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BINDING OF 2′,5′-DIDEOXYADENOSINE TO BRAIN MEMBRANES

COMPARISON TO P-SITE INHIBITION OF ADENYLATE CYCLASE

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Abstract—Membranes from rat cerebral cortex and striatum contain a relatively large number of high-affinity binding sites for [^{3}H]2′,5′-dideoxyadenosine, [^{3}H]adenine arabinoside, and [^{3}H]adenosine. The binding of [^{3}H]2′,5′-dideoxyadenosine and [^{3}H]adenine arabinoside was virtually unaffected by relatively specific agonists and antagonists for adenosine receptors, such as 2-chloroadenosine, N^6-phenylisopropyladenosine or theophylline. Binding of [^{3}H]adenosine was partially blocked by such receptor ligands. The specific binding of all three ligands was antagonized by a variety of adenosine analogs which inhibit adenylate cyclase by interaction with the so-called P-site associated with this enzyme. However, potencies of adenosine analogs as P-site inhibitors of adenylate cyclase and as antagonists of binding do not correlate well. 5′-Methylthioadenosine had high potency and efficacy versus binding of [^{3}H]2′,5′-dideoxyadenosine but had virtually no effect on activity of adenylate cyclase. 2-Fluoroadenosine was less potent than adenosine as an antagonist of specific binding of [^{3}H]2′,5′-dideoxyadenosine, while 2-fluoroderivatives of adenosine, adenine arabinoside and adenine xylofuranoside were more potent than the parent compounds as P-site inhibitors. The significance of the binding sites for [^{3}H]2′,5′-dideoxyadenosine remains unclear, but their presence complicates the use of [^{3}H]adenosine and certain analogs as ligands for adenosine membrane sites associated with adenylate cyclase.

Physiological functions of cells are regulated by a complex interplay of hormonal and other inputs. An important role for adenosine in such control mechanisms has emerged in recent years for many tissues and cell types [1]. At least two classes of adenosine-sensitive extracellular receptors that interact with adenylate cyclase have been defined: high-affinity A_2-receptors which are inhibitory to adenylate cyclase and low-affinity A_1-receptors which are stimulatory to adenylate cyclase [2–4]. Alkylxanthines such as theophylline are potent antagonists at both classes of adenosine receptors [5, 6]. In addition to extracellular receptors, adenosine interacts with an intracellular site associated with adenylate cyclase, resulting in a profound inhibition of the enzyme activity. Because of its rather strict requirement for the purine moiety of adenosine, this intracellular site has been referred to as the P-site [3, 7–9]. The P-site inhibition of cyclase is not blocked by alkylxanthines.

Research on the role of adenosine as a physiological regulator would benefit greatly from the development of specific ligands for the adenosine-binding sites. A number of laboratories have reported the results of studies on the binding of radioactive adenosine to membranes from fat, heart, and brain cells [10–13]. Relatively high densities of so-called specific binding sites were detected and the profile and/or extent of inhibition of binding by adenosine analogs and by alkylxanthines did not correlate well with known interactions of these compounds with adenosine receptors controlling cyclic AMP generation. Since adenosine obviously interacts with a variety of sites, attempts were made to develop ligands based on adenosine analogs which are relatively selective for specific adenosine sites. [^{3}H]2-Chloroadenosine and [^{3}H]N^6-substituted adenosines have proven to be selective high-affinity ligands for A_1-adenosine receptors [14–17]. Since 2′,5′-dideoxyadenosine and adenine arabinoside are relatively specific agonists for the P-site, the binding of [^{3}H]2′,5′-dideoxyadenosine and [^{3}H]adenine arabinoside to brain membranes has been investigated. The results indicate that, while specific and saturable binding of these ligands occurs, the sites are not identical with the inhibitory P-sites associated with adenylate cyclase in the same membranes.

MATERIALS AND METHODS

Materials. Formycin, 5′-azidoadenosine, guanine arabinoside, adenine 5′-deoxyxylofuranoside, aden-

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ine 3′-amino-3′-deoxyarabinoside, psicofuranine, and certain of the other adenosine analogs (*vide infra*) were from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. 2′,5′-Dideoxyadenosine was obtained from both that source and from ICN Chemical and Radioisotope Division, Irvine, CA. 2-Fluoroadenine xylofuranoside and 2-fluoroadenine arabinoside were from the Southern Research Institute, Birmingham, AL; adenosine 5′-carboxylate, 5′-ethylcarboxylate and 5′-cyclopropylcarboxamide were from Abbott Laboratories, North Chicago, IL. D- and L-N^6-phenylisopropyladenosine were from Boehringer-Mannheim GmbH, Mannheim, West Germany; 9-tetrahydro-2-furyladenine was from the Squibb Institute for Medical Research, Princeton, NJ; *S*-adenosylhomocysteine, zeatin riboside, kinetin riboside and 2′-deoxyadenosine-3′-phosphate were from the Sigma Chemical Co., Saint Louis, MO. A sample of decarboxylated *S*-adenosylmethionine was provided by Dr. Anthony Pegg, Department of Physiology, Pennsylvania State University, Hershey, PA. Sources of other adenosine analogs are as listed by Bruns [18]. The arabinoside compounds were all arabinofuranosides. The phosphodiesterase inhibitor 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724) was provided by Dr. W. E. Scott of Hoffmann-LaRoche, Nutley, NJ. Other compounds were from standard commercial sources.

[^{3}H]ATP (28.5 Ci/mmole) and [^{3}H]adenosine (21 Ci/mmole) were from Amersham/Searle, Arlington Heights, IL. The [^{3}H]adenine arabinoside (12 Ci/mmole) and [^{3}H]2′,5′-dideoxyadenosine (2.5 Ci/mmole) were exchange labeled by Amersham/Searle. After purification by thin-layer chromatography (silica gel, chloroform–methanol, 4:1), the compounds had a radiochemical purity of >90% based on thin-layer chromatographic analysis.

Membrane preparation. Sprague–Dawley rats (200 g, Taconic Farms, Germantown, NY) were killed by decapitation. The brains were rapidly removed and transferred to ice-cold Krebs–Ringer-bicarbonate–glucose buffer. The cerebral cortex and striatum were dissected free and washed with ice-cold 50 mM Tris–HCl buffer, pH 7.4. The tissues were then homogenized in 10 vol. of ice-cold Tris–HCl buffer with a polytron for 10 sec. Homogenization was then repeated for the same period. This suspension was centrifuged at 35,000 *g* for 20 min at 4°; the pelleted membrane fraction was washed with 10 vol of 50 mM Tris–HCl buffer and again centrifuged at 35,000 *g* for 20 min at 4°. The final pellet was suspended in about 10 vol. of 50 mM Tris–HCl buffer. In some experiments, the membrane suspension was divided so that one portion could be adjusted to 0.5 to 1 mg protein/ml for receptor binding assay, while the other portion was adjusted to about 2.5 mg protein/ml for assay of adenylate cyclase. Other treatments of membranes are described in Results and the legends of the figures and tables.

Assay of adenylate cyclase. Assay of adenylate cyclase activity was adapted from described methodology [7, 19]. The standard incubation mixture contained 50 mM Tris–HCl buffer (pH 7.4), 5 mM $MgCl_2$, 1 mM $MnCl_2$, 125 μM [^{3}H]ATP (2 μCi, 64 mCi/mmole), 10 mM creatine phosphate, 10 units creatine phosphokinase, 0.5 mM Ro 20-1724, and the membrane preparation in a final volume of 250 μl. This mixture was maintained at 4°. The incubation were initiated by the addition of 50 μl of brain membrane suspension (100–150 μg protein) and were terminated after 10 min at 37° by the addition of 500 μl of 10% trichloroacetic acid. All incubations were done in triplicate. After addition of 250 μl of 2 mM cyclic AMP, the mixture was centrifuged and then chromatographed first on Dowex 50 and then on neutral alumina, and [^{3}H]cyclic AMP was determined as described [19, 20]. The recovery of carrier cyclic AMP was 65–85%. Protein was measured by the method of Lowry *et al.* [21] using bovine serum albumin as standard. Reactions were linear with time and protein concentration under these conditions.

Assay of binding of radioactive purine ribosides. The membrane preparations were in 50 mM Tris–HCl buffer (pH 7.4) at about 0.5 mg protein/ml. In some cases membranes were preincubated in Tris buffer for 20 min at 30°. Incubations were initiated by addition of radioactive ligands and were usually at 25° for 40 min followed by rapid dilution with 5 ml of ice-cold 10 mM phosphate buffer (pH 7.4), filtration through Whatman (GF/B) filters on Millipore manifolds, and two washings with 5 ml of ice-cold 10 mM phosphate buffer. Filters were allowed to air dry and were then placed in 10 ml of Aquasol (New England Nuclear, Corp., Boston, MA), and radioactivity was determined by liquid scintillation spectrometry. Further details and variations on binding assays are contained in the legends to the figures and tables.

RESULTS

Binding of 2′,5′-dideoxyadenosine to rat brain membranes. A significant portion of the binding of [^{3}H]2′,5′-dideoxyadenosine to brain membrane preparations was displaceable by an excess of carrier 2′,5′-dideoxyadenosine or by another P-site agent, adenine arabinoside. Adenine arabinoside at 100 μM was used to define specific binding of [^{3}H]2′,5′-dideoxyadenosine. The proportion of specific binding was increased in membranes which were preincubated at 30°. This increase reached a maximum after about 20 min. The preincubation not only increased specific binding but also decreased nonspecific binding. The mechanism involved is unclear. It might involve proteolytic or lipolytic unmasking of sites and/or degradation of endogenous factors. At 100 nM [^{3}H]2′,5′-dideoxyadenosine, specific binding represented 30% of total binding in control rat cerebral cortical membranes (data not shown) and about 60% of total binding in membranes preincubated for 20 min at 30° (Fig. 1). At 25° specific binding of [^{3}H]2′,5′-dideoxyadenosine reached saturation at about 30 min (Fig. 2). Specific binding was very low at 0°. No evidence for significant degradation of [^{3}H]2′,5′-dideoxyadenosine was obtained. Kinetic analysis indicated that the density of specific binding sites for [^{3}H]2′,5′-dideoxyadenosine in rat brain membranes preincubated for 20 min at 30° was

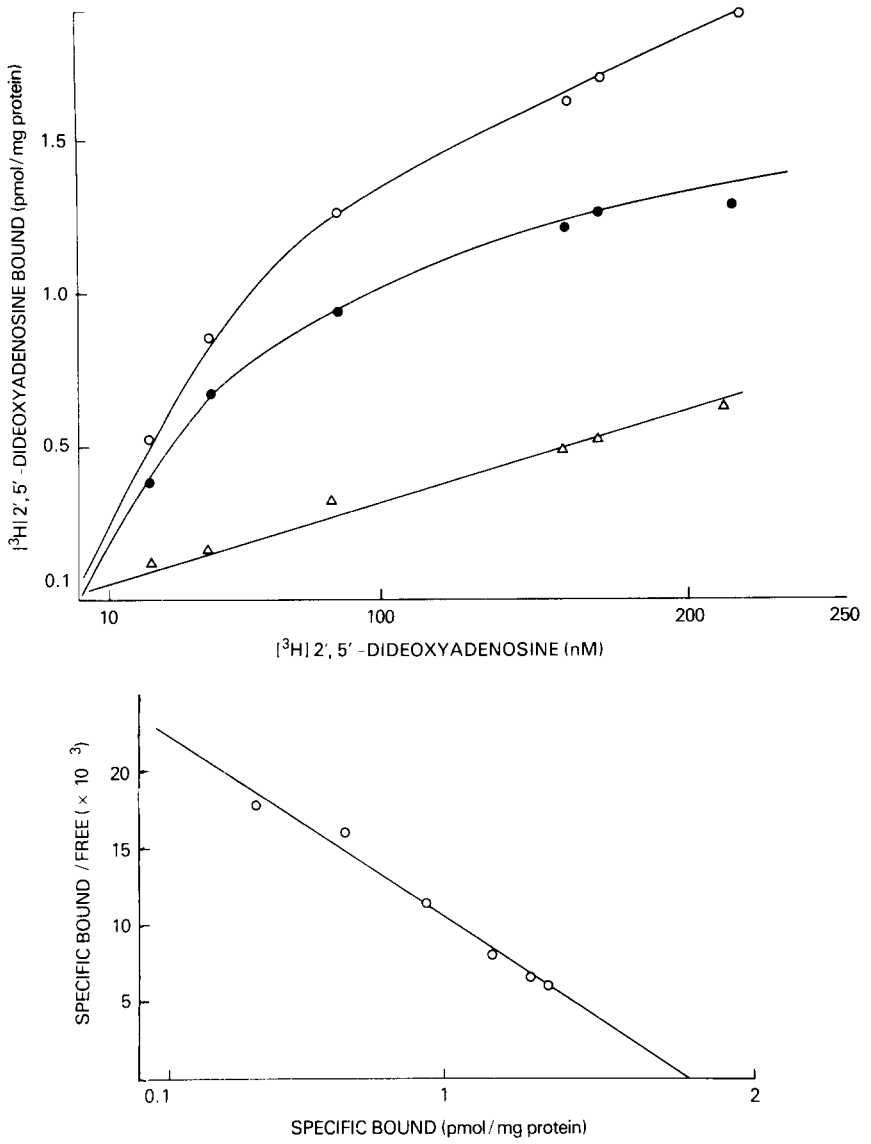

Fig. 1. Kinetic analysis of binding of [^{3}H]2′,5′-dideoxyadenosine to rat cerebral cortical membranes. Membranes were prepared and preincubated for 20 min at 30°. Incubations were initiated by addition of various concentrations (22–220 nM) of [^{3}H]2′,5′-dideoxyadenosine (2.5 Ci/mmole) in the presence or absence of 100 μM adenine arabinoside and terminated after 30 min at 25° by dilution with ice-cold buffer, followed by filtration and determination of bound radioactivity (see Materials and Methods). Total (○), specific (●), and nonspecific binding (△) are plotted in the upper figure. Nonspecific binding is defined as that in the presence of 100 μM adenine arabinoside. A Scatchard plot is shown in the lower figure. Data are means of triplicate determinations of one experiment with standard deviations of less than 2%.

about 1.8 pmoles/mg protein (Fig. 1). Scatchard analysis gave no indication of multiple binding sites. In membranes that had not been preincubated, the density of sites was significantly lower at about 0.6 pmole/mg protein (data not shown). K_D values for [^{3}H]2′,5′-dideoxyadenosine ranged from 75 to 90 nM in both control and preincubated membranes. A K_D value of 80 nM for specific binding of [^{3}H]2′,5′-dideoxyadenosine was calculated based on kinetics of binding ($K_1 = 3.4 \times 10^5\ M^{-1}\ min^{-1}$) and dissociation ($K_2 = 2.7 \times 10^{-2}\ min^{-1}$) (Fig. 2). Specific binding sites for [^{3}H]2′,5′-dideoxyadenosine were absent after boiling rat cerebral cortical membranes for 10 min (data not shown). A 15-min preincubation of membranes at 25° with 16 μg/ml of trypsin reduced specific binding by 95%. A similar preincubation with phospholipase A, C or D at 10 μg/ml reduced binding by 80, 49 and 97% respectively. Extraction of rat cerebral cortical membranes with 2 N sodium chloride for 4 hr at 25° reduced specific binding by 32% (data not shown). A preincubation with GppNHp had no significant effect on binding of [^{3}H]2′,5′-dideoxyadenosine. The densities of binding sites for [^{3}H]2′,5′-dideoxyadenosine were

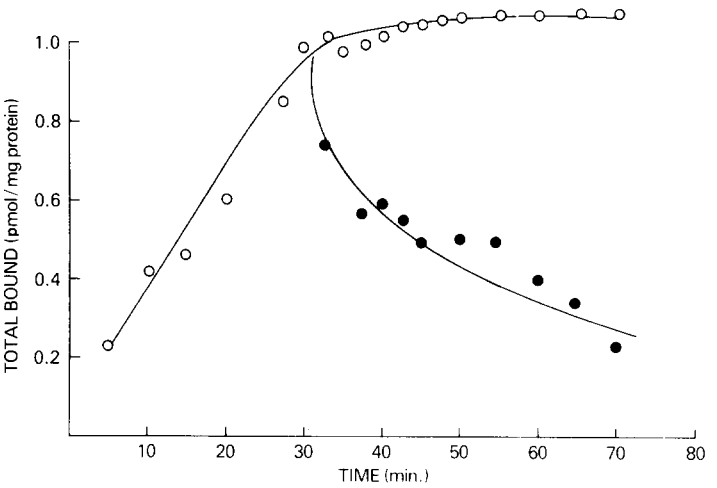

Fig. 2. Kinetics of binding and dissociation of [^{3}H]2′,5′-dideoxyadenosine to rat cerebral cortical membranes. Membranes were prepared and preincubated for 20 min at 30°. Incubation was initiated by addition of 100 nM [^{3}H]2′,5′-dideoxyadenosine (2.5 Ci/mmole) to a membrane preparation (1 mg protein/ml). After various times at 25°, 1-ml aliquots were diluted with ice-cold buffer followed by filtration and determination of bound radioactivity (○). At 30 min, 1 mM adenine arabinoside was added to one portion of the membrane suspension and bound radioactivity was determined in 1-ml aliquots at various times during dissociation of [^{3}H]2′,5′-dideoxyadenosine from specific binding sites (●). Data are means of triplicate determinations of one experiment with standard deviations of less than 3%.

highest in membranes from hypothalamus and striatum, intermediate in cerebral cortex and pons, and lowest in midbrain and cerebellum (Table 1).

Binding of adenine arabinoside to rat brain membranes. Specific binding of [^{3}H]adenine arabinoside to brain membrane preparations was in many respects similar to the specific binding of [^{3}H]2′,5′-dideoxyadenosine. Specific binding was defined by antagonism with 100 μM 2′,5′-dideoxyadenosine. The proportion of specific binding was increased in membranes that were preincubated for 20 min at 30°. At 50 nM, [^{3}H]adenine arabinoside specific binding represented 25–30% of total binding in control rat brain membranes, while representing about 70% of total binding in preincubated membranes (data not shown). Specific binding of 100 nM [^{3}H]adenine arabinoside reached saturation at about 20 min at 37° and at about 40 min at 25° (data not shown). Kinetic analysis of specific binding of adenine arabinoside to rat cerebral cortical membranes indicated a density of about 1.3 pmoles/mg protein in preincubated membranes and 0.3 pmole/mg protein in control membranes. The affinity constant was about 90 nM in both membranes. Scatchard analysis suggested the possible presence of two classes of binding sites. The density of binding sites for [^{3}H]adenine arabinoside in membranes from different brain regions correlated well with the densities of binding sites for [^{3}H]2′,5′-dideoxyadenosine (Table 1).

Binding of adenosine to brain membranes. [^{3}H]Adenosine, like [^{3}H]2′,5′-dideoxyadenosine and [^{3}H]adenine arabinoside, bound to many specific sites in rat cerebral cortical membranes (7 pmoles/mg protein) with an apparent affinity constant of 50–70 nM (data not shown). Scatchard analysis suggested the presence of only one class of binding sites.

Table 1. Binding of [^{3}H]2′,5′-dideoxyadenosine, [^{3}H]adenine arabinoside, and [^{3}H]adenosine to membranes from rat brain regions*

	Specific binding (fmoles/mg protein)		
	[^{3}H]2′,5′-Dideoxyadenosine	[^{3}H]Adenine arabinoside	[^{3}H]Adenosine
Hypothalamus	580	550	1150
Striatum	520	530	830
Cerebral cortex	276	260	600
Pons	210	200	330
Midbrain	130	130	430
Cerebellum	35	50	110

* Membrane preparations and incubations were as described in Materials and Methods with the concentration of the radioactive ligands used being nearly equal to the K_D value as follows: 150 nM [^{3}H]2′,5′-dideoxyadenosine (2.5 Ci/mmole), 100 nM [^{3}H]adenine arabinoside (12 Ci/mmole), and 70 nM [^{3}H]adenosine (21 Ci/mmole). Specific binding for the three ligands was defined, respectively, as that blocked with 100 μM adenine arabinoside, 100 μM 2′,5′-dideoxyadenosine, or 100 μM adenosine. Data are the means of three separate experiments with standard deviations of less than 2%.

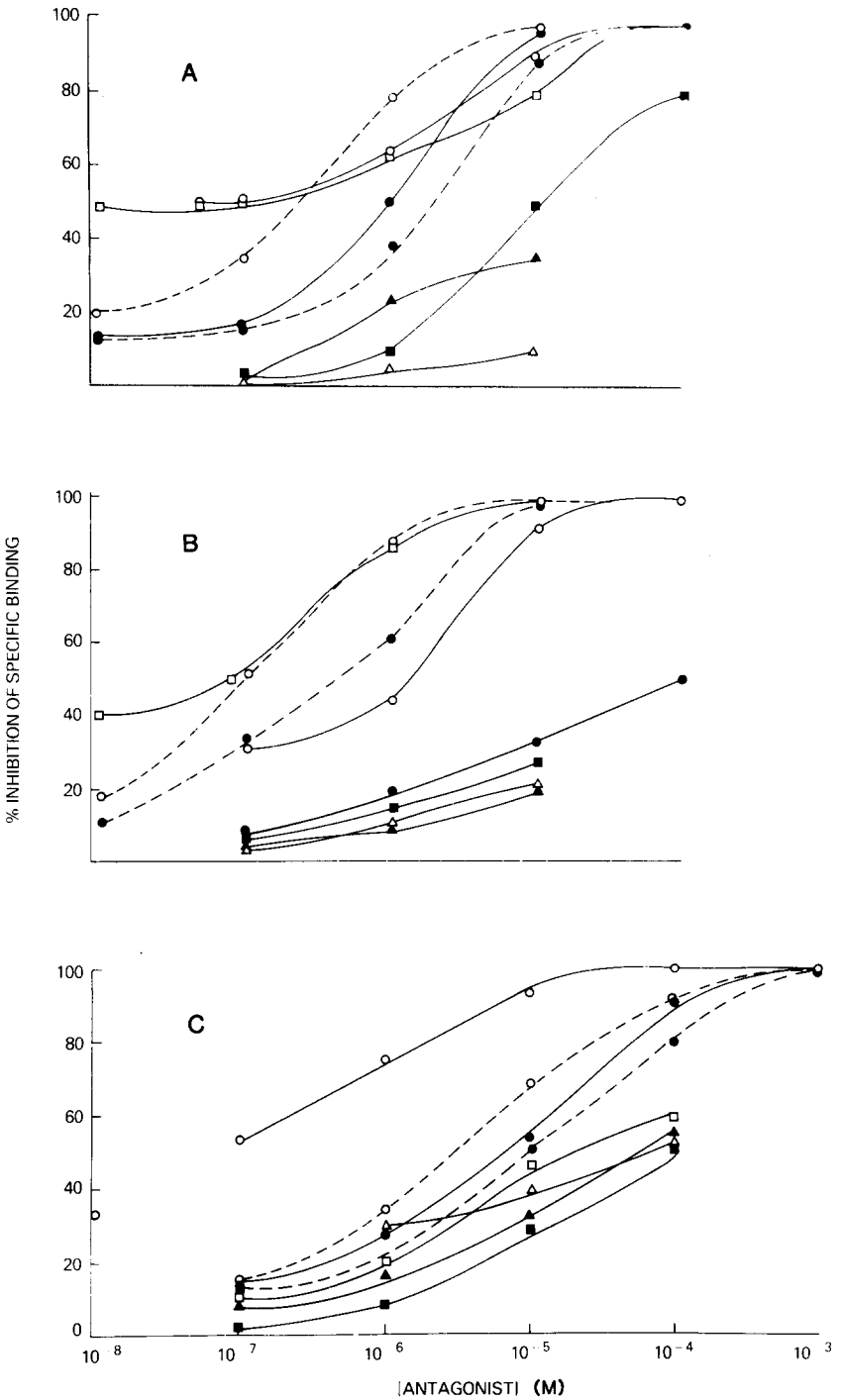

Fig. 3. Dose–response effects of adenosine analogs and theophylline on binding of (A) [^{3}H]adenine arabinoside, (B) [^{3}H]2′,5′-dideoxyadenosine, and (C) [^{3}H]adenosine in rat cerebral cortical membranes. Membrane suspensions (0.5 mg protein/ml) were prepared and then preincubated at 30° for 20 min, and binding was assayed as described in Materials and Methods. Incubations were for 30 min at 25° and were initiated by addition of (A) 100 nM [^{3}H]adenine arabinoside (12 Ci/mmole), (B) 100 nM [^{3}H]2′,5′-dideoxyadenosine (2.5 Ci/mmole), or (C) 100 nM [^{3}H]adenosine (21 Ci/mmole) and various concentrations of adenosine analogs or theophylline. Key: adenine arabinoside (●---●); adenosine (○—○); 2′,5′-dideoxyadenosine (○---○); adenosine 5′-cyclopropylcarboxamide (●—●); 5′-methylthioadenosine (□—□); theophylline (■—■); 2-chloroadenosine (▲—▲); and N^6-phenylisopropyladenosine (△—△). Specific binding was as defined in Results. Values are means of three or four experiments with standard deviations of less than 5%.

Table 2. Inhibition of specific binding of [^{3}H]2′,5′-dideoxyadenosine and [^{3}H]adenine arabinoside by adenosine analogs and theophylline in rat cerebral cortical membranes*

Antagonist	IC_{50} (μM) versus radioactive ligand [^{3}H]2′,5′-Dideoxyadenosine	[^{3}H]Adenine arabinoside	[^{3}H]Adenosine
Adenosine	2	0.5	0.1
L-N^6-Phenylisopropyladenosine	>100	>100	60
D-N^6-Phenylisopropyladenosine		>1000	1000
Adenosine 5′-cyclopropylcarboxamide	1000	10	7
5′-Methylthioadenosine	0.8	0.5	11
Adenine arabinoside	5	12	10
2′,5′-Dideoxyadenosine	1.0	2.5	3
2-Chloroadenosine	>100	>100	80
2-Fluoroadenosine	100		
2′-Deoxyadenosine	1000		
3′-Deoxyadenosine	500		
5′-Deoxyadenosine	9		
2′,3′-Dideoxyadenosine	50		
Adenine xylofuranoside	10		
9-(Tetrahydro-2-furyl)adenine	10		
Theophylline	>100	>100	100

* Membrane suspensions (0.5–1 mg protein/ml) were prepared and binding was assayed as described in Materials and Methods. Incubations were for 30 min at 25° and were initiated by addition of 100 nM [^{3}H]2′,5′-dideoxyadenosine (2.5 Ci/mmole), 100 μM [^{3}H]adenine arabinoside (12 Ci/mmole) or 100 nM [^{3}H]adenosine (21 Ci/mmole), and 0.01 to 100 μM concentrations of adenosine analogs and theophylline. Specific binding was as defined in Results. In some cases, concentrations of up to 1000 μM of an analog were tested. 8-Bromoadenosine and adenine had IC_{50} values of >1000 μM. Data obtained with striatal preparations were similar. The IC_{50} values were estimated from dose–response curves from three to four experiments. Calculation of inhibition was based on the following equation:

$$\% \text{ Inhibition} = 100\left[1 - \frac{(\text{binding with drug}) - (\text{nonspecific binding})}{(\text{total binding}) - (\text{nonspecific binding})}\right.$$

However, inhibition of binding by various analogs (Table 2) often gave biphasic curves suggestive of the presence of at least two sites (Fig. 3). Specific binding of [^{3}H]adenosine was defined by antagonism with 100 μM adenosine and at 50 nM [^{3}H]adenosine represented about 65% of total binding. Specific binding of 100 nM [^{3}H]adenosine reached saturation in about 30 min at 25° (data not shown). The effect of preincubation on binding of [^{3}H]adenosine was not investigated. Density of binding sites for [^{3}H]adenosine appeared greater in all brain regions than densities of sites for [^{3}H]2′,5′-dideoxyadenosine (Table 1).

Effects of adenosine analogs and theophylline on specific binding of [^{3}H]2′,5′-dideoxyadenosine, [^{3}H]adenine arabinoside and [^{3}H]adenosine in brain membranes. Adenosine analogs which antagonized binding of [^{3}H]2′,5′-dideoxyadenosine were the same analogs which antagonized binding of [^{3}H]adenine arabinoside, although there were some apparent differences in potencies (Table 2 and Fig. 3). The P-site agonists 2′,5′-dideoxyadenosine, adenine arabinoside, 9-(tetrahydro-2-furyl)adenine, and adenine xylofuranoside were potent antagonists of binding of [^{3}H]2′,5′-dideoxyadenosine and [^{3}H]adenine arabinoside. 2-Chloroadenosine, N^6-phenylisopropyladenosine and theophylline were virtually inactive. 5′-Methylthioadenosine was an extremely potent antagonist.

Binding of [^{3}H]adenosine was reduced by 2′,5′-dideoxyadenosine, adenine arabinoside and adenosine 5′-cyclopropylcarboxamide to an extent similar to the maximal reduction seen with adenosine (Table 2 and Fig. 3). Adenosine was, however, much more potent than 2′,5′-dideoxyadenosine which, in turn, appeared slightly more potent than adenine arabinoside and adenosine 5′-cyclopropylcarboxamide. 5′-Methylthioadenosine, 2-chloroadenosine, N^6-phenylisopropyladenosine and theophylline, even at 100 μM, reduced binding of [^{3}H]adenosine by only one-half of the maximal reduction seen with adenosine (Fig. 3). The data suggest that [^{3}H]adenosine may bind to more than one site, one of which corresponds to the site which binds [^{3}H]2′,5′-dideoxyadenosine while the other shows characteristics of an adenosine receptor, i.e. interacting with N^6-phenylisopropyladenosine, 2-chloroadenosine and theophylline. The results of binding studies with [^{3}H]adenosine and guinea pig cerebral cortical membranes [13, 22] might also be interpreted in terms of binding to both an adenosine receptor and a theophylline-insensitive site, i.e. the site to which 2′,5′-dideoxyadenosine binds.

Effects of adenosine analogs on adenylate cyclase in brain membranes. 2′,5′-Dideoxyadenosine at 100 μM had only weak inhibitory effects on adenylate cyclase activity in rat cerebral cortical or striatal membranes when assayed with 5 mM magnesium ions and 1 mM ATP (data not shown). Assays were, therefore, carried out under conditions which in other tissues [7] increase the potency of P-site agonists as inhibitors of adenylate cyclase. In the presence of high concentrations of magnesium (5 mM) and manganese (1 mM) ions, 2′,5′-dideoxyadenosine and

Table 3. Inhibitory effects of 2′,5′-dideoxyadenosine and adenine arabinoside on adenylate cyclase in membranes from various rat brain regions*

Region	Adenylate cyclase [pmoles cyclic AMP·min^{-1}·(mg protein)$^{-1}$] Basal	2′,5′-Dideoxyadenosine GppNHp	2′,5′-Dideoxyadenosine Basal	Adenine arabinoside GppNHp	Adenine arabinoside Basal	GppNHp
Cortex	79 ± 11	185 ± 11	9	5	70	40
Striatum	67 ± 2	151 ± 10	7	4	60	30
Hypothalamus	29 ± 1	125 ± 9	10	7	80	
Midbrain	35 ± 3	122 ± 7	9	5	80	
Pons	25 ± 1	115 ± 6	12	7	80	
Cerebellum	81 ± 4	150 ± 7	9	7	80	

* Membrane preparations from various regions and assay of adenylate cyclase activity were as described in Materials and Methods. Membranes were preincubated for 10 min at 37° in the presence or absence (basal) of 100 μM GppNHp, centrifuged, and washed once with 50 nM Tris–HCl buffer, pH 7.4. Incubations were for 10 min at 37° and were initiated by addition of the preincubated resuspended membrane preparations (see Materials and Methods). Protein concentrations were about 150 μg/250 μl of incubation solution. Values are the means ± S.E.M. for triplicate determinations of two experiments. The IC_{50} values were estimated from dose–response curves for five to six concentrations of 2′,5′-dideoxyadenosine or adenine arabinoside, each in triplicate in two separate experiments. A maximal inhibition of 92–100% with 1 mM 2′,5′-dideoxyadenosine and 85–97% with 5 mM arabinoside was observed in all brain areas both in the presence and absence of GppNHp.

Table 4. Inhibition of adenylate cyclase activity in rat cerebral cortical membranes by adenosine analogs*

Agents	IC_{50} (μM) Cortex
Adenosine	600
2-Fluoroadenosine	90
Formycin (7-deaza-8-azaadenosine)	400
2′-Deoxyadenosine	700
2′-*O*-Methyladenosine	400
3′-Deoxyadenosine	600
5′-Deoxyadenosine	600
2′,3′-Dideoxyadenosine	600
2′,5′-Dideoxyadenosine	10
2′-Deoxyadenosine-3′-monophosphate	80
Adenine 3′-amino-3′-deoxyarabinoside	100
Adenine arabinoside	100
2-Fluoroadenine arabinoside	30
Adenine xylofuranoside	70
2-Fluoroadenine xylofuranoside	20
Adenine 5′-deoxyxylofuranoside	70
9-(Tetrahydro-2-furyl)adenine	400

* Membrane preparations from rat cerebral cortex and assay of adenylate cyclase were as described in Materials and Methods. Incubations were for 10 min at 37° and were initiated by addition of the membrane preparation (150 μg protein). Basal activity of adenylate cyclase ranged from 40 to 100 pmoles cyclic AMP formed per min per mg protein. The IC_{50} values were estimated from dose–response curves for five to six concentrations of each compound with triplicate determinations in at least two separate experiments. Similar IC_{50} values were obtained with striatal preparations. The IC_{50} values, with the exception of adenosine, were not altered by the presence of 100 μM theophylline (see text). Less than 50% inhibition occurred with a 1 mM concentration of the following compounds: tubercidin (7-deazaadenosine), 8-azaadenosine, 1-methyladenosine, 2-chloroadenosine, L-N^6-phenylisopropyladenosine, D-N^6-phenylisopropyladenosine, N^6-dimethyladenosine, zeatin riboside, kinetin riboside, inosine, isoguanosine, guanosine, 8-bromoadenosine, 8-aminoadenosine, 8-dimethylaminoadenosine, psicofuranine, 2′,3′-isopropylidineadenosine, 2-aminoadenosine, the carbocyclic analog of adenosine, 3′-*O*-methyladenosine, 5′-azido-5′-deoxyadenosine, 5′-isobutylthioadenosine, adenosine-5′-carboxylic acid, adenosine 5′-cyclopropylcarboxamide, adenosine-5′-ethylcarboxylate, adenosine-5′-acetate, adenine 5′-methylthioarabinoside, 5′-methylthioadenosine, guanine arabinoside, adenine, *S*-adenosine-L-homocysteine, decarboxylated-*S*-adenosyl-L-methionine, theophylline, and 8-phenyltheophylline. None of these compounds at 100–1000 μM prevented the inhibition of adenylate cyclase activity in rat cerebral cortical membranes by 10 μM 2′,5′-dideoxyadenosine.

adenine arabinoside were relatively potent and very effective inhibitors of adenylate cyclase from various brain regions (Table 3).

A variety of adenosine analogs inhibited adenylate cyclase in cerebral cortical and striatal membranes (Table 4). The IC_{50} values were not altered by the presence of 100 μM theophylline except in striatal membranes, where adenosine was not inhibitory to activity of adenylate cyclase except in the presence of theophylline (data not shown). This is undoubtedly due to the presence of adenosine receptors which activate adenylate cyclase in striatal membranes and which are blocked by theophylline [23].

Adenosine receptors in cortical membranes have virtually no stimulatory effect on adenylate cyclase activity [23, 24].

The profile of inhibitory activity for the various analogs was consonant with that expected from interaction with P-sites described for peripheral tissues [7–9, 25]. However, 2′,5′-dideoxyadenosine, adenine arabinoside and certain other analogs appeared from 2- to 10-fold less potent in the preparations from brain (Table 4) than in hepatic preparations [8]. The potencies of 2′,5′-dideoxyadenosine, 2′-deoxyadenosine-3′-monophosphate, adenine arabinoside and adenine xylofuranoside were nearly equivalent in brain membranes (Table 4) and in thyroid membranes [9], while adenosine, 5′-deoxyadenosine and 2′-deoxyadenosine appeared significantly less potent in the brain preparation. Recently, adenosine, 2′-deoxyadenosine and 5′-deoxyadenosine were reported to inhibit rat striatal adenylate cyclase with K_i values of 50–70 μM [26]; such values are substantially lower than the values obtained in the present study. It should be noted that potencies of adenosine analogs at P-sites are strongly dependent on the conditions of assay of adenylate cyclase [7].

A 2-fluoro substituent significantly increased the potency of adenosine, adenine arabinoside and adenine xylofuranoside as P-site inhibitors. Other purine-modified analogs, such as 2-chloroadenosine, 2-aminoadenosine, N^6-phenylisopropyladenosine and 8-bromoadenosine had no inhibitory effect on adenylate cyclase activity (legend, Table 4). The 5′-methylthio, 5′-carboxylate and 5′-carboxamide analogs of adenosine had no inhibitory effect on adenylate cyclase activity. Analogs which alone had no effect on adenylate cyclase were tested in combination with 2′,5′-dideoxyadenosine. None of these antagonized the inhibitory effect of 2′,5′-dideoxyadenosine on adenylate cyclase activity even at concentrations as high as 1 mM (legend Table 4).

Salt extraction of rat cerebral cortical membranes had no effect on the magnitude of inhibition of adenylate cyclase by 2′,5′-dideoxyadenosine (data not shown). This is not unexpected since the P-sites are closely associated with the catalytic unit of adenylate cyclase and are still demonstrable in solubilized preparations [26]. Preincubation of rat cerebral cortical membranes with trypsin reduced the adenylate cyclase activity by 3-fold but the remaining activity was still inhibited 56% by 100 μM 2′,5′-dideoxyadenosine (data not shown).

DISCUSSION

A variety of adenosine analogs have been shown to be more or less selective agonists for the three major types of adenosine-sites associated with adenylate cyclase [3, 4]. The high selectivity of 2′,5′-dideoxyadenosine and adenine arabinoside for the so-called P-site which is tightly associated and inhibitory to adenylate cyclase was the basis for the selection of these analogs as possible ligands for this site. However, the present data on specific binding of [^{3}H]2′,5′-dideoxyadenosine and [^{3}H]adenine arabinoside to brain membranes are inconsistent with a correspondence of the ligand binding sites to the so-called P-sites. First, the number of binding sites for these adenosine analogs is many fold greater than would be expected of a site associated with adenylate cyclase. It appears unlikely that each adenylate cyclase molecule would have such large numbers of P-sites associated with it. Second, the density of binding sites in different brain regions shows a rather poor correlation with the specific activity of adenylate cyclase (compare Tables 1 and 3). The complete inhibition of adenylate cyclase in all brain regions by 2′,5′-dideoxyadenosine indicates an association of P-sites with all brain adenylate cyclases and suggests that the density of P-sites and levels of adenylate cyclase activity in different regions should show a correlation. Third, the specific binding sites for [^{3}H]2′,5′-dideoxyadenosine are very sensitive to trypsin treatment, while P-site inhibition of adenylate cyclase is virtually unaffected (see Results). Finally, salt extraction of membranes reduced specific binding sites for [^{3}H]2′,5′-dideoxyadenosine, while having little effect on P-site inhibition of adenylate cyclase (see Results).

The binding parameters for [^{3}H]2′,5′-dideoxyadenosine and [^{3}H]adenine arabinoside are in many respects similar, but there are some marked differences (Table 2). The most striking difference is the relatively marked effects of adenosine 5′-cyclopropylcarboxamide and theophylline on binding of the [^{3}H]adenine arabinoside as compared to the relatively small effects on binding of [^{3}H]2′,5′-dideoxyadenosine (Fig. 3). The carboxamide and theophylline apparently have no affinity for the P-site associated with adenylate cyclase (Table 4 and legend). This suggests that binding of [^{3}H]2′,5′-dideoxyadenosine is more consonant with selective interaction of P-sites than is the binding of [^{3}H]adenine arabinoside. However, 5′-methylthioadenosine had no apparent affinity for the P-site associated with adenylate cyclase (Table 4 and legend), but is was one of the most potent and completely effective antagonists of specific binding of [^{3}H]2′,5′-dideoxyadenosine (Fig. 3 and Table 2). Furthermore, 2-fluoroadenosine was much more potent than adenosine as a P-site inhibitor (Table 4), but it was much less potent than adenosine as an antagonist of specific binding of [^{3}H]2′,5′-dideoxyadenosine (Table 2). Clearly, the binding sites for [^{3}H]2′,5′-dideoxyadenosine and the P-sites associated with adenylate cyclase were not identical. However, inhibition curves (Fig. 3B) were suggestive of multiple sites for binding of [^{3}H]2′,5′-dideoxyadenosine even though Scatchard analysis was not. It is possible that some of the binding sites were indeed identical with P-sites.

5′-Methylthioadenosine is formed in tissues during polyamine synthesis through the action of the enzyme spermine synthetase [27]. 5′-Methylthioadenosine is a competitive inhibitor of the enzyme. However, catalytic sites on spermine synthetase did not contribute to binding of [^{3}H]2′,5′-dideoxyadenosine since decarboxylated *S*-adenosylmethionine at 100 μM, the substrate, had no effect on binding of [^{3}H]2′,5′-dideoxyadenosine (data not shown). Another enzyme which might contribute to binding of adenosine analogs is *S*-adenosylhomocysteinase, a so-called adenosine-binding protein [28, 29]. How-

ever, adenine and *S*-adenosylhomocysteine, two compounds which interact with this enzyme, had virtually no effect on binding of [^{3}H]2′,5′-dideoxyadenosine to brain membranes (legend, Table 4). Sites on adenosine deaminase do not appear to contribute significantly since the deaminase inhibitor 2′-deoxycoformycin [30] at 1 μM had no effect on binding of [^{3}H]2′,5′-dideoxyadenosine (data not shown).

It is not possible to do more than speculate on the nature of binding sites for [^{3}H]2′,5′-dideoxyadenosine in brain membranes. One possibility is that the specific binding sites for [^{3}H]2′,5′-dideoxyadenosine in brain membranes correspond, in part, to the intracellular binding sites for adenosine recently proposed to be involved in facilitated uptake of adenosine [31]. The effect of 2′,5′-dideoxyadenosine on facilitated uptake of adenosine has apparently not been studied. However, it also remains possible that the specific binding sites for [^{3}H]2′,5′-dideoxyadenosine are artifacts with no physiological significance. Regardless of the nature of the binding sites for [^{3}H]2′,5′-dideoxyadenosine, it would appear that such sites represent a significant portion of the sites to which adenosine binds in brain and probably other membranes. This conclusion is compatible with previous publications [10–13, 22] which indicate that a large portion of adenosine-binding sites in various membrane preparations exhibit properties inconsistent with those expected of adenosine receptors, i.e. not blocked or only partially blocked by theophylline. Thus, efforts to develop further ligands for adenosine receptors and the P-site should be directed towards compounds which have low affinity for the large pool of membrane sites which bind [^{3}H]2′,5′-dideoxyadenosine.

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