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Heterogeneity of Adenosine A_1 Receptor Binding in Brain Tissue

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SUMMARY

A possible heterogeneity of adenosine A_1 receptors in brain was evaluated by comparing drug specificity, regional distribution, and temperature influences on ^{3}H-labeled ligand binding in several mammalian species. Adenosine receptors were labeled with the agonists [^{3}H]N^6-cyclohexyladenosine ([^{3}H]CHA) and [^{3}H]N^6-phenylisopropyladenosine ([^{3}H]PIA) or the antagonist [^{3}H]1,3-diethyl-8-phenylxanthine ([^{3}H]DPX). The binding properties of adenosine analogues are similar in the species studied. In all cases, the N^6-substituted adenosine analogues have nanomolar affinity for [^{3}H]CHA binding sites with a marked stereoselectivity for the isomers of PIA. The xanthine derivative DPX manifests considerable differences in affinity for [^{3}H]CHA-labeled A_1 receptors of different species. DPX shows highest affinity for A_1 receptors in calf brain and lowest affinity for A_1 receptors in guinea pig and human brain. [^{3}H]DPX labels adenosine A_1 receptors in calf, rat, and rabbit cerebral cortical brain membranes. By contrast, very little specific [^{3}H]DPX binding is detected in human and guinea pig brain, and the limited level of binding is inhibited poorly by PIA or xanthines. The failure of [^{3}H]DPX to label adenosine A_1 receptors in human and guinea pig cortex may be related to the very low affinity of DPX for A_1 receptors in these species as labeled by [^{3}H]CHA. Temperature variations affect agonist and antagonist binding differentially. Adenosine derivatives have higher affinities for A_1 receptors at higher temperatures, whereas xanthine analogues have higher affinities at lower temperatures. Similar temperature effects on A_1 receptor binding occur in all species examined. Temperature effects can account for the differences in drug affinities at [^{3}H]agonist- and [^{3}H]antagonist-labeled sites. Temperature effects cannot account for the failure of [^{3}H]DPX to label A_1 receptors in guinea pig and human brain. Our thermodynamic analysis of temperature influences on A_1 receptor binding indicates that agonist binding is entropy-driven.

INTRODUCTION

Adenine nucleosides and nucleotides regulate numerous physiological processes via purinergic receptors (1). Adenosine appears to play a neuromodulatory role in brain tissue. It displays prominent neurophysiological actions (2) and influences adenylate cyclase activity via at least two types of membrane associated receptors. At A_1 adenosine receptors, adenosine inhibits adenylate cyclase activity (3, 4), whereas at A_2 adenosine receptors, adenosine stimulates adenylate cyclase activity (5, 6). Methylxanthines such as caffeine and theophylline block the actions of adenosine at A_1 and A_2 adenosine receptors.

Recently it has been possible to label adenosine receptors in brain (7, 8), fat cell membranes (9), and testes (10) with [^{3}H]adenosine analogues such as [^{3}H]CHA[3] (7, 10), [^{3}H]PIA (6), [^{3}H]-2-chloroadenosine (8), or a xanthine derivative, [^{3}H]DPX (7). Earlier studies of receptor binding of [^{3}H]CHA and [^{3}H]DPX in brain membranes (7) showed some evidence for heterogeneity of binding. In calf brain membranes, [^{3}H]DPX labeled adenosine A_1 receptors, whereas the structure-activity properties of [^{3}H]DPX-binding sites in the guinea pig were markedly different, bearing some resemblance to A_2 receptors. Although [^{3}H]CHA clearly labeled A_1 receptors in both calf and guinea pig brain, CHA and PIA displayed substantially lower affinities in guinea pig membranes than in calf membranes (7).

In our previous study (7), temperature variations af-

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[3] The abbreviations used are: CHA, N^6-cyclohexyladenosine; PIA, N^6-phenylisopropyladenosine; DPX, 1,3-diethyl-8-phenylxanthine; TRAC, tracazolate [ethyl-4-(butylamino)-1-ethyl-6-methyl-1*H*-pyrazolo[3,4-6]-pyridine-5-carboxylate]; IBMX, 3-isobutyl-1-methylxanthine.

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fected [^{3}H]CHA and [^{3}H]DPX binding differently, such that ligand binding was assessed at different temperatures in guinea pig and calf. We wondered whether differences in [^{3}H]CHA and [^{3}H]DPX binding properties in guinea pig and calf membranes might derive from variations in experimental condition.

Behavioral studies suggest a heterogeneity of A_1 receptors (11, 12). Some anticonvulsant properties of L-PIA in mice and rats are not blocked by theophylline and are not shared by CHA and 2-chloroadenosine.

To explore a possible heterogeneity of adenosine receptors, as well as to determine the effect that temperature might have on this heterogeneity, we have evaluated the drug specificity and temperature dependence of [^{3}H]CHA, L-[^{3}H]PIA, and [^{3}H]DPX binding in several species and in different brain regions. Although adenosine derivatives display little species variation in affinity for A_1 receptors, we report considerable species differences in the binding of the xanthine derivative DPX to A_1 adenosine receptors. We also have found evidence for thermodynamic differences between agonist and antagonist binding to adenosine receptors.

EXPERIMENTAL PROCEDURES

Materials. [*adenine*-2,8-^{3}H]CHA (11.5 Ci/mmole), 1,3-[*phenyl*-4-^{3}H]DPX (13.4 Ci/mmole), and L-[*adenine*-2,8-^{3}H]PIA (14.6 Ci/mmole) were obtained from New England Nuclear Corporation (Boston, Mass.); L-PIA and CHA were obtained from Boehringer Mannheim; D-PIA and DPX were gifts of Dr. James Briston, Warner-Lambert Company (Ann Arbor, Mich.). TRAC (ICI 136,753) was the gift of Dr. David H. McCurdy, ICI Americas Inc. IBMX was obtained from Sigma Chemical Company (St. Louis, Mo.). Unless otherwise stated, other materials were from standard sources. Solutions of xanthines and nucleosides were prepared and stored as described previously (7). Male Sprague-Dawley rats, male New Zealand White rabbits, and male Hartley guinea pigs were obtained from Charles River Laboratories (Wilmington, Mass.). Fresh calf brains were obtained from a local slaughterhouse within 30 min of the time of slaughter. Human cerebral cortex was obtained from Johns Hopkins Hospital autopsy specimens for which no brain abnormalities were reported and was kept on ice from the time of removal at autopsy until the time of preparation for receptor assays less than 6 hr later.

Methods. Brains were dissected into specific regions and the regions were homogenized in 10 volumes of 50 mM Tris-HCl buffer (pH 7.7) with a Brinkmann Polytron. The homogenates were centrifuged at 48,000 × *g* for 20 min at 4°, and the supernatants were discarded. The resulting pellets were washed by resuspension in 10 volumes of Tris buffer and homogenization and recentrifugation at 48,000 × *g* for 20 min. The membranes were washed a total of three times in this manner. Membranes were subsequently resuspended in 10 volumes of Tris buffer and incubated with adenosine deaminase (0.2 IU/ml) (Sigma, Type I) at 25° for 30 min; the homogenate was either frozen immediately and stored at −70° for at most 2 months or used immediately in receptor assays. No differences in binding characteristics were found between stored and fresh tissue.

For adenosine receptor assays, the fresh or thawed tissue homogenates were diluted again to 100 volumes of original tissue, and 2 ml of this suspension were incubated with [^{3}H]CHA, [^{3}H]DPX, or L-[^{3}H]PIA as indicated in figure and table legends. Nonspecific binding was normally determined in assays with 10 μM L-PIA. Incubations were terminated by vacuum filtration through Whatman GF/B glass-fiber filters, with three 5-ml buffer washes. Filter-bound ^{3}H activity was measured by scintillation spectrometry in 10 ml of Formula 947 (New England Nuclear Corporation).

RESULTS

Properties of [^{3}H]CHA binding. As previously observed, [^{3}H]CHA binds saturably and with high affinity to membranes of several mammalian species (Fig. 1; Table 1). Scatchard analysis indicates a single major component of binding in each species examined. However, affinities vary between species. For example, at 25°, [^{3}H]CHA has 8 times higher affinity for binding sites in calf cerebral cortex than in human cerebral cortex.

The drug specificity of [^{3}H]CHA binding to cerebral cortical membranes was determined in each species. Assays were carried out with concentrations between 1 and 2 nM [^{3}H]CHA, with nonspecific binding defined in the presence of 10 μM L-PIA. Respective total and nonspecific values for [^{3}H]CHA binding are approximately 4800 cpm and 200 cpm in calf, 2500 cpm and 150 cpm in rat, 3000 cpm and 200 cpm in rabbit, 1400 cpm and 150 cpm in guinea pig, and 1700 cpm and 300 cpm in human.

In all five species examined, the drug-inhibition pattern of [^{3}H]CHA binding reflects labeling of adenosine A_1 receptors. Thus, in each species, a marked stereoselectivity for the isomers of PIA is apparent at [^{3}H]CHA binding sites. In all cases, L-PIA has at least 10-fold higher affinity for [^{3}H]CHA binding sites than its isomer D-PIA, consistent with the pattern of drug potencies at A_1 adenosine receptors (7). In addition, the absolute affinities of L-PIA and CHA are in the nanomolar range, consonant with the potencies these N^6-substituted adenosine analogues have for inhibiting adenylate cyclase via A_1 adenosine receptors in fat cells (4) or brain membranes (3).

A detailed comparison of the drug specificity of [^{3}H]CHA binding sites in various regions of calf and rat brain as well as rabbit, guinea pig, and human cerebral cortex is shown in Table 1. There does not appear to be any major difference in the drug specificity of the [^{3}H]CHA binding sites between the various brain regions within the same species. For example, in calf brain, each brain region examined displays the same rank order potency of drugs and similar degrees of stereospecificity for PIA isomers at [^{3}H]CHA binding sites. In rat brain as well, the various regions studied show essentially identical drug specificity of [^{3}H]CHA binding sites.

However, there are striking species differences. The greatest variation in affinity for [^{3}H]CHA binding sites between the various species examined occurs for DPX. DPX has a 2 nM K_i for A_1 adenosine receptors in calf brain, but shows 200–300 times lesser affinity at A_1 receptors in guinea pig and human cerebral cortex.

The large difference in DPX affinity for [^{3}H]CHA-labeled A_1 adenosine receptors found between species

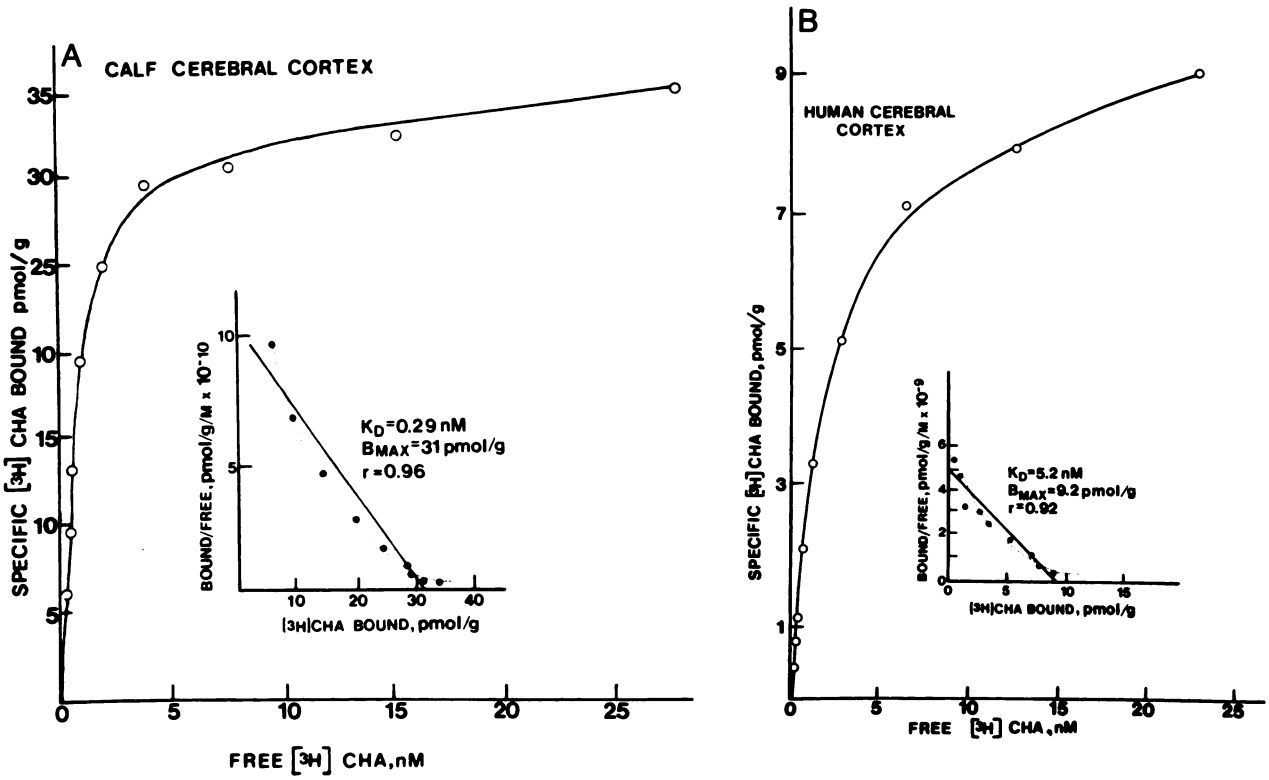

FIG. 1. *Saturation of [^{3}H]CHA binding in calf (A) and human (B) cerebral cortex*

Specific [^{3}H]CHA (picomoles) bound per gram of tissue (wet tissue weight) is plotted against free [^{3}H]CHA concentration. Total and nonspecific binding values were determined by triplicate incubations in the absence and presence of 10 μM L-PIA, respectively. Specific binding is defined as the difference between the mean total and mean nonspecific binding. From Scatchard plots of these binding isotherms (*inset*), the inferred dissociation constant (K_D) and maximal binding capacity (B_{max}) of a single component model fit by linear regression are shown. The correlation coefficients (r) of the best-fit line to the data indicate the presence of only one major component of binding. Each experiment was replicated twice.

contrasts with the relatively small species differences in affinity of L-PIA and CHA. For example, there is only a 6-fold difference in the affinity of L-PIA for A_1 receptors of calf and guinea pig, and only a 4-fold difference for CHA. IBMX and TRAC show less species variation than DPX in their affinities for A_1 receptors.

Drug specificity of [^{3}H]DPX binding. Earlier studies evaluated [^{3}H]DPX binding to brain membranes of only two species, calf and guinea pig (7). In bovine brain, [^{3}H]DPX binding displayed the characteristics of adenosine A_1 receptors. By contrast, in guinea pig brain, lower levels of specific binding were detected. Adenosine derivatives

TABLE 1

Drug specificity of [^{3}H]CHA binding in mammalian brain

IC_{50} values were determined using 1–2 nM [^{3}H]CHA and six to eight concentrations of each inhibitor assayed in triplicate for 90 min at 25° with 20 mg of tissue (wet weight) in 2 ml of 50 mM Tris-HCl buffer (pH 7.7). K_i values were calculated as described (13, 14), using the relationship $K_i = IC_{50}/(1 + L/K_D)$, where L is the [^{3}H]CHA concentration in the assay and K_D is the equilibrium dissociation constant determined by Scatchard analysis of a saturation experiment performed in parallel with the inhibition study (see Table 5). Data are presented as means ± standard error of the mean of three or four determinations or the mean of two or three values which varied less than 25%. Nonspecific binding was determined in the presence of 10 μM L-PIA.

Region	K_i, 25° (nM)					
	L-PIA	D-PIA	CHA	DPX	TRAC	IBMX
Calf cortex	0.24 ± 0.03	4.37 ± 0.29	0.68 ± 0.07	1.6 ± 0.52	215 ± 23.5	3937 ± 425
Calf cerebellum	0.35 ± 0.03	6.26 ± 0.23	0.93 ± 0.04	1.9 ± 0.25	185 ± 15	3925 ± 176
Calf hippocampus	0.27 ± 0.01	3.85 ± 0.38	0.57 ± 0.08	2.37 ± 0.13	217 ± 7.5	4875 ± 1262
Calf striatum	0.25 ± 0.02	4.5 ± 1.01	0.60 ± 0.05	2.05 ± 0.55	198 ± 23	3750 ± 50
Rat cortex	0.65 ± 0.08	40.5 ± 11.5	0.79 ± 0.03	38.3 ± 7.27	308 ± 30.1	2450 ± 511
Rat cerebellum	0.64 ± 0.05	35.0 ± 2.8	0.80 ± 0.15	31.5 ± 3.53	387 ± 16.6	1647 ± 1164
Rat hippocampus	0.70 ± 0.05	34.0 ± 3.0	0.83 ± 0.18	25.7 ± 0.36	265 ± 25	2450 ± 353
Rat striatum	0.60 ± 0.05	32.0 ± 5.6	0.83 ± 0.08	28.7 ± 1.30	337 ± 88	3500 ± 1750
Rabbit cortex	0.28	37	1.2	82	2900	3100
Guinea pig cortex	1.4	46	2.7	550	990	1100
Human cortex	1.1	38	3.3	450	5200	960

TABLE 2

Comparison of the drug specificity of [^{3}H]CHA, L-[^{3}H]PIA, and [^{3}H]DPX binding in calf cerebral cortical membranes

IC_{50} values were determined using 1–2 nM [^{3}H]CHA and L-[^{3}H]PIA or 2–3 nM [^{3}H]DPX and six to eight concentrations of each inhibitor assayed in triplicate for 90 min at 25° with 20 mg or tissue (wet weight) in 2 ml of 50 mM Tris-HCl buffer (pH 7.7). K_i values were calculated as described in Table 1. Results shown are means ± standard error of the mean of four experiments. Nonspecific binding was determined in the presence of 10 μM L-PIA.

Drug	K_i, 25° (nM)		
	[^{3}H]CHA	L-[^{3}H]PIA	[^{3}H]DPX
L-PIA	0.24 ± 0.03	0.23 ± 0.03	0.21 ± 0.05
D-PIA	4.37 ± 0.29	5.60 ± 0.44	8.10 ± 0.85
CHA	0.68 ± 0.07	0.69 ± 0.09	0.81 ± 0.07
DPX	1.60 ± 0.52	3.13 ± 0.58	1.40 ± 0.60
TRAC	215 ± 23	174 ± 16	179 ± 16
IBMX	3837 ± 425	4000 ± 305	3499 ± 1946

were up to 1000 times weaker in guinea pig brain than in calf brain and had a drug specificity with some resemblances to adenosine A_2 receptors but with major differences. To clarify these discrepancies, we evaluated properties of [^{3}H]DPX binding at 0° to brain membranes of five species (Tables 2 and 3). The drug specificity of [^{3}H]DPX binding was determined by drug inhibition experiments using between 2 and 3 nM [^{3}H]DPX at 0° with nonspecific binding determined in the presence of 10 μM L-PIA. Respective total and nonspecific values for [^{3}H]DPX binding are approximately 6900 cpm and 2200 cpm in calf, 3200 cpm and 900 cpm in rat, 3000 cpm and 1000 cpm in rabbit, 2000 cpm and 1700 cpm in guinea pig, and 2300 cpm and 2100 cpm in human.

Although the absolute affinities of the adenosine analogues are somewhat lower at [^{3}H]DPX sites labeled at 0°, the over-all drug specificity of [^{3}H]DPX sites in rat, rabbit, and calf brain appears characteristic of A_1 receptors. Thus, in these three species, adenosine derivatives display nanomolar potencies with stereoselectivity for PIA isomers. The extent of stereoselectivity for PIA isomers varies among the species, with only an 18-fold difference in calf brain but a 140-fold difference in rabbit brain. The extent of PIA isomer stereoselectivity in rat, rabbit, and calf brain for [^{3}H]DPX binding parallels such differences observed with [^{3}H]CHA (Tables 1 and 2).

TABLE 3

Drug specificity of [^{3}H]DPX binding in mammalian cerebral cortex

IC_{50} values were determined using 2–3 nM [^{3}H]DPX and six to eight concentrations of each inhibitor assayed in triplicate for 90 min at 0° with 20 mg tissue (wet weight) in 2 ml of 50 mM Tris-HCl buffer (pH 7.7). K_D values for [^{3}H]DPX, determined in parallel saturation experiments, were used to calculate K_i values for each inhibitor as described in Table 1. Results shown are means of two experiments which differed less than 25%. Nonspecific binding was determined in the presence of 10 μM L-PIA.

Species	K_i, 0° (nM)					
	L-PIA	CHA	D-PIA	DPX	TRAC	IBMX
Calf	1.3	3.5	23	0.65	71	1400
Rat	26	30	2220	3.6	40	750
Rabbit	33	197	4800	5.1	61	460

The drug specificities of A_1 adenosine receptors in calf cortex labeled either with [^{3}H]CHA, L-[^{3}H]PIA, or [^{3}H]DPX, all at 25°, are shown in Table 2. This analysis was carried out for two reasons. It demonstrates that [^{3}H]DPX-labeled sites display molar potencies for drugs nearly identical with those for [^{3}H]CHA-labeled sites, when the incubation temperature is the same for each [^{3}H]ligand. This demonstration is useful since, for [^{3}H]DPX sites labeled at 0°, some lowering of the potencies for adenosine analogues is observed. Also, the sites labeled with L-[^{3}H]PIA appear to have the same potencies for the drugs examined as the [^{3}H]CHA- and [^{3}H]DPX-labeled sites. It appears that these three ligands label the same types of adenosine receptors in calf cortex.

In contrast to the straightforward labeling of A_1 receptors by [^{3}H]DPX in rat, calf, and rabbit, it is difficult to obtain meaningful binding of [^{3}H]DPX to human and guinea pig cerebral cortex membranes (Table 4). Even with 10 μM PIA we do not detect substantial reduction of total [^{3}H]DPX binding in human and guinea pig brain membranes. At 250 nM DPX or 2 μM IBMX, only 25–33% reduction of total [^{3}H]DPX binding is observed in these two species, suggesting that [^{3}H]DPX is a poor ligand for binding studies in these two species.

Temperature influences on adenosine receptor binding. In initial studies of adenosine receptor binding we found that [^{3}H]CHA binding was enhanced at higher temperatures, whereas [^{3}H]DPX binding was favored by lower temperatures (7). The [^{3}H]DPX binding studies in guinea pig brain membrane were conducted at 0°, since at higher temperatures no specific binding could be detected (7). Since adenosine derivatives display reduced receptor affinity at lower temperatures, their poor displacement of [^{3}H]DPX at 0° in human and guinea pig brain might derive simply from temperature effects. According to this notion, [^{3}H]DPX would label A_1 receptors in guinea pig and human brain, as it does in other species, but the adenosine derivatives would display a more pronounced reduction in affinity at lower temperatures in guinea pig and human than in rat, calf, and rabbit. To explore this possibility, we evaluated the influence of temperature upon [^{3}H]CHA and [^{3}H]PIA binding in various species (Table 5; Fig. 2). In all species examined, [^{3}H]CHA and L-[^{3}H]PIA display lower affinity at 0° than at 25°, and the extent of reduced affinity is similar for all

TABLE 4

Drug effects on [^{3}H]DPX binding in human and guinea pig cerebral cortex

Assays were performed in triplicate at 0° for 90 min. The assay mixture contained 2.5 nM [^{3}H]DPX, the indicated concentrations of inhibitor, and 20 mg of tissue (wet weight) in a final volume of 2 ml of 50 mM Tris-HCl (pH 7.7). Each assay tube contained 50,000 cpm of [^{3}H]DPX, and typically between 2000 and 2400 cpm bound to tissue. Only 200–300 cpm bound to the filter itself. In contrast, total binding to 20 mg of bovine cortex was 6500–6900 cpm with 1800–2200 cpm bound in the presence of 10 μM L-PIA. Results are means of triplicate determinations from a single experiment.

	% Inhibition of total [^{3}H]DPX binding		
	L-PIA (10 μM)	DPX (250 nM)	IBMX (2 μM)
Human	18%	25%	33%
Guinea pig	5%	26%	4%

TABLE 5

Temperature influences on agonist affinities at A_1 adenosine receptors in cerebral cortex

K_D values were determined from Scatchard analysis of saturation isotherms using nine concentrations of [^{3}H]CHA or L-[^{3}H]PIA between 0.05 nM and 25 nM assayed in triplicate for 90 min at 0° or 25° with 20 mg of tissue (wet weight) in 2 ml of 50 mM Tris-HCl buffer (pH 7.7). Nonspecific binding was determined in the presence of 10 μM L-PIA. Results are means of two experiments which differed less than 25%.

Species	K_D (nM)			
	[^{3}H]CHA		L-[^{3}H]PIA	
	0°	25°	0°	25°
Calf	3.3	0.29	1.1	0.24
Rat	9.0	0.7	3.4	0.54
Rabbit	5.4	0.9	4.7	0.59
Guinea pig	22	1.8	8.2	1.1
Human	14	2.3	4.8	1.0

species evaluated. This suggests that the very weak displacement by adenosine derivatives of [^{3}H]DPX binding at 0° in guinea pig and human brain cannot be explained simply by greater temperature influences in these species.

Thermodynamic analysis of the temperature-dependent affinities of agonist and antagonist have revealed fundamental differences in the interaction of agonists and antagonists with receptors (15, 16). The temperature dependence of agonist and antagonist interactions varies at several neurotransmitters. At dopamine (15) and *beta*-adrenergic receptors (16), agonists become more potent at lower temperatures. Our findings suggest that at adenosine receptors agonists are more potent at higher temperatures and antagonists more potent at lower temperatures. To explore this phenomenon in greater detail, we evaluated the potencies of a series of adenosine agonists and antagonists in inhibiting [^{3}H]CHA and [^{3}H]DPX binding at 0°, 10°, 20°, 25°, and 30° (Fig. 3; Table 5). At both [^{3}H]CHA and [^{3}H]DPX binding sites the adenosine derivatives are more potent at higher temperatures whereas the xanthines and related agents are more potent at lower temperatures. Van't Hoff plots are linear within this temperature range (Fig. 3), indicating the absence of major phase transitions of the membranes influencing receptor affinities and permitting simple calculations of the enthalpy and entropy of the over-all binding reaction (Table 6).

The adenosine derivatives each display an unfavorable positive enthalpy of binding, whereas all of the antagonists display a favorable negative enthalpy. The entropy values for both the adenosine derivatives and the xanthines are positive but are substantially greater for the adenosine derivatives.

Regional distribution of [^{3}H]CHA and [^{3}H]DPX binding. If there are differences in adenosine receptors in the various species, one might anticipate variations in regional distribution as well as drug specificity. Likewise, if both [^{3}H]CHA and [^{3}H]DPX label A_1 adenosine receptors, then the regional variation in the levels of [^{3}H]CHA binding should correspond to the regional variations in [^{3}H]DPX binding. Accordingly, we compared the regional variation in levels of [^{3}H]CHA and [^{3}H]DPX binding in calf, rat, and rabbit brains (Fig. 4).

The regional distribution of [^{3}H]CHA and [^{3}H]DPX binding are similar within a given species. Regions with relatively high levels of [^{3}H]CHA binding, such as the hippocampus, generally also have relatively high levels of [^{3}H]DPX binding. The only possible discrepancy occurs in rabbit thalamus-hypothalamus, where [^{3}H]DPX

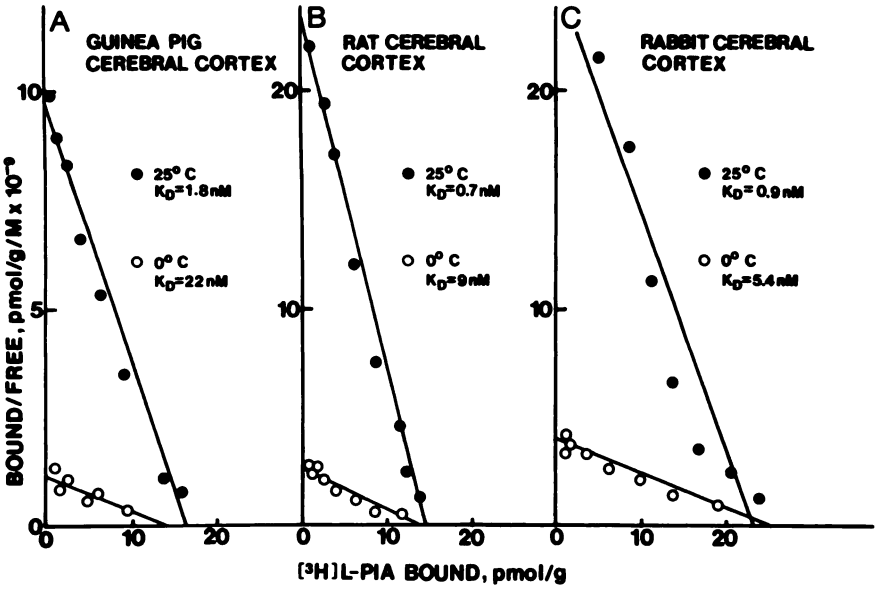

FIG. 2. *Temperature influences on the saturation of L-[^{3}H]PIA binding in guinea pig (A), rat (B), and rabbit (C) cerebral cortex*

Specific L-[^{3}H]PIA binding was defined as the difference between total and nonspecific L-[^{3}H]PIA binding determined in triplicate incubations of various L-[^{3}H]PIA concentrations with 20 mg of tissue in the absence and presence of 10 μML-PIA, respectively. Assays were conducted at 25° or 0° for 2 hr. The ratio of bound L-[^{3}H]PIA [picomoles of L-[^{3}H]PIA bound per gram of tissue (wet weight)] to free L-[^{3}H]PIA (molar) is plotted against the bound L-[^{3}H]PIA (picomoles per gram). Scatchard transforms were fit by linear regression to a single component model to determine the apparent dissociation constant (K_D) at each temperature. The maximal binding capacity (B_{max}) was not affected by temperature.

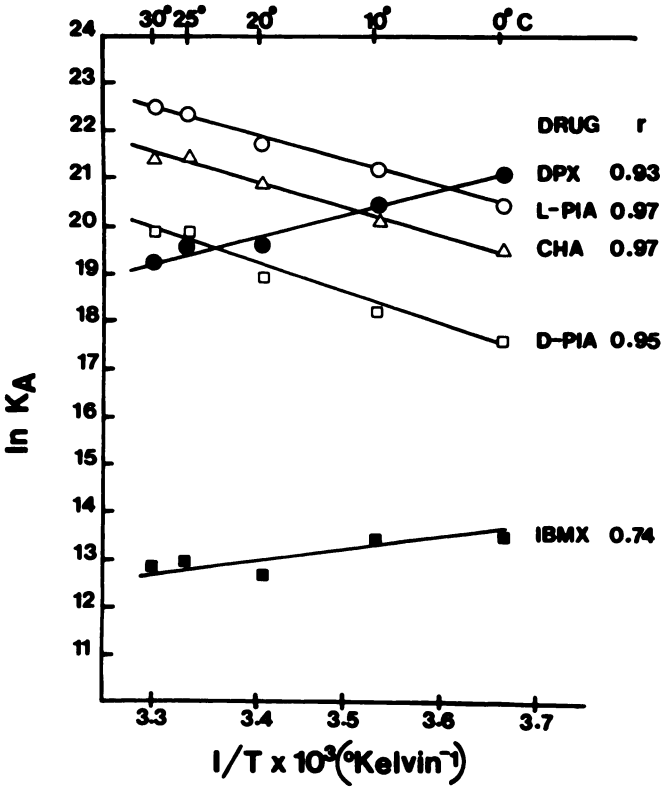

FIG. 3. *Van't Hoff plots of the influence of temperature on the association constants (K_A) of agonists and antagonists to the adenosine A_1 receptor in bovine cortex*

K_A values determined at various temperatures are plotted against $1/T$ (°Kelvin^{-1}) for three agonists and two antagonists. K_A values were calculated from inhibition constants ($K_A = 1/K_i$) determined from inhibition studies at the five temperatures indicated. IC_{50} values for each inhibitor were determined from Hill plots of the inhibition of [^{3}H]CHA binding at 30°, 25°, and 20°, and from the inhibition of [^{3}H]DPX binding at 10° and 0°. Scatchard plots of saturation isotherms of [^{3}H]CHA at 30°, 25°, and 20° and of [^{3}H]DPX at 10° and 0°, performed in parallel with the inhibition studies, provided direct determination of the K_D of [^{3}H]CHA and [^{3}H]DPX at each temperature. K_i values for each inhibitor at each temperature were calculated from the IC_{50} value as described (17, 18), using the formula $K_i = IC_{50}/(1 + L/K_D)$, where L is the concentration of either [^{3}H]CHA (1.6 nM) or [^{3}H]DPX (2.5 nM) and K_D is the dissociation constant for the tritiated label. The plots of K_A against $1/T$ were fit by linear regression. The correlation coefficients of each line are shown. The slope of the best-fit line is equal to $-\Delta H°/R$. Entropy (ΔS) is calculated from the relationship $\ln K_A = -\Delta H°/RT + \Delta S°/R$.

binding is proportionally greater than [^{3}H]CHA binding. These findings support the notion that in these three species both [^{3}H]CHA and [^{3}H]DPX predominantly label A_1 receptors.

The pattern of regional variations for adenosine receptor binding differs with the various species. The most pronounced regional differences occur in the rabbit. In rabbit, the highest levels of binding occur in the hippocampus and the lowest levels of binding occur in the cerebellum and brain stem, representing only about 20–25% of hippocampal levels. By contrast, the cerebellum displays the highest levels of binding in rat for both [^{3}H]CHA and [^{3}H]DPX, whereas the cerebellum has intermediate levels in bovine brain. In all three species, the hippocampus displays the highest, or nearly the highest, levels of A_1 receptors labeled by [^{3}H]CHA or [^{3}H]DPX and the cerebral cortex displays 70–80% of hippocampal levels.

TABLE 6

Agonists and antagonists display differences in thermodynamic parameters of binding to A_1 adenosine receptors in bovine brain

Assays were carried out as described in Fig. 3. The changes in free energy ($\Delta G°$), enthalpy ($\Delta H°$), and entropy ($\Delta S°$) of binding at 25° were calculated from the slopes and intercepts of the lines determined in Fig. 3, as described by Weiland *et al.* (16).

	$\Delta G°$ (Kcal/mole)	$\Delta H°$ (Kcal/mole)	$\Delta S°$ (entropy units)
Agonists			
L-PIA	−13.0	+10.5	+79
D-PIA	−11.7	+11.7	+83
CHA	−12.5	+13.2	+82
Antagonists			
DPX	−11.5	−10.5	+ 3.4
IBMX	− 7.6	− 4.5	+10.5

DISCUSSION

Earlier reports suggested heterogeneity of adenosine receptors labeled with [^{3}H]DPX and [^{3}H]CHA (7). One goal of the present study was the elaboration of this finding through comparison of the properties of [^{3}H]DPX and [^{3}H]CHA in several species and in various brain regions within a species. Our evaluation of a series of adenosine analogues and xanthine derivatives demonstrates the conservation of several properties of A_1 adenosine receptors in all species examined, but highlights some species differences.

In all species, adenosine analogues display nanomolar affinity for [^{3}H]CHA-binding sites with a marked stereoselectivity for the isomers of PIA, characteristic of A_1 receptors. Adenosine A_1 receptors were proposed on the basis of inhibition of adenylate cyclase activity in tissues such as fetal brain cultures (5), adult brain membranes (3), and fat cell membranes (4). Only very limited studies of drug specificity in these systems have been carried out. It appears that, in all systems, the N^6-substituted adenosine analogues are more potent than adenosine. In addition, stereoselectivity between L-PIA and D-PIA is a common feature. Beyond this, however, other studies have not focused on detailed differences in adenosine A_1 receptors from different sources.

Of the drugs examined, DPX shows the most pronounced species differences in competing for [^{3}H]CHA binding sites, 250-fold less affinity in guinea pig and human than in calf brain. Similar species variation in the binding properties occur for [^{3}H]DPX to brain membranes.

In calf, rat, and rabbit, [^{3}H]DPX labels typical A_1 adenosine receptors, with high affinity of adenosine derivatives and stereoselectivity for PIA isomers. On the other hand, very little specific binding of [^{3}H]DPX is detected in guinea pig and human brain membranes. It seems likely that the failure to detect substantial [^{3}H]DPX binding to A_1 receptors in these species is related to the low affinity of DPX for A_1 receptors as labeled by [^{3}H]CHA in guinea pig and human brain.

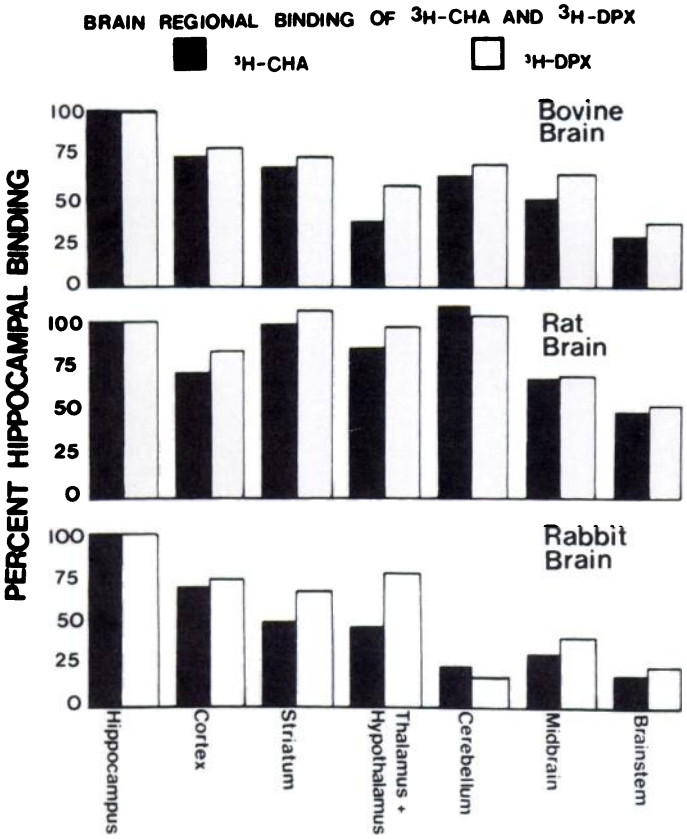

FIG. 4. *Brain regional binding of [^{3}H]CHA and [^{3}H]DPX*

Homogenates of various brain regions pooled from two to five animals each at 10 mg of tissue (wet weight) per milliliter in a total volume of 2 ml of 50 mM Tris-HCl buffer (pH 7.7) were incubated with either [^{3}H]CHA (1 nM) or [^{3}H]DPX (2.5 nM) in parallel in the absence and presence of 10 nM L-PIA to determine total and nonspecific binding, respectively. Specific binding to each region is defined as the difference between total and nonspecific binding and is expressed as a percentage of the specific binding to the hippocampus. Values are the means of two separate experiments, performed in triplicate, which varied less than 10%.

TRAC is a pyrazolopyridine derivative similar in structure to etazolate and cartazolate. These three drugs display some benzodiazepine-like pharmacologic properties and interact with benzodiazepine receptors (17). TRAC displaces [^{3}H]CHA and [^{3}H]DPX binding more potently than does IBMX in all species except human (Tables 1 and 2), whereas cartazolate and etazolate show negligible effects at adenosine receptors at 50 μM (data not shown). CHA, PIA, and DPX fail to affect benzodiazepine receptors labeled with [^{3}H]flunitrazepam at 100 μM concentration. The pyrazolopyridines have structural similarities to xanthines and N^6-adenosine analogues. TRAC was used in our screen of adenosine receptors in different species because of its relatively high potency for A_1 receptor relative to other more commonly known xanthines.

Our analysis of temperature influences on adenosine receptor binding highlights the marked differences between the effects of temperature on agonist and antagonist interactions with adenosine A_1 receptors. Agonist affinities increase at higher temperatures whereas antagonist affinities are enhanced at low temperatures. Van't Hoff analysis indicates that binding of agonists to A_1 receptors is entropy-driven. Each agonist displays a remarkably large and positive enthalpy ($\Delta H°$) of binding in comparison to values reported for agonists at other receptors (16). This large unfavorable contribution to the over-all energy of binding is overcome by an energetically favorable entropic contribution to the energy of binding. Thus, although the over-all free energy of binding ($\Delta G°$) at 25° of L-PIA to calf A_1 adenosine receptors is −13 kcal/mole, the enthalpic contribution to this is a highly unfavorable +10 kcal/mole. However, the large increase in entropy contributes nearly −23 kcal/mole to the over-all binding reaction. Clearly, agonist binding to adenosine receptors is entropy-driven. In contrast, both enthalpy and entropy appear to contribute favorably to the binding of antagonists to adenosine A_1 receptors. Findings very similar to this have been obtained in preliminary experiments on rat cortical A_1 adenosine receptors. Since all of the antagonists evaluated are xanthines, one must be cautious about generalizing these results to other, as yet unidentified, adenosine antagonists.

This pattern of temperature influences on adenosine A_1 receptors differs from that which has been observed with dopamine and *beta*-adrenergic receptors (15, 16). For *beta*-adrenergic receptors, the binding of full agonists results in a favorable negative enthalpy change but in an unfavorable entropy change, exactly opposite to the pattern for adenosine agonists. At *alpha*$_2$-noradrenergic receptors, agonist binding appears to be favored at lower temperatures, similar to the situation for *beta*-receptors and dopamine receptors (18). On the other hand, at opiate receptors, agonists demonstrate higher binding affinities at 25° than at 0° (19), similar to the situation for adenosine receptors.

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