Biochemical Pharmacology, Vol. 35, No. 5, pp. 847–856, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00

A NEW CLASS OF ADENOSINE RECEPTORS IN BRAIN

CHARACTERIZATION BY 2-CHLORO[^{3}H]ADENOSINE BINDING

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(*Received* 27 *September* 1984; *accepted* 15 *August* 1985)

Abstract—Micromolar concentrations of adenosine and its analogs have profound depressant effects on neuronal firing and synaptic transmission in many brain areas. Using the adenosine agonist 2-chloro[^{3}H]-adenosine (Cl[^{3}H]Ado), we have identified a distinct class of micromolar-affinity adenosine binding sites in rat forebrain membranes. Specific Cl[^{3}H]Ado binding was reversible and saturable with an apparent K_D of 9.1 μM and a B_{max} of 61 pmoles/mg protein. The present studies were conducted using washed brain membrane fractions *not* treated with adenosine deaminase. Specific Cl[^{3}H]Ado binding under these conditions was insensitive to (-)-N^6-(*R*-phenylisopropyl)adenosine ((-)PIA) and treatment with 3 mM *N*-ethylmaleimide, unlike high-affinity A1 adenosine receptor binding. Treatment of membranes with adenosine deaminase revealed an additional population of binding sites sensitive to (-)PIA. Inhibition of Cl[^{3}H]Ado binding by adenosine analogs exhibited an order of potency ClAdo > 5′-*N*-ethylcarboxamide adenosine (NECA) > (-)PIA which differs from that of both A1 and A2 adenosine receptors. The potent A1 and A2 receptor antagonist 8-phenyltheophylline had no significant effect on binding up to 10 μM. Specific binding, however, was inhibited by the adenosine antagonists 8(*p*-sulfophenyl)theophylline, isobutylmethylxanthine, theophylline, and caffeine. Micromolar Cl[^{3}H]Ado binding was highly selective for adenosine agonists and antagonists. These results suggest that the micromolar-affinity Cl[^{3}H]Ado binding sites may represent a novel central purinergic receptor, distinct from the A1 and A2 adenosine receptors involved in the regulation of adenylate cyclase.

Adenosine and its nucleotides have gained increased recognition in recent years as important modulators of both central and peripheral synaptic transmission [1–3]. Adenosine and related purines are released from stimulated nerve tissue in micromolar concentrations [4, 5]. Micromolar concentrations of adenosine and the metabolically-stable analog 2-chloroadenosine (ClAdo)† have profound depressant actions on synaptic transmission and the spontaneous firing of neurons in many areas of the brain [6–10]. These effects are generally believed to result from presynaptic inhibition of neurotransmitter release, possibly through a reduction in membrane calcium permeability [1, 2, 11–14]. Recently, however, micromolar concentrations of adenosine have been reported to increase postsynaptic K^+ conductances in several nerve preparations [15–17].

Adenosine and its analogs are effective regulators of adenylate cyclase in brain and other tissues causing inhibition at nanomolar concentrations or stimulation at micromolar concentrations via what has been termed the A1 and A2 adenosine receptors [2, 18–21]. A1 receptors in brain have been identified in receptor binding studies using the metabolically-stable synthetic analogs 2-chloro[^{3}H]adenosine, N^6-cyclohexyl[^{3}H]adenosine, and (-)-N^6-(*R*-phenylisopropyl)[^{3}H]adenosine [22–25]. The affinities of these binding sites are in the low nanomolar range and the relative potencies of adenosine analogs in displacing binding are consistent with interactions primarily at A1 receptors. A2 adenosine receptors have been identified in *N*-ethylmaleimide-treated membranes from rat striatum using the potent A2 agonist 5′-*N*-ethylcarboxamide[^{3}H]adenosine [26]. Membranes lacking adenosine-stimulated adenylate cyclase activity, such as from the hippocampus [27], do not contain A2 receptor binding activity [26].

Despite considerable investigation, the exact adenosine receptor subtypes and mechanisms responsible for electrophysiological depression in different brain regions have not been clarified. The best evidence for the involvement of a specific receptor type comes from studies of the CA1 region of the rat hippocampal slice, where the potencies of certain synthetic adenosine analogs correlate well with their activities at A1 receptors [28, 29]. However, inhibition of adenylate cyclase via A1 receptors has only been observed in brain membrane fractions and never in intact slice preparations [18]. Moreover, in the cerebral cortex, electrophysiological data are inconsistent with the involvement of A1 adenosine receptors [30]. More importantly, the issue of what receptor mediates the actions of the endogenous

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† Abbreviations: ClAdo, 2-chloroadenosine; (-)PIA, (-)-N^6-(*R*-phenylisopropyl)adenosine; (+)PIA, (+)-N^6-(*S*-phenylisopropyl)adenosine; NECA, 5′-*N*-ethylcarboxamide adenosine; NCPCA, 5′-*N*-cyclopropylcarboxamide adenosine; ADA, adenosine deaminase; NEM, *N*-ethylmaleimide; Gpp(NH)p, 5′-guanylyl-imidodiphosphate; 8PT, 8-phenyltheophylline; EHNA, erythro-9-[3-(2-hydroxynonyl)]adenine; DMSO, dimethylsulfoxide; and HEPES, *N*-2-hydroxyethylpiperizine-*N*′-2-ethanesulfonic acid.

ligand adenosine has not been addressed. For example, inhibition of cAMP accumulation in brain membranes by adenosine has not been reported since adenosine deaminase is routinely included in assays of A1 receptor activity [31]. As noted above, adenosine and its analogs have a number of biochemical and electrophysiological effects in the CNS. This suggests that the actions of adenosine analogs may be mediated by multiple receptor subtypes in different brain regions.

The present studies were initiated to investigate the existence of membrane-bound adenosine receptor(s) in brain that may be responsible for some of the physiological actions of adenosine on nerve cell firing. We have employed 2-chloro[^{3}H]adenosine (Cl[^{3}H]Ado) as a ligand in these experiments, since it is a water-soluble, metabolically-stable analog of adenosine and a potent depressant of synaptic transmission in brain. Moreover, since the ability of adenosine and ClAdo to inhibit evoked potentials in brain are manifest at micromolar concentrations of these compounds [7–10], we have specifically investigated the binding of Cl[^{3}H]Ado in this pharmacologically-active concentration range. Our results demonstrate the existence of a novel class of micromolar-affinity adenosine binding sites in brain membranes that is distinct from the A1 and A2 adenosine receptors associated with adenylate cyclase.

METHODS

Materials. All compounds were obtained from Sigma except: (-)-N^6-(*R*-phenylisopropyl)adenosine ((-)PIA), (+)-N^6-(*S*-phenylisopropyl)adenosine ((+)PIA), adenosine deaminase, and N^6-cyclohexyladenosine from Boehringer-Mannheim; 5′-*N*-cyclopropylcarboxamide adenosine (NCPCA) from Abbott Laboratories; 5′-*N*-ethylcarboxamide adenosine (NECA) and 8(*p*-sulfophenyl)theophylline from Research Biochemicals; erythro-9-[3-(2-hydroxynonyl)]adenine (EHNA) from Burroughs Wellcome; and α,β-methylene 5′-ADP and 3′,5′-cyclic AMP from P-L Biochemicals. 2-Chloro[^{3}H]-adenosine (Cl[^{3}H]Ado) (9–18 Ci/mmole) was obtained from Moravek Biochemicals.

Membrane preparation. A crude synaptic membrane fraction was prepared from rat brain for use in binding assays. Freshly excised rat forebrains were homogenized in 10 vol. of 0.32 M sucrose buffered with 10 mM HEPES-NaOH, pH 7.0, and containing 0.3 mM phenylmethylsulfonyl fluoride to inhibit endogenous protease activity. This homogenate was centrifuged at 2000 *g* for 10 min to obtain the nuclear (P1) pellet, and the supernatant fraction was then centrifuged at 12,400 *g* for 20 min. The resulting pellet (P2) was resuspended in 20 mM HEPES-NaOH, pH 7.0, with 1 mM $MgCl_2$ to the original homogenate volume and centrifuged at 35,300 *g* for 10 min. This procedure was repeated three times to remove endogenous adenosine from the membrane preparations. For some experiments, the supernatant fraction from the 12,400 *g* spin was centrifuged at 100,000 *g* for 1 hr to obtain a microsomal membrane fraction (P3). The final membrane suspension was aliquoted and quick-frozen with dry ice-acetone. Membrane preparations retained full binding activity when stored for several weeks at −20°. Protein was determined by the method of Bradford [32] using bovine serum albumin as a standard. Almost identical results were obtained by the method of Lowry *et al.* [33] using the same standard.

Cl[^{3}H]*Ado binding assay.* Incubation mixtures for the determination of Cl[^{3}H]Ado binding routinely consisted of 20 mM HEPES, pH 7.0, 1 mM $MgCl_2$, 0.1 to 0.3 μM Cl[^{3}H]Ado (9–18 Ci/mmole), and membrane protein (1.0 to 2.5 mg/ml) in a total volume of 0.4 ml. Reactions were initiated by the addition of tissue, and terminated after 10 min at 20° by pipetting 0.1 ml of the incubation mixture onto each of three Whatman GF/B filters subjected to vacuum with a Millipore model 1225 sampling manifold. Unbound radioactivity was removed by four 5-ml washes of ice-cold 20 mM HEPES-NaOH, pH 7.0, with 1 mM $MgCl_2$ using a Manostat pipettor. The washing procedure was accomplished in less than 5 sec. Filters were placed in vials containing 4 ml of Formula 963 scintillation fluid (New England Nuclear) and allowed to stand overnight to elute the filter-bound radioactivity. Samples were then mixed and counted in a Beckman LS2800 scintillation counter at an efficiency of 53%. All samples were performed in duplicate incubations for a total of six filter replicates. Nonspecific binding was routinely defined as the radioactivity bound in the presence of 1 mM unlabeled ClAdo and represented 50–60% of total binding.

For some experiments, Cl[^{3}H]Ado binding was measured using a modification of the microcentrifugation technique described by Herrup and Shooter [34]. Briefly, reactions were carried out as described above and allowed to incubate for 10 min at 20°. At the end of this time, 0.1 ml of the incubation mixture was layered onto 0.6 ml of 0.6 M sucrose in a microcentrifuge tube. The tubes were then centrifuged at 13,000 rpm for 2 min in a Beckman Microfuge 11. The tubes were removed and immediately frozen with a dry ice–acetone bath. The tips of the tubes containing the brain membrane pellets were severed with a razor blade and placed into 4 ml of Formula 963. Samples were kept overnight to allow the pellets to dissolve and then mixed and counted.

Under standard assay conditions, the concentration of receptor binding sites was less than 2% of the dissociation constant (K_D = 9.1 μM) determined from equilibrium binding experiments. Less than 5% of the radioligand was bound at equilibrium when concentrations of Cl[^{3}H]Ado from 0.1 to 10,000 μM were employed.

Drug solutions. All compounds were solubilized and diluted in distilled water except: (-)PIA, (+)PIA, N^6-cyclohexyladenosine, N^6-phenyladenosine, and 8-phenyltheophylline (8PT) in DMSO; isobutylmethylxanthine in dilute NaOH. Final reaction concentrations of DMSO were 1%. Control incubations received an equivalent volume of distilled water or other solvent.

RESULTS

Characterization of a micromolar-affinity Cl[^{3}H]Ado binding site in brain membranes. Since adeno-

sine and ClAdo depress synaptic transmission in brain in micromolar concentrations [7–10], initial experiments were performed to identify Cl[^{3}H]Ado binding sites in rat brain synaptic membranes with affinities in this pharmacologically-active concentration range. Binding affinity constants were determined by displacement of bound Cl[^{3}H]Ado with increasing concentrations of unlabeled ClAdo (Fig. 1). Data obtained from such displacement curves were analyzed using the model-fitting computer program LIGAND [35]. Nonspecific binding was defined as a program-fitted parameter, computed as the limiting bound/free ratio at infinitely high ligand concentrations. Data points were analyzed for one-, two-, and three-site binding models. The two-site binding model provided the best statistical fit to the data. Kinetic parameters calculated for the two binding sites were K_{D_1} = 11 μM, B_{max_1} = 61 pmoles/mg protein, and K_{D_2} = 7,300 μM, B_{max_2} = 14,470 pmoles/mg. Two additional experiments performed at fewer ligand concentrations than in Fig. 1 provided values of K_{D_1} = 5.2 μM, B_{max_1} = 69 pmoles/mg, K_{D_2} = 823 μM, B_{max_2} = 3,646 pmoles/mg, and K_{D_1} = 12 μM, B_{max_1} = 114 pmoles/mg, K_{D_2} = 1,048 μM, B_{max_2} = 3,273 pmoles/mg. Although the displacement binding data are consistent with the presence of two major classes of Cl[^{3}H]Ado binding sites, we cannot at this time exclude the involvement of negative cooperativity.

To confirm the results of displacement binding studies, saturation experiments were performed using Cl[^{3}H]Ado concentrations in the range of 0.1 to 15 μM. Nonspecific binding was defined in the presence of 1 mM unlabeled ClAdo in order to isolate the higher-affinity micromolar binding site identified in displacement studies. Specific Cl[^{3}H]Ado binding was saturable over the concentration range studied while nonspecific binding increased linearly

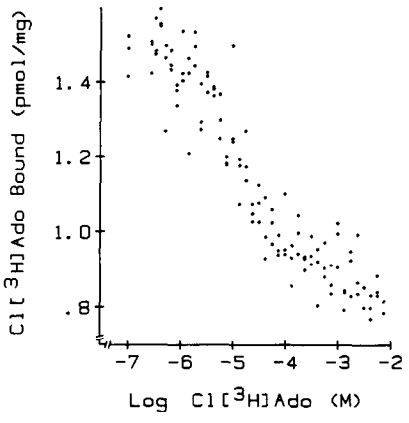

Fig. 1. Displacement of Cl[^{3}H]Ado binding (0.1 μM) to brain membranes by increasing concentrations of unlabeled ClAdo (0.18 to 10,000 μM). Binding was measured using a standard filtration technique (see Methods). Shown are the results of triplicate determinations at forty different ligand concentrations. Data were analyzed by the model-fitting LIGAND computer program (see text). Similar results were obtained in two additional experiments performed at fourteen different ligand concentrations over the same range.

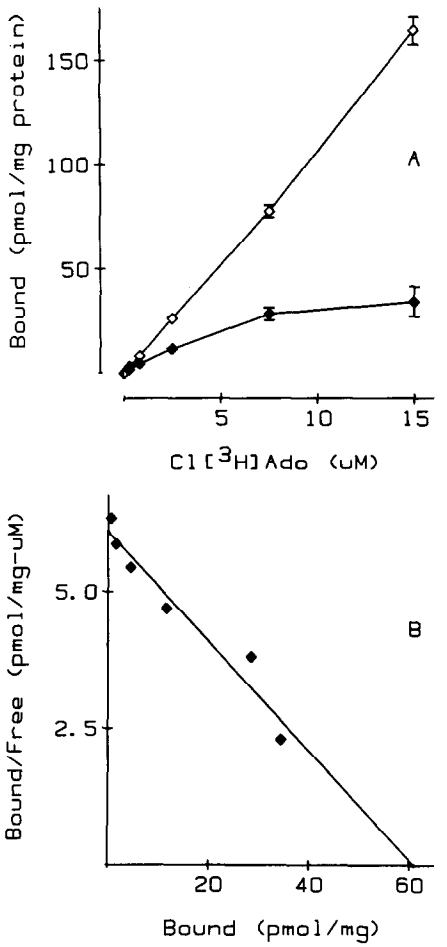

Fig. 2. (A) Saturation of specific Cl[^{3}H]Ado binding to brain membranes. Membranes were incubated under standard conditions with various Cl[^{3}H]Ado concentrations (0.1 to 15 μM). Specific binding (◆) was determined as the difference between total binding and nonspecific binding (◇), defined in the presence of 1 mM unlabeled ClAdo. Data are the means ± S.E.M. (N = 6). (B) Scatchard plot of saturation binding data. Line was determined by linear regression analysis, r = −0.97. K_D = 10 μM and B_{max} = 61 pmoles/mg protein. Similar results were obtained in three additional experiments.

(Fig. 2A). Scatchard plots obtained from these saturation curves (Fig. 2B) provided a K_D of 9.1 ± 1.1 μM and a B_{max} of 61 ± 3.1 pmoles/mg (± S.E.M., N = 4) in good agreement with the results of displacement experiments for the higher-affinity micromolar site (Fig. 1). The K_D for this Cl[^{3}H]Ado binding site is in the same concentration range as that required for ClAdo inhibition of evoked synaptic potentials [7, 10] and neurotransmitter release [36, 37] in brain, and for inhibition of Ca^{2+} conductances in cultured neurons derived from dorsal root ganglion [11] and in superior cervical ganglion neurons [12]. All further data relate to this higher-affinity binding site, since its K_D is in the pharmacologically-relevant concentration range.

Properties of Cl[^{3}H]*Ado binding to brain membranes.* The properties of micromolar-affinity Cl[^{3}H]-Ado binding to rat brain synaptic membranes were

Table 1. Effects of various treatments on Cl[^{3}H]Ado binding to brain membranes

Treatment	Specific binding (fmoles/mg)	Relative binding
Control, no addition	423 ± 71	1
ADA, 2 units/ml	738 ± 98*	1.75
ADA, 20 units/ml	838 ± 73†	1.98
NEM, 3 mM	385 ± 69	0.91
NEM, 30 mM	253 ± 83‡	0.60
Control, 1% DMSO	502 ± 11	1
(-)PIA, 10 μM	472 ± 94	0.94
ADA, 2 units/ml	1002 ± 69§	2.00
ADA, 2 units/ml + (-)PIA, 10 μM	640 ± 77‖	1.27

Brain membranes (2.5 mg/ml) were incubated for 30 min at 20° with the indicated concentrations of adenosine deaminase (ADA) or *N*-ethylmaleimide (NEM). At the end of the incubation period, ADA-treated samples were placed on ice. NEM-treated membranes were rinsed twice in 15 vol. of ice-cold buffer (20 mM HEPES-NaOH, pH 7.0, with 1 mM $MgCl_2$) by resuspension and centrifugation at 35,300 *g* for 10 min. Specific Cl[^{3}H]Ado binding to brain membranes was then determined under standard conditions using 0.1 μM Cl[^{3}H]Ado and 1 mM unlabeled ClAdo to define nonspecific binding. For the second set of reactions, the effects of 10 μM (-)PIA on specific Cl[^{3}H]Ado binding to control membranes and ADA-treated membranes were assessed. Control specific binding was determined in the presence of 1% DMSO since (-)PIA was dissolved in DMSO and diluted 100-fold in binding reactions. Results are the means ± S.E.M. for six determinations (twelve for controls). Essentially identical results were obtained in an additional experiment using a different membrane preparation.

* $P < 0.02$ compared to control, Student's *t*-test (two-tailed).

† $P < 0.01$ compared to control.

‡ $P > 0.1$ compared to control.

§ $P < 0.001$ compared to control.

‖ $P < 0.01$ compared to reaction without (-)PIA.

examined using Cl[^{3}H]Ado concentrations of 0.1 to 0.3 μM. Under these conditions, specific Cl[^{3}H]Ado binding, defined as the difference between total Cl[^{3}H]Ado binding and the binding in the presence of 1 mM unlabeled ClAdo, represented 40–50% of total binding. Maximal displacement of Cl[^{3}H]Ado binding was similar using a number of compounds including ClAdo, adenosine, NECA, and isobutylmethylxanthine, indicating that Cl[^{3}H]Ado was binding to a general population of receptor sites responsive to adenosine agonists and antagonists.

The present studies were conducted using crude synaptic membrane fractions washed several times with buffer to remove endogenous adenosine but *not* treated with adenosine deaminase. It has been demonstrated by several laboratories [22, 23] that pretreatment of washed brain membrane preparations with adenosine deaminase (and its inclusion in binding assays), to metabolize residual adenosine to inosine, is essential for detecting nanomolar-affinity binding to A1 adenosine receptors. Since the objective of our experiments was to characterize the properties of micromolar-affinity adenosine receptors, we routinely employed untreated membrane fractions in order to restrict Cl[^{3}H]Ado binding to these micromolar-affinity binding sites.

Table 1 shows the effects of various treatments on Cl[^{3}H]Ado binding to washed brain membranes. In agreement with the studies described above, we were able to demonstrate a significant enhancement of specific Cl[^{3}H]Ado binding using adenosine deaminase. At 2 units/ml, an increase in binding of 0.32 pmole/mg was observed which is approximately equal to the maximal binding capacity for A1 receptors [22, 23]. This is consistent with the fact that nanomolar-affinity A1 receptors should be saturated at 0.1 μM Cl[^{3}H]Ado. Ten-fold higher levels of adenosine deaminase (20 units/ml) produced some additional increase in specific binding. A 10 μM concentration of (-)PIA had no effect on specific Cl[^{3}H]-Ado binding in untreated membranes but inhibited the increase in binding produced by adenosine deaminase by 73%.

These findings suggest that the enhancement of binding produced by adenosine deaminase is due to the uncovering of nanomolar-affinity A1 receptor sites. Furthermore, these observations indicate that specific Cl[^{3}H]Ado binding in untreated washed brain membranes is to a class of binding sites distinct from the A1 adenosine receptors. This is supported by the fact that treatment of washed brain membranes with 3 mM *N*-ethylmaleimide (NEM), which completely prevents A1 receptor binding [26], had no significant effect on specific Cl[^{3}H]Ado binding. At much higher concentrations (30 mM) a modest inhibition of binding was observed (Table 1), although this was not statistically significant. Furthermore, the non-hydrolyzable GTP analog 5'-guanylyl-imidodiphosphate (Gpp(NH)p) inhibits the binding of adenosine agonists to A1 receptors with an IC_{50} of about 1 μM [22, 24]. This compound had no significant effect on micromolar Cl[^{3}H]Ado binding up to concentrations of 1 mM.

Specific Cl[^{3}H]Ado binding was linear with membrane protein concentration up to 3.0 mg/ml and was

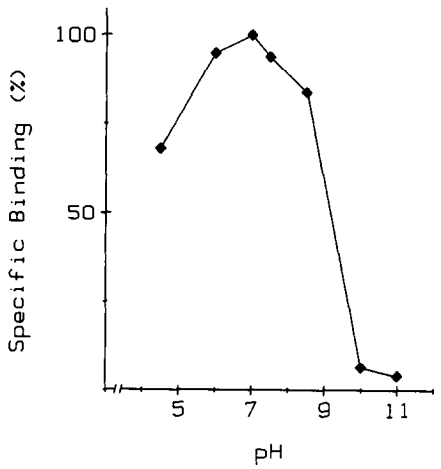

Fig. 3. pH dependency of specific Cl[^{3}H]Ado binding to brain membranes. Specific Cl[^{3}H]Ado binding was determined in the presence of 50 mM Tris-acetate buffer using 0.3 μM Cl[^{3}H]Ado and 1 mM unlabeled ClAdo to define nonspecific binding. Data are expressed as the percentage of maximal specific binding.

sensitive to trypsin. The optimal pH for specific Cl[^{3}H]Ado binding was 7.0 (Fig. 3). Specific binding was maximal at 0°, reduced about 30 and 60% at 20° and 37°, and completely inactivated by boiling (100°) (Fig. 4). A similar enhancement of agonist binding at lower temperatures has been observed for benzodiazepine [38], α-adrenergic [39], and β-adrenergic receptors [40]. Physiological concentrations of Ca^{2+}, Mg^{2+}, Na^+, K^+, and Cl^- had no significant effect on specific Cl[^{3}H]Ado binding when studied in membranes prepared in buffers lacking these ions.

Subcellular and regional distribution of Cl[^{3}H]*Ado binding in brain.* The subcellular distribution of specific Cl[^{3}H]Ado binding (Table 2) is consistent with a role for the micromolar-affinity Cl[^{3}H]Ado binding sites in the regulation of synaptic function in brain, since a high level of binding was observed in the enriched synaptosomal fraction. The microsomal fraction also displayed a high level of specific binding. The nuclear and mitochondrial fractions demonstrated considerably less binding while the crude myelin fraction exhibited intermediate binding activity. It is uncertain whether the binding in crude myelin represents contamination by microsomes or the presence of intrinsic adenosine receptors. A similar degree of enrichment in the synaptosomal compartment has been demonstrated in binding studies of A1 adenosine receptors [23], GABA receptors [42], and acidic amino acid receptors [43, 44]. Significant Cl[^{3}H]Ado binding was observed in all brain regions with the highest levels of specific binding present in the hippocampus and striatum (Table 3).

Kinetics of Cl[^{3}H]*Ado binding to brain membranes.* Figure 5 shows the time course of association of specific Cl[^{3}H]Ado binding to brain membranes. Specific binding reached equilibrium rapidly with an association $t_{\frac{1}{2}}$ of approximately 10 sec. An association rate constant (k_{+1}) of $8.4 \times 10^3\ M^{-1}\ sec^{-1}$ was estimated using the pseudo-first order method [45].

Dissociation of Cl[^{3}H]Ado binding was studied by volumetric dilution as well as by the addition of an excess of unlabeled ClAdo (Fig. 6). Specific Cl[^{3}H]-Ado binding dissociated almost instantaneously (90% at 10 sec) when excess ClAdo (1 mM) was added, while dissociation induced by 100-fold dilution of the incubation with buffer exhibited a $t_{\frac{1}{2}}$ of about 30 sec (k_{-1} = 0.023 sec^{-1}). In both cases, complete dissociation of specific binding was observed. In no instance was a slow component of dissociation observed ($t_{\frac{1}{2}}$ = 23 min) as previously reported for nanomolar-affinity binding of Cl[^{3}H]-Ado to A1 receptors in brain membranes [22]. The acceleration of dissociation in the presence of unlabeled ClAdo could be explained by negative cooperativity, although other interpretations are equally plausible such as the "retention effect" [46].

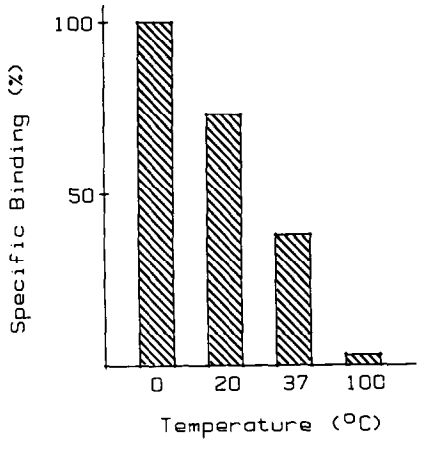

Fig. 4. Temperature dependence of Cl[^{3}H]Ado binding to brain membranes. Reactions were incubated under standard conditions for 10 min at the indicated temperature. For 100°, membranes were boiled for 5 min and then assayed for specific Cl[^{3}H]Ado binding at 20°. Specific Cl[^{3}H]Ado binding was determined using 0.1 μM Cl[^{3}H]Ado and 1 mM unlabeled ClAdo to define nonspecific binding. Data are expressed as the percentage of maximal specific binding and are the means of three separate experiments.

Table 2. Subcellular distribution of Cl[^{3}H]Ado binding

Fraction	Relative binding
Brain homogenate	1.0
Nuclear (P1)	1.1
Crude synaptosomal (P2)	1.9
Mitochondria	0.74
Myelin	1.7
Synaptosomes	3.1
Microsomal (P3)	3.1

Nuclear (P1), crude synaptosomal (P2), and microsomal (P3) fractions were prepared from brain homogenates (see Methods). Subfractions enriched for mitochondria, myelin, and synaptosomes were isolated from P2 preparations using a discontinuous Ficoll-sucrose gradient technique [41]. All fractions were washed as described in Methods for the crude synaptosomal fraction. Specific Cl[^{3}H]Ado binding was measured under standard conditions using 0.1 μM Cl[^{3}H]Ado and 1 mM unlabeled ClAdo to define nonspecific binding. Data are expressed as the specific binding per mg of protein relative to the crude homogenate (422 fmoles/mg). Results are representative of three individual experiments.

Table 3. Regional distribution of Cl[^{3}H]Ado binding

Region	Relative binding
Whole brain	1.0
Striatum	1.2
Hippocampus	1.2
Cortex	0.74
Midbrain-thalamus	0.67
Pons-medulla	0.67
Cerebellum	0.63

Crude synaptic membrane fractions were prepared from the pooled brain regions of twelve rats (see Methods). Specific binding was determined under standard conditions using 0.1 μM Cl[^{3}H]Ado and 1 mM unlabeled ClAdo to define nonspecific binding. Data are expressed as the specific binding per mg of protein relative to whole brain (520 fmoles/mg). Results are representative of three individual experiments.

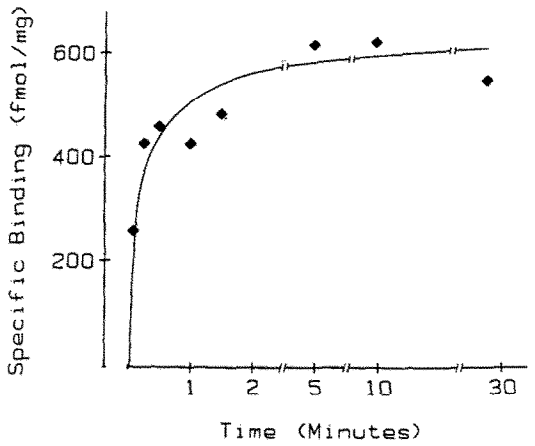

Fig. 5. Time course of association of specific Cl[^{3}H]Ado binding to brain membranes. Membranes (20°, 2.5 mg/ml) were combined with 0.1 μM Cl[^{3}H]Ado at time 0 and assayed for specific binding at various times. Nonspecific binding was defined in the presence of 1 mM unlabeled ClAdo. Data are the means of three to six individual determinations. An association rate constant (k_{+1}) of $8.4 \times 10^3\ M^{-1}sec^{-1}$ was estimated by dividing the initial rate (5 sec) by the free ligand concentration (0.1 μM) and receptor concentration (150 nM) [45]. Similar results were obtained in two additional experiments.

From the kinetic rate constants determined above, an independent estimate of the dissociation constant of 2.7 μM was obtained from the relationship $K_D = k_{-1}/k_{+1}$ [45]. This is in reasonable agreement with

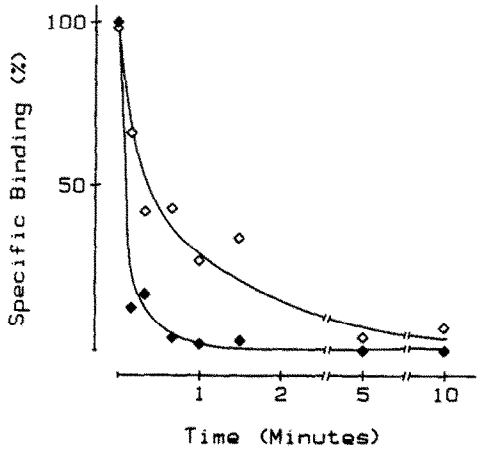

Fig. 6. Dissociation of specific Cl[^{3}H]Ado binding from brain membranes. Membranes (2.5 mg/ml) were incubated with 0.1 μM Cl[^{3}H]Ado for 10 min at 20°. At the end of this time (time 0), dissociation was initiated by the addition of excess unlabeled ClAdo (1 mM) (◆) or by the addition of a 100-fold excess of incubation buffer (◇). For the latter, some reaction aliquots were given an immediate dilution and filtration (time 0). Specific binding was then determined at various times up to 10 min. Nonspecific binding was determined in parallel reactions conducted in the presence of 1 mM unlabeled ClAdo. Data are expressed as the percentage of specific binding at time 0 and are the means of three to six individual determinations. Similar results were obtained in two additional experiments.

Table 4. Effects of adenosine analogs and related compounds on Cl[^{3}H]Ado binding

Compound	IC_{50} (μM)
2-Chloroadenosine	15.3 ± 1.9
NCPCA	65.3 ± 15.8
5'-AMP	126 ± 33
Isobutylmethylxanthine	138 ± 18
Adenosine	145 ± 22
NECA	152 ± 19
3'-5'-Cyclic AMP	181 ± 34
Adenine	392 ± 52
EHNA	431 ± 68
Theophylline	472 ± 73
α,β-Methylene 5'-ADP	513 ± 115
8(p-Sulfophenyl)theophylline	582 ± 109
2'-Deoxyadenosine	583 ± 94
S-Adenosyl-L-homocysteine	767 ± 151
Caffeine	1020 ± 136
Hypoxanthine	>1000
Inosine	>1000
Cytidine	>1000
Uridine	>1000
Thymidine	>1000
Guanosine	>1000
Gpp(NH)p	>1000
Papaverine	>1000
N^6-Phenyladenosine	>1000
(−)PIA	>1000
(+)PIA	>1000
N^6-Cyclohexyladenosine	>1000

Displacement of Cl[^{3}H]Ado binding was measured under standard conditions using 0.1 to 0.3 μM Cl[^{3}H]Ado and 1 mM unlabeled ClAdo to determine specific binding. The IC_{50} values for the various compounds were determined by using four to eleven concentrations of each compound and linear regression analysis of the logit transformation of the binding inhibition data [49]. Results are the mean ± S.E.M. for three to eight separate experiments. None of the compounds tested had significant effects on nonspecific binding. 8-Phenyltheophylline had no significant effect on specific binding up to 10 μM.

The following compounds inhibited Cl[^{3}H]Ado binding less than 25% when tested at 1 mM: glutamate, N-methyl-D-aspartate, glycine, atropine, naloxone, dopamine, epinephrine, norepinephrine, clonidine, γ-aminobutyric acid, serotonin, amitriptyline, chlorpromazine, and verapamil. Less than 25% inhibition was also observed with diazepam (250 μM), nitrendipine (100 μM), bicuculline (250 μM), quinuclidinyl benzilate (250 μM) and tetrodotoxin (50 μM).

the values obtained from both displacement and saturation experiments (Figs. 1 and 2). It should be noted that similarly rapid time courses of association and dissociation have been reported for micromolar-affinity glutamate receptor binding in rat brain synaptic membranes [47, 48].

Structural specificity of Cl[^{3}H]*Ado binding.* A number of adenosine analogs and related compounds were tested for their abilities to displace specific Cl[^{3}H]Ado binding to brain membranes (Table 4). Several adenosine agonists and antagonists such as NECA and isobutylmethylxanthine (IBMX) effectively displaced Cl[^{3}H]Ado binding in a concentration-dependent manner (Fig. 7). Inhibition of Cl[^{3}H]Ado binding (0.1 μM) by these compounds

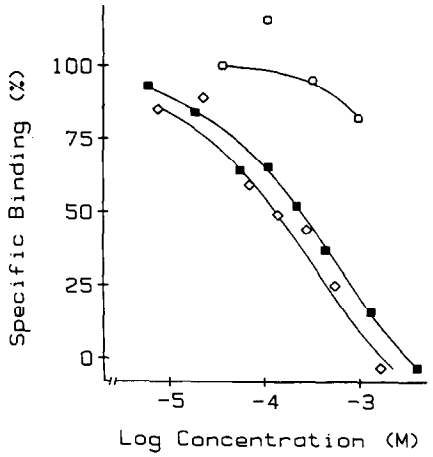

Fig. 7. Inhibition of specific Cl[^{3}H]Ado binding to brain membranes by NECA (◇), IBMX (◆), and (-)PIA (○). Specific Cl[^{3}H]Ado binding was determined under standard conditions using 0.1 μM Cl[^{3}H]Ado and 1 mM unlabeled ClAdo to define nonspecific binding. Data are expressed as the percentage of specific binding in the absence of added compounds, and are the means of three separate experiments.

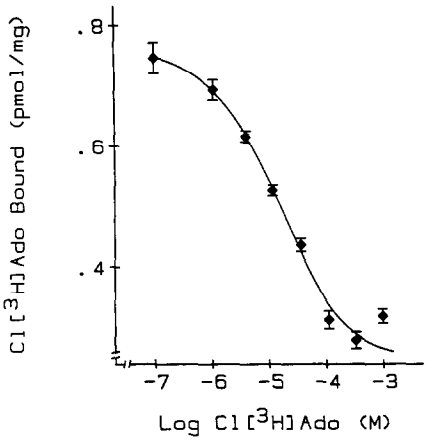

Fig. 8. Displacement of Cl[^{3}H]Ado binding (0.1 μM) to brain membranes by increasing concentrations of unlabeled ClAdo (1–1000 μM). Binding was measured using a microcentrifugation technique (see Methods). Data are the means ± S.E.M. (N = 6). Similar results were obtained in one additional experiment.

was monophasic and complete, suggesting the presence of only one major binding component. Hill coefficients for NECA and isobutylmethylxanthine displacement curves were 1.1 and 0.97 respectively (Fig. 7). NECA was 10-fold less potent than ClAdo in inhibiting binding, although the reverse potency is observed for stimulation of cAMP accumulation via A2 adenosine receptors [18]. The potent A1 receptor agonist (-)PIA weakly displaced specific Cl[^{3}H]Ado binding at the concentrations tested (Fig. 7). 8-Phenyltheophylline (8PT), a potent antagonist of A1 and A2 adenosine receptors [2, 50] had no significant effect on micromolar Cl[^{3}H]Ado binding up to its limit of solubility of 10 μM. A variety of neurotransmitter agonists and antagonists and centrally-active drugs failed to demonstrate effective displacement of micromolar Cl[^{3}H]Ado binding (Table 4).

Effectiveness of filtration assay for studying Cl[^{3}H]-*Ado binding to brain membranes. Comparison to microcentrifugation technique.* The experiments above were quantitated using a conventional vacuum filtration technique (see Methods). The ability of a filtration assay to detect specific radioligand binding to a receptor is determined by the separation and washing time and the $t_{\frac{1}{2}}$ of dissociation for the receptor binding site in question. In the vacuum filtration system we employed, the total time required to separate the fluid phase of a 0.1 ml incubation sample and deliver four ice-cold 5-ml buffer washes was less than 6 sec. From Table 5, it is apparent that one 5-ml buffer wash was highly effective in reducing the amount of free drug adhering to the filter and brain membrane sample. The higher level of specific membrane binding observed with no buffer washes may represent a considerable amount of very low affinity

Table 5. Filtration assay for Cl[^{3}H]Ado binding

	Counts/min bound					
	+ Membrane			− Membrane		
Washes	Total	Nonspecific	Specific	Total	Nonspecific	Specific
0	132,306	115,192	17,114	94,162	93,402	760
1	16,753	10,337	6,416	2,888	3,357	−469
2	12,291	6,125	6,166	1,398	1,000	398
4	10,547	4,920	5,627	588	978	−390

Brain membranes (2.5 mg/ml) were allowed to bind with 0.1 μM Cl[^{3}H]Ado for 10 min at 20° (+ membrane). Parallel incubations were conducted in the absence of brain membranes (− membrane). Reactions were then terminated by filtration of 0.1 ml of sample onto a Whatman GF/B filter followed by the indicated number of ice-cold washes with 20 mM HEPES-NaOH, pH 7.0, containing 1 mM $MgCl_2$. Nonspecific binding was defined as the Cl[^{3}H]Ado bound in the presence of 1 mM unlabeled ClAdo. Specific binding is the difference between total and nonspecific binding. Values represent the means of triplicate determinations.

binding which is rapidly lost during the first wash. Since specific binding was only slightly reduced with four washes compared to one wash, we routinely employed four washes because of the improved consistency in the replicate filters. It should be noted that the observed specific binding of Cl[^{3}H]Ado to rat brain membranes could not be attributed to "specific" binding to the filters themselves since no "specific" binding of Cl[^{3}H]Ado could be demonstrated in the absence of brain membranes (Table 5). Based on the dissociation $t_{\frac{1}{2}}$ for Cl[^{3}H]Ado binding of 30 sec (Fig. 6), it can be calculated that less than 15% of the specific Cl[^{3}H]Ado bound will have dissociated in the 6 sec required to complete the sample separation and washing. Thus, we have demonstrated that this filtration assay can effectively detect Cl[^{3}H]Ado binding in the micromolar concentration range.

To confirm the effectiveness of the filtration assay for studying micromolar-affinity Cl[^{3}H]Ado binding, we examined the binding of Cl[^{3}H]Ado to brain membranes using a microcentrifugation technique (see Methods). Centrifugation assays are often employed to investigate micromolar-affinity receptor binding since they provide more rapid separation times than most filtration systems [45]. We found that displacement curves for Cl[^{3}H]Ado binding using the microcentrifugation assay (Fig. 8) were highly comparable to those obtained using the filtration assay (Fig. 1), in both the apparent affinity of micromolar Cl[^{3}H]Ado binding (about 10 μM) and the maximal amount of displaceable Cl[^{3}H]Ado binding. Nonspecific binding was lower in the centrifugation assay which can be attributed in part to the nonspecific binding to the glass fiber filters in the filtration assay (Table 5).

DISCUSSION

This report demonstrates the presence of a distinct class of micromolar-affinity adenosine binding sites in brain membranes that may represent a functional central adenosine receptor. The affinity of these binding sites is in the same concentration range as that required for depression of evoked synaptic potentials [7–10], inhibition of neurotransmitter release [36, 37, 51, 52], inhibition of Ca^{2+} conductances [11, 12], and stimulation of K^+ conductances [13, 14] in nerve preparations by adenosine and its analogs. Moreover, adenosine and related purines are released endogenously from stimulated brain slices in micromolar concentrations that are sufficient to effectively agonize these binding sites [5]. Therefore, the affinity of these adenosine binding sites is in the pharmacologically- and physiologically-relevant concentration range.

Adenosine analogs regulate adenylate cyclase in brain tissue through what has been termed the A1 and A2 adenosine receptors [2]. Our experiments indicate that the micromolar-affinity Cl[^{3}H]Ado binding sites in brain membranes are distinct from both the A1 and A2 receptors. A1 receptors have been well characterized in receptor binding studies using a variety of radioactive adenosine agonists [22–25]. These studies have demonstrated that nanomolar-affinity A1 receptor binding can only be detected in brain membranes treated with adenosine deaminase to completely degrade residual adenosine [22, 23]. A1 receptor binding in these membrane preparations is completely inactivated by treatment of brain membranes with 3 mM NEM [26] and is inhibited by (-)PIA, 8PT, and Gpp(NH)p [22, 24]. In our experiments using brain membranes not treated with adenosine deaminase, micromolar Cl[^{3}H]Ado binding was unaffected by (-)PIA, 8PT, Gpp(NH)p, or NEM treatment. Moreover, we were able to demonstrate the appearance of a (-)PIA sensitive class of Cl[^{3}H]Ado binding sites by treating our membrane fractions with adenosine deaminase (Table 1). Presumably these are the A1 receptor binding sites previously characterized. Taken together, the above findings indicate that the micromolar-affinity Cl[^{3}H]Ado binding sites described here are a separate class of binding sites that can be studied independently of A1 adenosine receptors through the use of brain membranes *not* treated with adenosine deaminase.

A2 adenosine receptors have been characterized extensively by the effects of adenosine analogs on cAMP accumulation in brain slices and homogenates [2, 18]. The order of potency for several agonists at A2 receptors is NECA> ClAdo> (-)PIA [2]. 8PT is the most potent antagonist of adenosine-elicited accumulations of cAMP with an IC_{50} of approximately 6 μM [50]. In the present studies, we found the order of potency for inhibiting micromolar Cl[^{3}H]Ado binding to be ClAdo > NECA > (-)PIA (Table 4). 8PT had no significant effect on specific Cl[^{3}H]Ado binding up to 10 μM. These results indicate that the micromolar-affinity Cl[^{3}H]Ado binding sites are pharmacologically distinguishable from A2 adenosine receptors.

On the other hand, Yeung and Green [26] recently reported the apparent binding of [^{3}H]NECA to A2 receptors in striatal membranes with a K_D value of 16.9 nM and a B_{max} of 188 fmoles/mg protein. The membranes were treated with NEM to inactivate the A1 receptor binding component. NECA was 3 to 4-fold more potent than ClAdo in inhibiting [^{3}H]-NECA binding in accordance with the relative potencies of these drugs in stimulating adenylate cyclase at A2 receptors. Moreover, 8PT inhibited binding with an IC_{50} of 3.3 μM. A2 receptors could not be demonstrated in hippocampal membranes using [^{3}H]NECA in agreement with previous reports indicating a lack of A2 receptors in membranes prepared from this tissue [27]. This is in contrast to the present data demonstrating an enrichment of Cl[^{3}H]Ado binding in hippocampal membranes (Table 3). The results of Yeung and Green [26] indicate that A2 receptors can be labeled in brain membranes containing adenosine-sensitive adenylate cyclase. However, the density of these A2 receptor sites is approximately 0.3% of the density for the micromolar Cl[^{3}H]Ado binding sites and thus would represent only a small contribution to the overall binding observed in the present experiments.

The Cl[^{3}H]Ado binding site is also distinct from the low-affinity intracellular P adenosine receptor which is inhibitory to adenylate cyclase [2]. 2′-Deoxyadenosine is an effective agonist at the P site whereas ClAdo has little or no activity. 2′-Deoxy-

adenosine, however, was approximately 40-fold less potent than ClAdo in displacing Cl[^{3}H]Ado binding (Table 4). Moreover, P site inhibition of adenylate cyclase is not antagonized by theophylline [2] in contrast to Cl[^{3}H]Ado binding.

The exact receptor(s) and mechanism(s) responsible for depression of neuronal firing and synaptic transmission in the CNS by adenosine are not known. Support for an A1-type receptor comes from studies of transmission in the CA1 region of the hippocampal slice [28, 29]. In these experiments, adenosine analogs inhibit evoked potentials at nanomolar concentrations with a potency order (-)PIA > NECA > ClAdo. However, inhibition of cAMP accumulation by adenosine agonists (the measure of A1 receptor activity) has never been observed in intact slice preparations [18]. Furthermore, the IC_{50} for adenosine of 29 μM is considerably higher than those for the other compounds tested [28]. Adenosine itself has not been shown to inhibit adenylate cyclase activity in brain membranes since adenosine deaminase is routinely included in these assays [31].

Studies of electrophysiological depression in the cerebral cortex are inconsistent with the involvement of A1 adenosine receptors [30]. When applied to spontaneously active neurons, the potent A1 agonist (-)PIA depressed less than half of the cells tested with a very prolonged time course similar to its actions on CA1 cells [28]. NECA depressed 70% of the neurons tested in a rapidly reversible manner. However, NECA was somewhat less potent than adenosine or 5'-AMP in depressing the firing of cerebral cortical neurons. The effects of NECA and (-)PIA were similar whether applied by microiontophoresis or pressure ejection. These complex findings suggest that the majority of cerebral cortical neurons, unlike CA1 neurons, are insensitive to A1 receptor agonists. Cortical cells, however, contain adenosine receptors that respond similarly to NECA, adenosine, and 5'-AMP. Such receptors are unlikely to be A2 adenosine receptors, since NECA is approximately ten times more potent than adenosine in stimulating adenylate cyclase activity [2].

The relative potencies of various adenosine analogs in displacing specific Cl[^{3}H]Ado binding to brain membranes (Table 4) are consistent with the electrophysiological depression of cerebral cortical neurons described above and elsewhere [1, 53, 54]. For example, NECA, adenosine, and 5'-AMP were effective inhibitors of Cl[^{3}H]Ado binding with approximately equal potencies. On the other hand, (-)PIA was a poor inhibitor of Cl[^{3}H]Ado binding (Fig. 7). Phillis and co-workers [1, 53, 54] have characterized the depressant effects of a wide range of adenosine analogs on the firing of cerebral cortical neurons. In their studies, ClAdo was ten to twenty times more potent than adenosine in agreement with our findings that ClAdo was ten times more potent than adenosine in displacing Cl[^{3}H]Ado binding (Table 4). The depressant actions of adenosine and 5'-AMP were antagonized by 8(*p*-sulfophenyl)-theophylline, isobutylmethylxanthine, theophylline, and caffeine [1, 54]. All of these alkylxanthines effectively and completely inhibited specific Cl^{3}H]Ado binding to brain membranes (Fig. 7, Table 4). Compounds with little or no depressant activity, such as guanosine, cytidine, inosine, and *S*-adenosyl-L-homocysteine [1], had weak effects on Cl[^{3}H]Ado binding. It should be noted that the pharmacology of Cl[^{3}H]Ado binding does not correlate with any known enzyme involved in adenosine metabolism [55]. In addition, the potent adenosine uptake inhibitor papaverine [56] only weakly inhibited Cl[^{3}H]Ado binding at a concentration of 1 mM (Table 4).

The presence of micromolar-affinity binding sites for adenosine agonists has been described in rat liver [57] and human platelet [58] membranes. In platelet membranes, Huttemann *et al.* [58] observed both high- and low-affinity binding sites for [^{3}H]NECA with K_D values of 0.16 and 2.9 μM respectively. The properties of high-affinity binding correlated with activation of adenylate cyclase in these membranes by adenosine analogs. Schutz *et al.* [57] found similar binding sites for [^{3}H]NECA in liver membranes. Because of the large capacity of low-affinity [^{3}H]-NECA binding sites (15–30 pmoles/mg), Schutz *et al.* considered these sites to represent non-receptor binding. However, it should be noted that comparable binding capacities have been reported for [^{3}H]glutamate binding to brain membranes [43, 48, 59, 60]. Available data indicate that these high-capacity [^{3}H]glutamate binding sites represent physiological glutamate receptors in brain [43, 48, 59, 60]. The Cl[^{3}H]Ado binding sites described in this paper have a density similar to that of [^{3}H]glutamate binding sites in brain. Our experiments indicate that these Cl[^{3}H]Ado binding sites may represent a physiological class of adenosine receptors that modulate neuronal activity in brain. Whether the Cl[^{3}H]Ado binding sites identified in brain exist in peripheral tissues and represent the sites previously described [57, 58] requires further investigation.

In summary, the experiments presented in this report have provided evidence for a new class of adenosine binding sites in brain. The pharmacological properties of these adenosine binding sites are distinct from those of the A1 and A2 adenosine receptors associated with adenylate cylase. The good correlation between the specificity of these binding sites and the electrophysiological depression of cortical neurons by adenosine analogs suggests a functional role for these binding sites. Further studies will be necessary to determine the precise relationship of this new class of adenosine binding sites to the central actions of adenosine.

Acknowledgements—The helpful discussions of Drs. John F. Tallman, William C. Taft, Jack R. Cooper and Edward Hawrot are appreciated. We are grateful to Walter Mashman for technical assistance. J. H. C. is the recipient of a Medical Scientist Training Program fellowship from the National Institutes of General Medical Sciences. This work was supported by United States Public Health Service Grant NS13532, Research Career Development Award 5-K05 NS-245 from the National Institute of Neurological Communicative Disorders and Stroke, and Air Force Office of Scientific Research Grant 82-0284.

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