylate cyclase in fibroblasts (6). This endogenous product of polyamine synthesis has substantial affinity for both [^{3}H]CHA and [^{3}H]DPX sites, suggesting that it may be an endogenous modulator at adenosine receptors.

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