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CHARACTERISTICS OF HIGH-AFFINITY [^{3}H]ADENOSINE BINDING TO RAT BRAIN SYNAPTOSOMES AND TURKEY ERYTHROCYTE MEMBRANES

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High affinity binding sites for [^{3}H]adenosine in rat brain and in turkey erythrocytes can be identified by binding experiments. Displacement experiments using a number of adenosine analogs indicate that these high affinity sites do not represent the R-type adenosine receptors which mediate activation of adenylate cyclase, although the binding is theophylline sensitive. Similarly, the binding of [^{3}H]adenosine is not to the P-site, which mediates inhibition of adenylate cyclase, since the high affinity binding persists in the presence of 2′,5′-dideoxyadenosine. Furthermore, these results remain qualitatively similar also in the presence of dipyridamole which blocks adenosine transport sites. We conclude that theophylline sensitivity does not indicate that [^{3}H]adenosine binding sites correspond to adenosine recepors coupled to adenylate cyclase.

Introduction

Theophylline-sensitive adenosine binding sites have been reported in rat fat cell membranes [1], in the dog heart microsomal fraction [2], and in crude or purified membranes from rat [3] or guinea-pig [4] brain. Adenosine-stimulated adenylate cyclases have been found in rat striatal homogenates by Prémont et al. [5] and in rat caudate nucleus membranes by Braun and Levitzki [6], who also found a similarly activated enzyme in turkey erythrocyte membranes. The binding constant of adenosine to the high affinity site in brain membranes (0.5 to 1.7 μM) [3,4] and its inhibitory constant for theophylline (11 μM) as well as its localization to membranes derived from the synaptosomal fraction [4], suggested that binding of adenosine to this site may be responsible for the adenosine mediated increase in cyclic AMP levels, first seen in brain slices by Sattin and Rall [7], and subsequently found in many other tissues (for references see Ref. 8).

Direct stimulation of adenylate cyclase by adenosine has also been shown in cell cultures of neural origin such as mouse neuroblastoma NS20 cells, where K_a values for adenosine and its non-metabolizable analog 2-chloroadenosine were 67.6 and 6.7 μM respectively [9], while theophylline inhibited with K_i 35 μM. In glial cultures of perinatal mouse brain [10] adenosine had both stimulatory and inhibitory effects on cyclic AMP levels, the effects being distinguished both by the concentrations of adenosine required and the relative potencies of various analogs.

In the present work analogs of adenosine have been used to further characterize the properties of the adenosine binding site in both rat brain and turkey erythrocyte membranes. To eliminate binding to the adenosine uptake system(s), which are active in mammalian brain [11] and also operate in isolated synaptosomes [12], binding studies were carried out in the presence of the uptake inhibitor dipyridamole. 2′,5′-Dideoxyadenosine, which like

Abbreviations: EHNA, erythro-9-(2-hydroxy-3-nonyl) adenine; IBMX, 3-isobutyl-1-methylxanthine.

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other deoxyadenosines also inhibits adenosine uptake [13], was included primarily to prevent binding to the intracellular adenylate cyclase P-site which mediates inhibition of the the enzyme [14] and has recently been shown to be present in rat brain membranes [15]. Some experiments on turkey erythrocyte membranes were carried out in the additional presence of adenine to eliminate binding to any residual cytoplasmic adenine analog binding protein [16] present in the preparation.

Materials and Methods

Membrane preparation

Turkey erythrocyte membranes were prepared and stored as previously described [17]. Rat cortex synaptosomal membranes or caudate membranes were prepared by homogenizing tissue from animals killed by decapitation in 10 volumes 0.32 M sucrose, containing 4 mM $MgCl_2$, using a Dounce homogenizer, and centrifuging at $3000 \times g$ for 10 min in a Sorvall centrifuge to sediment nuclei and cell debris. The pellet was washed once and the supernatant centrifuged at $10000 \times g$ for 20 min to sediment the crude mitochondrial fraction (P_2). In the case of cortical material this was re-suspended in 0.32 M sucrose, and 10 ml amounts layered onto a gradient of 12 ml 0.8 M sucrose and 12 ml 1.2 M sucrose, followed by centrifugation for 90 min at $95000 \times g$ in an SW-27 rotor, using a Spinco model L ultracentrifuge. The interface between the sucrose layers (P_2B or synaptosomal fraction), or the P_2 fraction for caudate, was collected and washed twice in 50 mM Tris, 10 mM $MgCl_2$, pH 7.4, and stored frozen under liquid nitrogen in 1-ml aliquots.

Adenosine binding assay

Adenosine binding was determined as described by Newman et al. [4]. The assay medium routinely contained 20 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$ and 0.1 μM [2,5′,8-^{3}H]adenosine in a total volume of 0.2 ml. The reaction was started by addition of the tissue preparation to be assayed, containing up to 300 μg protein, and allowed to continue for 60 min at 0°C in the case of rat brain membranes, or 40 min at 37°C for turkey erythrocyte membranes. The contents of each tube were then diluted by addition of 4 ml ice-cold assay buffer, and immediately filtered by vacuum on Whatman GF/C filters, 2.5 cm diameter. Each tube was then washed a further four times with an equal volume of the same buffer and the filters dried in an oven at 80°C before counting. Non-specific binding was determined in parallel incubation of tubes containing an additional 0.1 mM non-radioactive adenosine, and subtracted from the total to give values for specific binding. Specific binding was only obtained for turkey erythrocyte membranes when incubation was performed at 37°C, while in rat brain membranes stored at 4°C specific binding was the same, whether incubations were performed at 0°C or 37°C (c.f. Schwabe et al. [3] who found that only non-specific binding increased with temperature in rat brain membranes, while there was a slight decrease in specific binding).

The identity of the bound radioactivity was checked by thin-layer chromatography, as described by Newman et al. [4]. The filters were eluted with 0.25% trichloroacetic acid containing 8 mM adenosine, and the eluate run on silica plates in *n*-butanol/ethyl acetate/methanol/25% NH_4OH (7:4:3:6, v/v). The R_F values obtained were 0.61 for adenosine, 0.43 for inosine and 0.06 for 5′-AMP, corresponding to those obtained by Schwabe et al. [3]. The fraction of bound radioactivity which co-chromatographed with adenosine was unchanged by the incubation conditions of the membranes, and was 87.8% in a control incubation, 79.8% in the presence of dipyridamole and dideoxyadenosine (each 100 μM) and 90.7% in the additional presence of 100 μM N^6-phenylisopropyladenosine.

Materials

[2,5′,8-^{3}H]Adenosine, 42 Ci/mmol, was obtained from the Radiochemical Centre, Amersham, U.K. N^6-Methyladenosine was from P-L Biochemicals, Inc., Milwaukee, WI, U.S.A.; 2′,5′-dideoxyadenosine was from ICN Pharmaceuticals, Cleveland, OH, U.S.A. Dipyridamole was from Dr. Karl Thomae GmbH, Biberach an der Riss, F.R.G. N^6-Phenylisopropyladenosine, 2-chloroadenosine, 2′-deoxyadenosine, theophylline and 3-isobutyl-1-methylxanthine were from Sigma

Chemical Co., St. Louis, MO, U.S.A., and EHNA was a gift of Burroughs Wellcome Co., Research Triangle Park, NC, U.S.A.

Results

Rat brain membranes

Binding of [2,5′,8-^{3}H]adenosine to rat cortex synaptosomal membranes was saturable (Fig. 1), and Scatchard analysis revealed the existence of two classes of binding sites (Table I) with K_d values 0.3 μM and 10 μM, respectively, corresponding to previous findings in both rat and guinea-pig [3,4]. The effects of various analogs on binding to each of these sites were determined at [^{3}H]adenosine concentrations of 0.1 μM and 5.7 μM, respectively. N^6-Methyladenosine and 2-chloroadenosine were the most potent analogs at 0.1 μM [^{3}H]adenosine in both cerebral cortex and caudate membranes (Figs. 2a and 3a), while N^6-phenylisopropyladenosine was less active, and theophylline and 2′,5′-dideoxyadenosine were relatively inactive. Both inosine and 2′-deoxyadenosine, however, caused considerable displacement of [^{3}H]adenosine binding. In rat caudate membranes at 5.7 μM [^{3}H]adenosine (Fig. 3b), the most potent displacing analogs were inosine and 2′,5′-dideoxyadenosine, while N^6-methyladenosine was less active and theophylline had very weak activity.

The maximal binding capacities of both high and low affinity sites in rat cortex were reduced by approximately 60% by 50 μM dipyridamole and 100 μM 2′,5′-dideoxyadenosine (Fig. 1b, Table I), but addition of these agents caused very little difference to the pattern of displacement of [^{3}H]adenosine by the various analogs. The concentration of N^6-methyladenosine, required to give half-maximal displacement of 0.1 μM [^{3}H]adenosine binding to rat cortex membranes, was 20 μM in the presence of either 250 μM 2′,5′-dideoxyadenosine alone or 100 μM 2′,5′-dideoxyadenosine + 50 μM dipyridamole, where binding in the ab-

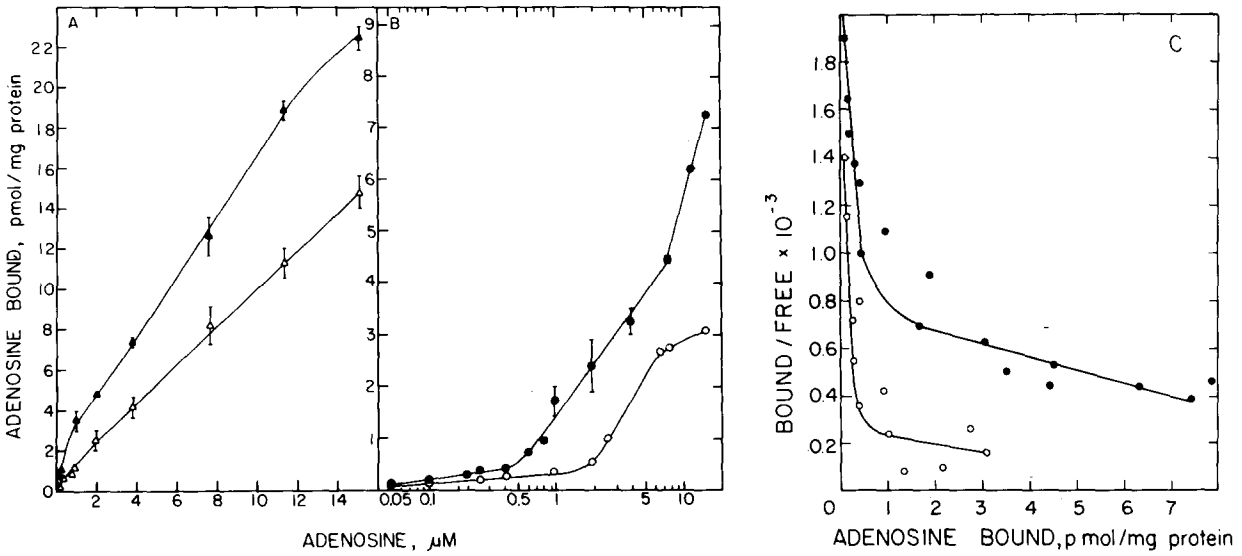

Fig. 1. Saturation analysis of [^{3}H]adenosine binding to rat cortex synaptosomal membranes. Synaptosome membranes (42 to 106 μg protein) were incubated with 20 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$ and various concentrations of [^{3}H]adenosine from 0.05 μM to 19 μM for 60 min at 0°C. Results are individual values or mean ± S.D. of three observations. (A) ▲——▲, total binding; △——△, non-specific binding determined in the additional presence of 1 mM non-radioactive adenosine. (B) ●——●, specific binding derived from the difference between total and non-specific binding in (A); ○——○, specific binding in an experiment in which tubes for determination of both total and non-specific binding contained in addition 50 μM dipyridamole and 100 μM 2′,5′-dideoxyadenosine. (C) Scatchard analysis of the data from Fig. 1 (B). Mean affinity constants and maximal binding capacities for the preparations under the various conditions of incubation derived from this analysis are given in Table I.

TABLE I

BINDING CONSTANTS OF [^{3}H]ADENOSINE FOR RAT CORTEX SYNAPTOSOMAL MEMBRANE PREPARATION

Experimental conditions were as in the legend to Fig. 1. Values are mean ± S.D. of the number of observations in parentheses.

Addition	High affinity		Low affinity	
	K_d (μM)	B_{max} (pmol per mg protein)	K_d (μM)	B_{max} (pmol per mg protein)
None	0.33 ± 0.15 (4)	2.78 ± 0.68 (4)	10.8 ± 3.0 (4)	10.5 ± 2.7 (3)
50 μM dipyridamole + 100 μM 2′,5′-dideoxyadenosine	0.25 ± 0.07 (2)	0.43 ± 0.02 (2)	7.4 ± 4.8 (2)	3.4 ± 1.0(2)
50 μM dipyridamole + 100 μM 2′,5′-dideoxyadenosine, + 100 μM theophylline	0.14 ± 0.04 (2)	0.37 ± 0.07 (2)	9.3 ± 5.7 (2)	4.7 ± 1.1 (2)

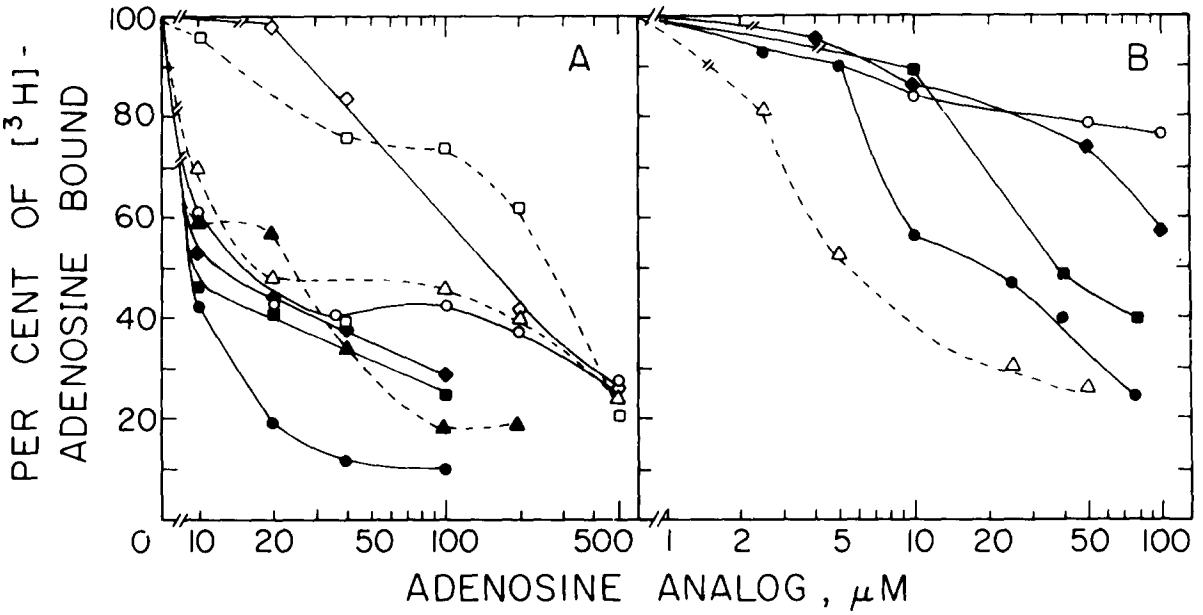

Fig. 2. Displacement of the binding of 0.1 μM [^{3}H]adenosine from rat cortex synaptosomal membranes by various adenosine analogs. (A) Synaptosome membranes (114 μg protein) were incubated with 20 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 0.1 μM [2,5′,8-^{3}H]adenosine and unlabeled compounds, as shown below, for 60 min at 0°C. Binding in the absence of added agents after correction for counts bound in the presence of 0.1 mM unlabeled adenosine was 1.14 ± 0.07 pmol/mg protein (mean ± S.D. of three observations), and results are calculated as percentages of this value. ●———●, adenosine bound in the presence of N^6-methyladenosine; ◆———◆, N^6-phenylisopropyladenosine; ■———■, 2-chloroadenosine; ▲— — —▲, 2′-deoxyadenosine; △— — —△, inosine; ○———○, theophylline; □— — —□, 3-isobutyl-1-methylxanthine; ◇———◇, 2′,5′-dideoxyadenosine. (B) Synaptosome membranes (144 μg protein) were incubated as above in the additional presence of 50 μM dipyridamole and 100 μM 2′,5′-dideoxyadenosine. Mean binding with these agents was 0.53 pmol/mg protein or 57% of binding in their absence, and the results are given as percentages of this value.

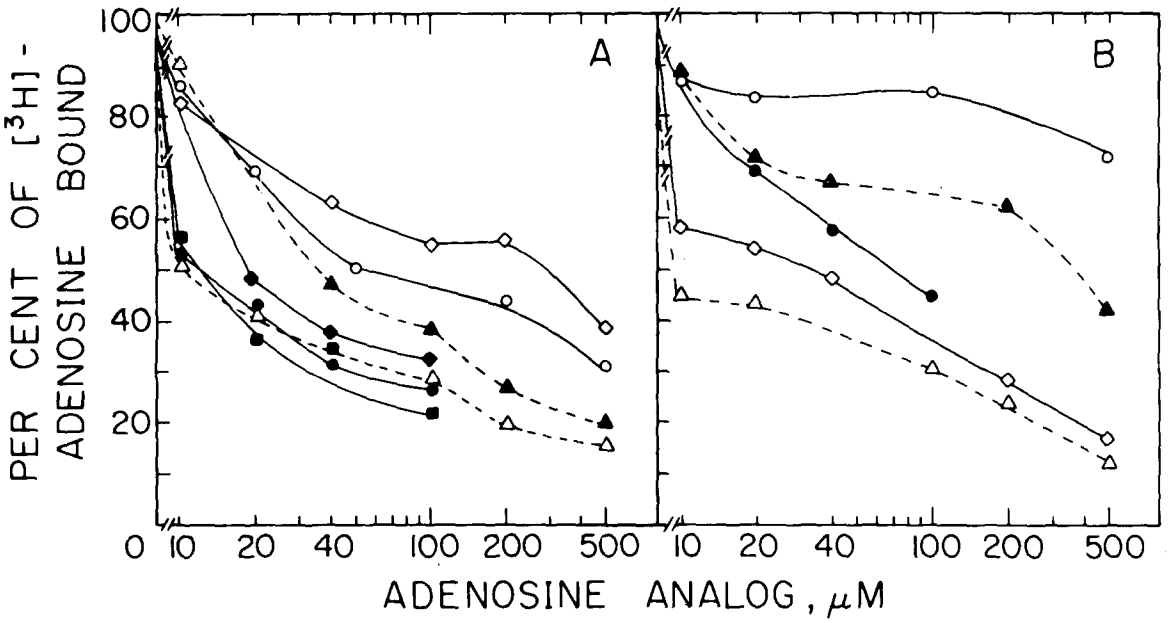

Fig. 3. Displacement of the binding of [^{3}H]adenosine from rat caudate membranes. (A) Binding assays were performed as in Fig. 2(A), except that 36 μg of rat caudate membrane protein was used. Binding in the absence of added agents was 1.88 pmol/mg protein. (B) Rat caudate membranes (27 μg protein) were incubated with 20 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 5.7 μM [2,5′,8-^{3}H]adenosine and unlabeled analogs, as shown in the legend to Fig. 2, for 60 min at 0°C. Binding in the absence of added agents after correction for counts bound in the presence of 5 mM unlabeled adenosine was 82.6 pmol/mg protein, and results are shown as percentages of this value.

sence of other analogs was 29 ± 3% and 47 ± 6% of control, respectively (mean ± S.D. of three observations in each case), compared with 12 μM when no agents were added (Fig. 2b). Addition of dipyridamole and dideoxyadenosine, however, dramatically reduced the effect of theophylline, which had almost no activity on displacement of binding of 0.1 μM [^{3}H]adenosine (Fig. 2b) and failed to affect the K_d or B_{max} values for either the high or low affinity site (Table I). The adenosine deaminase inhibitor EHNA also had no effect on either binding of [^{3}H]adenosine or its displacement by inosine at low adenosine concentrations. Binding in the presence of 25 μM EHNA was 0.47 pmol/mg protein and 6.53 pmol/mg protein at 0.1 μM [^{3}H]adenosine and 5.7 μM [^{3}H]adenosine, respectively, compared to control values of 0.43 and 5.54 pmol/mg protein. 0.5 mM inosine reduced specific binding to 16.2% of control in the absence of EHNA and to 17.9% in its presence at 0.1 μM [^{3}H]adenosine, and to 14.6% of control without EHNA and 21.5% of control with EHNA at 5.7 μM [^{3}H]adenosine. The increased binding with EHNA at high adenosine concentrations corresponds to previous findings [4] of a requirement for EHNA to show the low-affinity site in unwashed membranes.

Turkey erythrocyte membranes

Binding of [^{3}H]adenosine to turkey erythrocyte membranes was saturable where both non-linear regression analysis of the saturation curve and Scatchard analysis showed a single binding site, with a dissociation constant of about 3 μM (Fig. 4). Binding at 0.1 μM [^{3}H]adenosine was 42 ± 5% of control in the presence of 50 μM 2′,5′-dideoxyadenosine and 53 ± 6% of control with 100 μM dipyridamole + 50 μM 2′,5′-dideoxyadenosine (mean ± S.D.), although the relative displacement potencies of the various analogs were again unchanged by addition of these agents. Concentrations of N^6-methyladenosine, required to give half-maximal displacement of 0.1 μM

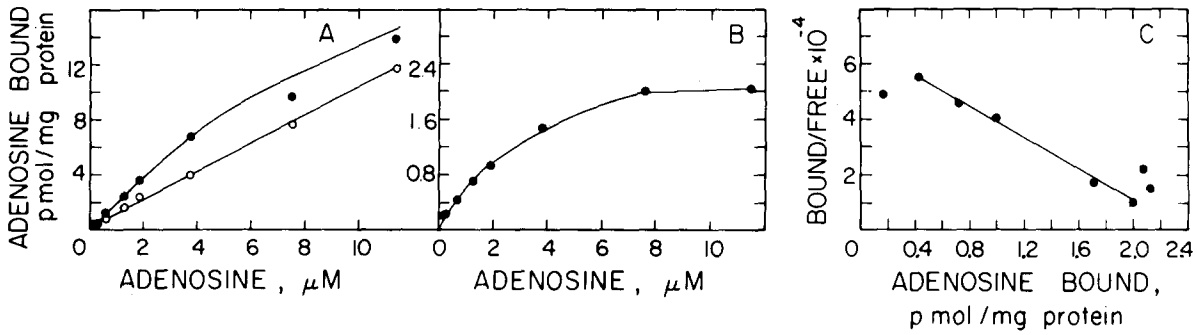

Fig. 4. Saturation analysis of [^{3}H]adenosine binding to turkey erythrocyte membranes. Turkey erythrocyte membranes (240 μg protein) were incubated with 20 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$ and various concentrations of [^{3}H]adenosine from 0.05 to 11.4 μM for 40 min at 37°C. (A) ●——●, total binding; ○——○, non-specific binding determined in the additional presence of 1 mM non-radioactive adenosine. (B) Specific binding derived from the difference between total and non-specific binding in (A). (C) Scatchard analysis of the data in (B). Computed values were K_d 3.25 μM and B_{max} 2.66 pmol/mg protein.

[^{3}H]adenosine binding, were 3.5 μM in the absence of added agents, 7 μM in the presence of 50 μM dipyridamole and 2.3 μM with 50 μM dipyridamole + 50 μM 2′,5′-dideoxyadenosine, but inosine was almost equipotent in all cases (Fig. 5). In the presence of dipyridamole and dideoxyadenosine, and also in the additional presence of 0.2 mM adenine (Figs. 5b and 5c), 2-chloroadenosine was more potent than N^6-methyladenosine, while 2′-deoxyadenosine was also very active, and theophylline and N^6-phenylisopropyladenosine showed little activity. K_i values for both preparations for the various analogs are shown in Table II.

Discussion

The present results cast doubt on the proposal of Schwabe et al. [3] that the high and low affinity binding sites for [^{3}H]adenosine in rat brain repre-

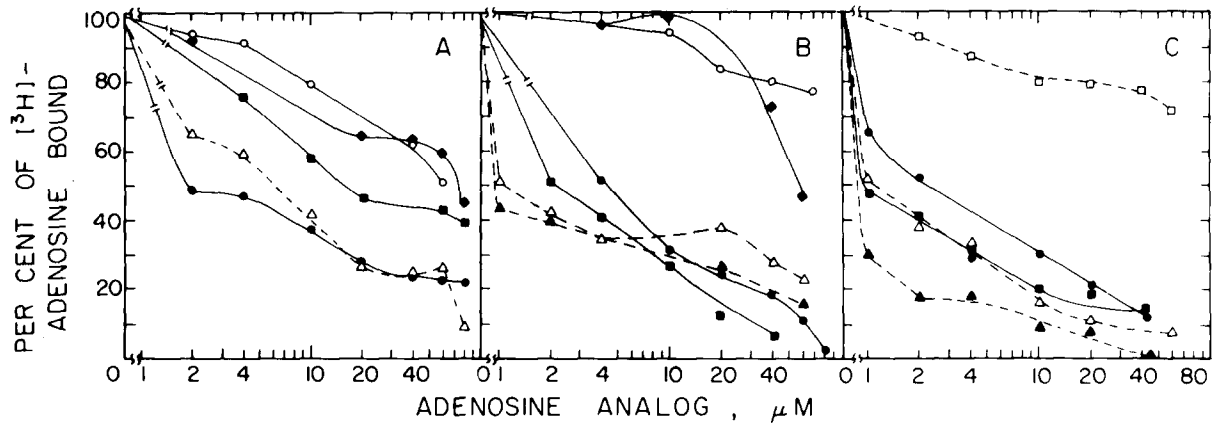

Fig. 5. Displacement of the binding of 0.1 μM [^{3}H]adenosine from turkey erythrocyte membranes. (A) Turkey erythrocyte membranes (≃200 μg protein) were incubated in an assay medium, as described in the legend to Fig. 2, for 40 min at 37°C. Binding in the absence of added agents was 164±38 fmol/mg protein (mean±S.D. of seven observations), and results are given as percentages of this value. (B) As (A), except that incubations were performed in the presence of 50 μM dipyridamole and 50 μM 2′,5′-dideoxyadenosine. Mean binding with these agents was 72±17 fmol/mg protein (mean±S.D. of five observations) or 44% of binding in their absence. (C) As (B), except that incubations were performed in the additional presence of 0.2 mM adenine. Total binding was 50.7 fmol/mg protein or 33% of binding in the absence of any added agents.

TABLE II

K_i VALUES

Experimental conditions were as in the legends to Figs. 2 and 5.

Analog	Turkey erythrocyte		Rat cortex synaptosomal	
	No addition	+50 μM dipyridamole +50 μM 2′,5′-dideoxyadenosine	No addition	+50 μM dipyridamole +100 μM 2′,5′-dideoxyadenosine
N^6-Methyladenosine	3.4	2.2	6.0	15.0
N^6-Phenylisopropyl-adenosine	70.8	56.8	10.1	
2-Chloroadenosine	10.8	3.9	10.8	27.8
Theophylline	66.8		12.0	
Inosine	6.8	1.9	14.3	4.1
2′-Deoxyadenosine	n.d. *		31.6	
2′,5′-Dideoxyadenosine	n.d.		108.3	
IBMX	n.d.	48.5	195.0	

* n.d., not determined.

sent, respectively, the R-site and P-site receptors for adenosine-sensitive adenylate cyclase. Although both the binding constant for adenosine to the high affinity site and the K_i value for its displacement by theophylline agree closely with the values of 0.5 μM for adenosine activation and 20 μM for theophylline inhibition of adenylate cyclase observed by Prémont et al. [5] in rat striatum, the relative efficacies of N^6-phenylisopropyladenosine and N^6-methyladenosine in displacement of [^{3}H]adenosine binding were in inverse order to their potencies as agonists for adenosine-sensitive adenylate cyclase. Furthermore, inosine, which has no activity on adenylate cyclase, was very active even after elimination of the adenosine uptake site by dipyridamole, or addition of EHNA to block any residual deaminase in the preparation. The results obtained with dipyridamole agree with those of Schwabe et al. [3] who obtained 30% inhibition of [^{3}H]adenosine binding at 100 μM, but contrast with the findings in fat cell membranes [1], where it had no effect.

Similar results were obtained for displacement of [^{3}H]adenosine binding from the low affinity site of rat caudate, where the P site analog 2′,5′-dideoxyadenosine was more active than any of the R site analogs but less active than inosine. In the turkey erythrocyte system, in which N^6-phenylisopropyladenosine was equipotent to adenosine in stimulation of adenylate cyclase [6], it was considerably less active than inosine in displacement of bound adenosine. A possible explanation for these results is that the increase in cyclic AMP levels observed with adenosine in brain slices and other systems is not due to direct stimulation of adenylate cyclase but represents a process secondary to interaction of adenosine with a pre-synaptic receptor mediating inhibition of neurotransmitter release [18]. Support for this concept is provided by the fact that Prémont et al. [5] were unable to demonstrate adenosine-activated adenylate cyclase in rat cerebral cortex homogenates, while inosine was active in inhibition of noradrenalin release from rat cerebral cortex slices, an action presumed to occur at a pre-synaptic adenosine receptor [19].

The antagonism of the binding by theophylline could be explained by its interaction with such a receptor, which would account for its inhibition of the action of adenosine on cell firing [20] as well as neurotransmitter release. However, the virtual disappearance of this effect in the presence of dipyridamole and dideoxyadenosine suggests that theophylline has a complex range of actions, among which inhibition of adenosine uptake and inhibition of the adenosine P site cannot be discounted. These effects have both been reported in brain tissue [13,15], although at high (mM) concentrations, and imply that theophylline sensitivity can-

not be considered an effect specific for adenosine receptors coupled to adenylate cyclase.

During the preparation of this manuscript several reports appeared [21–23] in which modified analogs of adenosine were themselves labelled and used to characterize adenosine receptors in brain tissue by binding studies. These results support our conclusion that receptors directly coupled to adenylate cyclase are difficult to identify using [^{3}H]adenosine.

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