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Solubilization of an Adenosine Uptake Site in Brain

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Abstract: Procedures are described for the solubilization of adenosine uptake sites in guinea pig and rat brain tissue. Using [^{3}H]nitrobenzylthioinosine ([^{3}H]NBI) the solubilized site is characterized both kinetically and pharmacologically. The binding is dependent on protein concentration and is saturable, reversible, specific, and high affinity in nature. The K_D and B_{max} of guinea pig extracts are 0.13 ± 0.02 n*M* and 133 ± 18 fmol/mg protein, respectively, with linear Scatchard plots obtained routinely. Similar kinetic parameters are observed in rat brain. Adenosine uptake inhibitors are the most potent inhibitors of [^{3}H]NBI binding with the following order of potency, dilazep > hexobendine > dipyridamole. Adenosine receptor ligands are much less potent inhibitors of binding, and caffeine is without effect. The solubilized adenosine uptake site is, therefore, shown to have virtually identical properties to the native membrane site. The binding of the adenosine A_1 receptor agonist [^{3}H]cyclohexyladenosine ([^{3}H]CHA) to the solubilized brain extract was also studied and compared with that of [^{3}H]NBI. In contrast to the [^{3}H]NBI binding site [^{3}H]CHA binds to two apparent populations of adenosine receptor, a high-affinity site with a K_D of 0.32 ± 0.06 n*M* and a B_{max} of 105 ± 30 fmol/mg protein ,and a lower-affinity site with a K_D of 5.50 ± 0.52 n*M* and B_{max} of 300 ± 55 fmol/mg protein. The pharmacology of the [^{3}H]CHA binding site is consistent with that of the adenosine receptor and quite distinct from that of the uptake ([^{3}H]NBI binding) site. Therefore, we show that the adenosine uptake site can be solubilized and that it retains both its binding and pharmacologic properties in the solubilized state. **Key Words:** Solubilized adenosine uptake sites—Nitrobenzylthioinosine—Adenosine receptors. **Verma A. et al.** Solubilization of an adenosine uptake site in brain. *J. Neurochem.* **45**, 596–603 (1985).

Adenosine and several of its metabolically stable analogs, such as cyclohexyladenosine (CHA) and L-phenylisopropyladenosine (L-PIA), have been found to exert potent effects on nervous tissue activity. These compounds depress nerve cell firing (Phillis et al., 1979), inhibit the stimulus-evoked release of several neurotransmitters (Fredholm and Hedqvist, 1980), modulate adenylate cyclase activity (Daly, 1979; Van Calker et al., 1979; Patel et al., 1981), and induce sedation when administered in vivo (Haulica et al., 1973; Crawley et al., 1982).

Adenosine is released in a depolarization-induced, calcium-dependent manner (Stone, 1981) and its effects are thought to be mediated by specific, high-affinity, cell-surface recognition sites that have been described in brain using radiolabeled stable adenosine analogs (Bruns et al., 1980; Patel et al., 1982*a*). Termination of adenosine action is thought to be regulated primarily by a facilitated reuptake mechanism involving specific elements in the cell membrane (Paterson, 1979; Bender et al., 1980; Barberis et al., 1981). The activity of this adenosine uptake mechanism is important since agents that inhibit this process have been shown to potentiate the neuromodulatory effects of adenosine (Huang and Daly, 1974; Phillis et al., 1979; Crawley et al., 1983; Phillis and Wu, 1983*a*). Inhibition of adenosine uptake in peripheral tissue is thought to be the mechanism of action of vasoactive drugs such as dilazep and dipyridamole (Sano, 1974). Such an inhibition may also be partially responsible for the actions of centrally active drugs such as benzodiazepines and phenothiazines (Phillis and Wu, 1983*a*).

Recent investigations examining adenosine uptake sites directly have employed binding studies using [^{3}H]nitrobenzylthioinosine ([^{3}H]NBI), which is a potent, selective inhibitor of adenosine uptake

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Abbreviations used: CHA, cyclohexyladenosine; NBI, nitrobenzylthioinosine; L-PIA, L-phenylisopropyladenosine; PMSF, phenylmethylsufonyl fluoride.

into both nervous (Barberis et al., 1981) and non-nervous tissue (Pickard and Paterson, 1972; Brajeswar et al., 1975). [^{3}H]NBI has been shown to bind specifically, reversibly, and with high affinity to adenosine uptake sites in erythrocytes (Jarvis and Young, 1980), HeLa cells (Lauzon and Paterson, 1977), and brain (Marangos et al., 1982). Hammond et al., in studies using human erythrocytes (Hammond et al., 1982) and guinea pig cortical tissue (Hammond and Clanachan, 1982, 1983), have shown that displacement of [^{3}H]NBI binding by a test compound was indicative of that compound's ability to block adenosine uptake into these tissues. Thus, the measurement of site-specific binding of [^{3}H]NBI seems to offer a convenient probe for studying molecular interactions at adenosine uptake sites just as the stable radioactive adenosine analogs do for adenosine receptors.

One of the fundamental approaches in understanding the biochemistry of these membrane-bound sites is their solubilization in a functional state. Several recent studies have used this approach to examine adenosine receptors in brain (Gavish et al., 1982; Bruns et al., 1983; Nakata and Fujisawa, 1983). Using [^{3}H]NBI and cyclohexyl[^{3}H]adenosine ([^{3}H]CHA) as probes, we now report successful solubilization of both adenosine uptake sites and receptors from guinea pig brains. [^{3}H]NBI-labeled uptake sites are characterized and are seen to retain pharmacological and binding kinetic properties similar to those described in membrane bound preparations.

MATERIALS AND METHODS

Membrane preparation

Frozen rat or guinea pig whole brains were suspended in 10 volumes of cold 50 m*M* Tris-HCl buffer (pH 7.5) containing 10 m*M* EDTA, 100 *M* phenylmethysulfonyl fluoride (PMSF), and 1 m*M* 1,10-phenathroline using a Brinkman Polytron (speed 5 for 30 s). The suspension was centrifuged at 30,000 *g* for 20 min and the resulting pellets were suspended in 10 volumes of 50 m*M* Tris-HCl (pH 7.5) and spun again as above. The following pellets were resuspended in Tris buffer and incubated with 2 U/ml of adenosine deaminase at room temperature for 30 min. The suspensions were centrifuged again and the final pellets were stored at −70°C. Pellets thus stored were found to be stable for several weeks.

Detergent solubilization

Solubilization of membrane pellets was accomplished using the method described by Bruns et al. for solubilizing various receptors (Bruns et al., 1983). Briefly, 1 g (tissue wet weight) of membrane pellet was thawed and suspended in 20 volumes of 50 m*M* Tris-HCl and centrifuged at 30,000 *g* for 20 min. Using a pasteur pipet, the washed pellet was finely resuspended in 2 ml of solubilization buffer containing 10 m*M* Tris-HCl, 1 m*M* dithiothreitol (DTT), and either 20% glycerol or no glycerol at 0°C. Two milliliters of solubilization buffer with 1% CHAPS detergent was added, and the suspension was swirled gently for 30 s and centrifuged at 145,000 *g* for 1 h in a Beckman L8-55 ultracentrifuge using a 45-Ti rotor. The supernatant was removed with a pasteur pipet and passed through a Millipore 0.22 μ*M* filter. The filtrate was either assayed directly for binding or stored at −20°C. To test the effect of glycerol on stability of the solubilized extracts, the preparation extracted without any glycerol was adjusted to contain either 5 or 20% glycerol. Subsequently, all binding experiments were done with supernatant that had been adjusted to contain 20% glycerol after the extraction.

[^{3}H]NBI binding assay

Solubilized extracts (0.3–0.4 mg of protein) were incubated in the presence of [^{3}H]NBI (17 Ci/mmol, Moravek Biochem) in 50 m*M* Tris-HCl buffer (pH 7.4) (final volume of 0.5 ml) for 30 min at 22°C. Assays were terminated by rapid vacuum filtration through Whatman GF-B filters that had been presoaked for 1–24 h in 0.3% polyethylenimine (Sigma). The filters were subsequently washed quickly with 4 × 3 ml washes of ice-cold Tris buffer. Filters were air-dried and counted in 10 ml of Redi-Solv scintillation fluid (Beckman). Nonspecific binding was determined by adding 5 μ*M* NBI (Calbiochem) in the assay and routinely represented 10% or less of the total binding at 0.7 n*M* [^{3}H]NBI. Specific binding was determined by subtracting the nonspecific binding values from total binding values. Scatchard analyses were performed by incubating the soluble extracts with [^{3}H]NBI concentrations ranging from 0.05 to 2.5 n*M*. Inhibition studies were done by incubating the extracts in the presence of six different concentrations of inhibitor and 0.7 n*M* [^{3}H]NBI. All binding data are expressed as specific binding and all assays were performed in triplicate. K_I values were calculated using the relationship $K_I = IC_{50}/(1 + [L]/K_D)$.

[^{3}H]CHA binding assay

[^{3}H]CHA binding to soluble extracts was measured in a manner similar to that described above for [^{3}H]NBI binding, except that the incubation was performed at 22°C for 2 h. Nonspecific binding was determined by incorporating 50 μ*M* CHA (Calbiochem). Scatchard analysis was performed by incubating extracts with [^{3}H]CHA (25 Ci/mmol) concentrations ranging from 0.40 to 25.0 n*M*. Inhibition studies were done using 5 n*M* [^{3}H]CHA. For calculating K_I values the K_D value for [^{3}H]CHA binding was taken as 1 n*M* rather than using the component high- and low-affinity K_D values. All protein determinations were done using the Biorad protein assay.

Verapamil was a gift of Knoll Pharmaceuticals and nimodipine of Miles Pharmaceuticals. In experiments utilizing nimodipine, procedures were performed in darkened rooms. Dilazep was a gift of Hoffmann La Roche (Nutley), CHA was obtained from Calbiochem, and L-PIA and all other chemicals used were obtained from Sigma.

RESULTS

Binding characteristics

The site-specific binding of [^{3}H]NBI to soluble extractions from guinea pig and rat brains was proportional to the protein concentration in the assay over the range of 0.04–0.60 mg protein/ml as shown

in Fig. 1. Saturation of binding was achieved above a protein concentration of 0.60 mg/ml. In all experiments performed, final protein concentrations ranged between 0.30 and 0.40 mg/ml.

Figure 2 shows the effect of glycerol in stabilizing the activity of solubilized guinea pig extractions. Preparations extracted with 20% glycerol in the solubilization buffer and those that were reconstituted with 20% glycerol after extraction in glycerol-free buffer showed no loss of specific binding activity over a 2-week period. Reconstituting glycerol-free extractions with 5% glycerol led to a loss of about 30% of the initial specific binding after a 2-week period. In the absence of glycerol, preparations were found to retain only 50% of the initial specific binding after 1 week and after 2 weeks almost 90% of the activity was lost. Preparations extracted with 20% glycerol included in the solubilization buffer were cloudy or translucent in appearance, even after filtration through 0.22-μm filters whereas the preparations to which glycerol was added only after the extraction procedure were completely clear (data not shown).

The time course for the association and dissociation of [^{3}H]NBI binding to guinea pig brain solubilized extractions is shown in Fig. 3a. The specific binding of 0.45 n*M* [^{3}H]NBI was 50% complete after approximately 3 min and the binding reaction equilibrium was attained by 20 min. The observed forward rate constant (k_{obs}) calculated from the slope of the line in Fig. 3b was 0.143 min^{-1} and the dissociation rate constant (k_{-1}), obtained from the slope of the line in Fig. 3c was 0.046 min^{-1}. K_1, as determined using the equation $K_1 = (k_{obs} - k_{-1})/$ ([^{3}H]NBI) (Williams et al., 1976), was 2.2×10^8 min^{-1} M^{-1}. An estimate of the equilibrium dissociation constant (K_D) obtained as the ratio k_{-1}/k_1 was 0.21 n*M*.

The saturation isotherm and Scatchard analysis of [^{3}H]NBI binding to soluble guinea pig brain extracts are shown in Fig. 4. The specific binding was saturable with an apparent K_D of 0.13 ± 0.02 n*M* and B_{max} value of 150 ± 35 fmol/mg protein. Similar plots were obtained in examining [^{3}H]NBI binding to soluble extracts from rat brain with a K_D value of 0.12 ± 0.03 n*M* and B_{max} of 133 ± 18 fmol/mg protein. All Scatchard plots performed for [^{3}H]NBI showed monophasic profiles indicating the presence of only one class of binding sites.

Kinetic analyses performed for the binding of the adenosine receptor agonist [^{3}H]CHA to soluble guinea pig brain extracts are shown in Fig. 5. Saturable, specific binding of [^{3}H]CHA, in contrast to [^{3}H]NBI binding, revealed biphasic Scatchard plot,

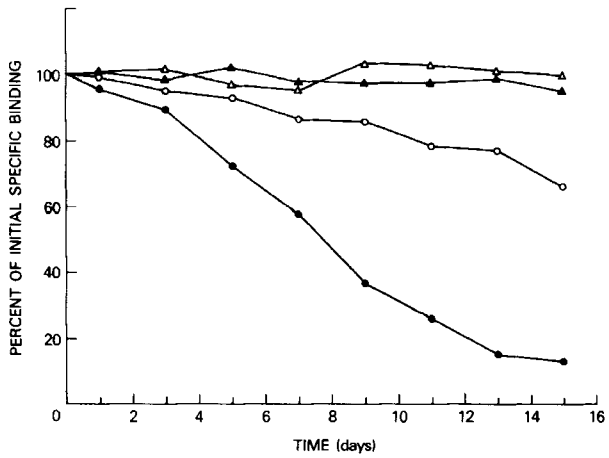

FIG. 2. Effect of glycerol on the stability of [^{3}H]NBI binding to soluble guinea pig brain extracts. Preparations were extracted with (△) and without 20% glycerol present in the solubilization buffer. Those extracted without any glycerol were reconstituted with either 0% (●), 5% (○), or 20% (▲) glycerol after extraction and stored in aliquots at −20°C. Assays for specific binding were done as described in Materials and Methods for 2 weeks. Each point reflects the percent initial (day 0) specific binding remaining after various storage times. The experiment was repeated twice with similar results.

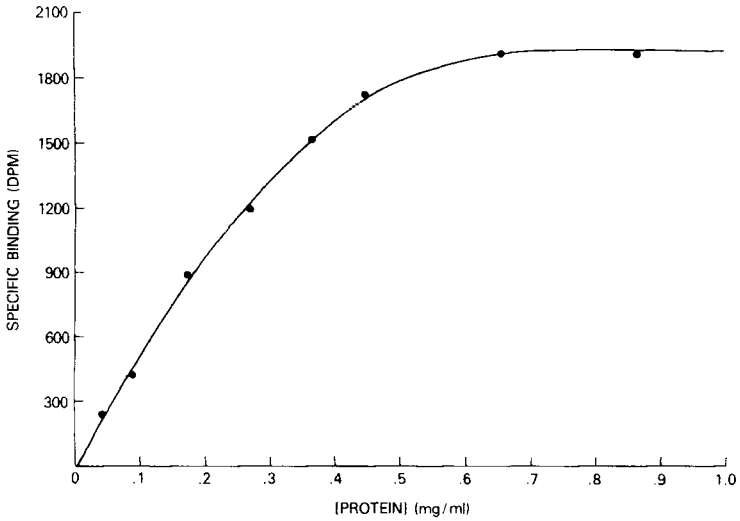

FIG. 1. Effect of protein concentration on the site-specific binding of [^{3}H]NBI to guinea pig soluble brain extracts. [^{3}H]NBI (0.7 n*M*) was incubated for 30 min at 22°C in 50 m*M* Tris-HCl buffer (pH 7.5) adjusted to contain the indicated protein concentrations (abscissa) in the presence and absence of 5 μ*M* NBI. Site-specific binding of [^{3}H]NBI (ordinate) was determined as described in Materials and Methods. Each point is the average of two experiments performed in triplicate.

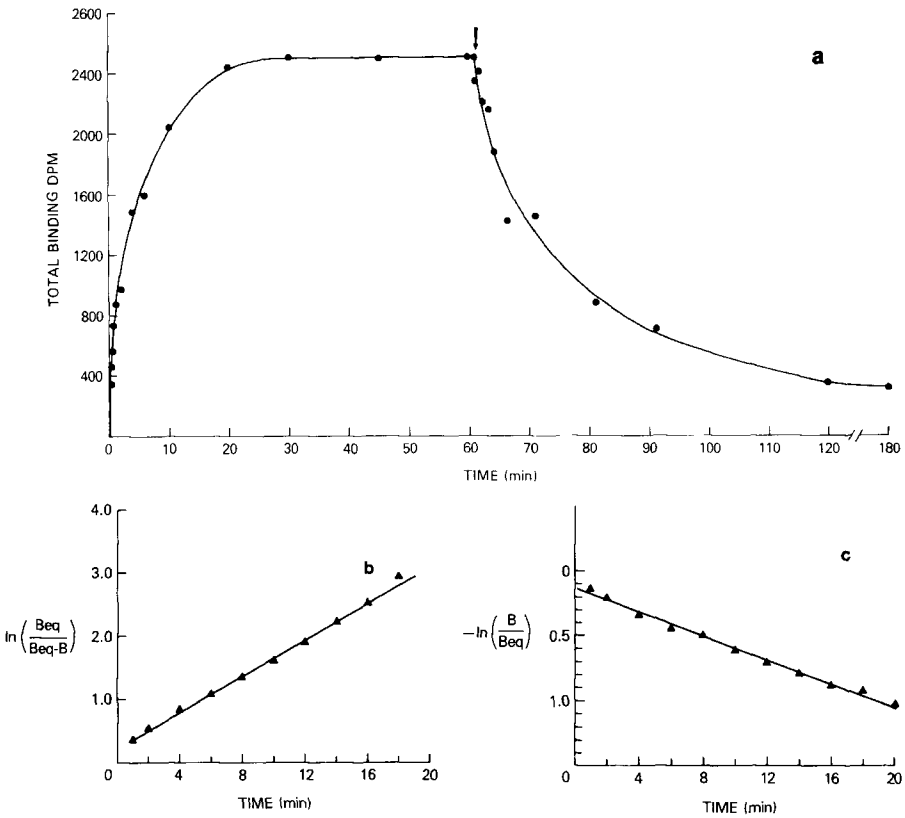

FIG. 3. Reversibility of [^{3}H]NBI binding to soluble guinea pig extracts. **a:** Solubilized extracts were incubated with [^{3}H]NBI (0.45 n*M*) with aliquots (0.3 mg) filtered at the indicated times. Unlabeled NBI (5 μM) was added at the point shown by the arrow; each data point is the total binding. The experiment was repeated three times with very similar results. **b:** Kinetic analysis of association of [^{3}H]NBI binding. **c:** Dissociation of [^{3}H]NBI binding. The dissociation constant (k_{-1}) was calculated from the slope of the line in c as 0.046 min^{-1}. The rate of association (k_{obs}) was calculated from the slope of the line in b as 0.143 min^{-1} and K_I was determined using the equation $K_I = (k_{obs} - k_{-1})/[^3H]NBI$ as 2.2×10^8 min^{-1} M^{-1}. Beq represents specific binding at equilibrium and B represents specific binding at time *t*.

suggesting heterogeneous high- and low-affinity binding sites for [^{3}H]CHA. Apparent K_D values for the two [^{3}H]CHA binding sites were 0.32 ± 0.06 n*M* and 5.50 ± 0.52 n*M*, and B_{max} values were found to be 105 ± 30 and 300 ± 55 fmol/mg protein, respectively. Preincubation of membrane pellets with adenosine deaminase prior to solubilization did not influence [^{3}H]NBI binding constants whereas [^{3}H]CHA binding showed a strict dependence on this procedure (data not shown).

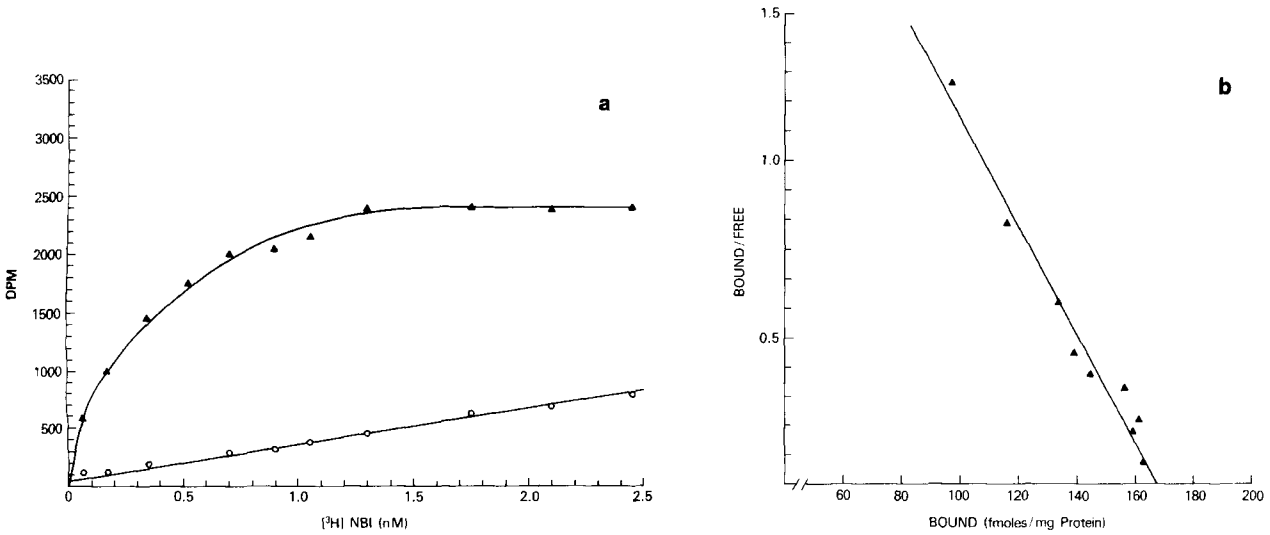

FIG. 4. Saturation isotherm and Scatchard plot for the binding of [^{3}H]NBI to soluble guinea pig brain sites. **a:** Soluble extracts (0.4 mg/ml protein) were incubated with graded concentrations of [^{3}H]NBI at 22°C in the absence and presence (○) of 5 μM NBI. Specific binding (▲) is defined as the total binding minus the nonspecific binding component. This plot is typically representative of eight similar experiments performed in triplicate. **b:** The Scatchard plot shows a single class of binding sites (linear regression line) with a K_D of 0.13 n*M* ± 0.06 and a B_{max} of 150 ± 35 fmol/mg protein.

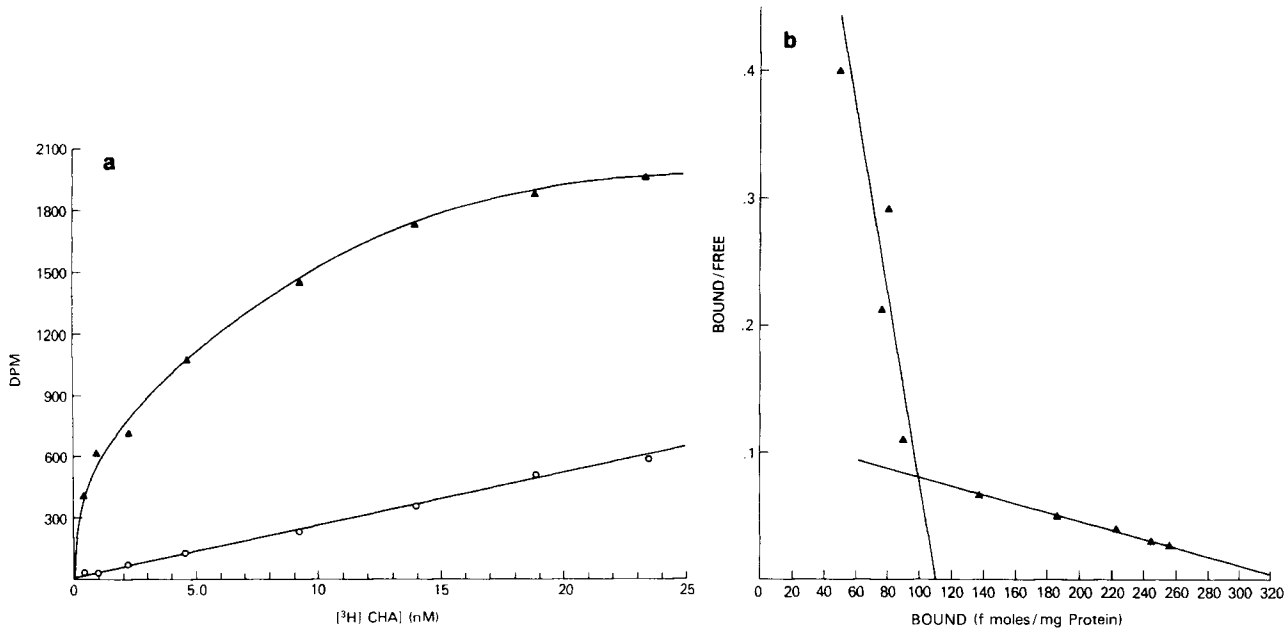

FIG. 5. Saturation isotherm and Scatchard plot for the binding of [^{3}H]CHA to soluble guinea pig brain sites. Soluble extracts (0.4 mg/ml protein) were incubated with graded concentrations of [^{3}H]CHA at 22°C and pH 7.5. **a:** Specific (total minus nonspecific) and nonspecific binding is expressed in dpm. The saturation plot is typical of four experiments performed in triplicate. **b:** The biphasic Scatchard plot is one of four experiments performed. K_D values for the two soluble binding sites were determined to be 0.32 ± 0.06 n*M* and 5.5 ± 0.52 n*M*. B_{max} values for the high- and low-affinity sites were 105 ± 30 fmol/mg protein and 300 ± 55 fmol/mg protein, respectively.

Pharmacology of binding

Table 1 shows the pharmacology of [^{3}H]NBI binding in rat and guinea pig brain extracts and that of [^{3}H]CHA binding in guinea pig brain extracts. NBI was a potent displacer of [^{3}H]NBI binding in both guinea pig and rat preparations with K_I values in the low nanomolar range. The potent adenosine uptake inhibitors dilazep, hexobendine, and dipyridamole were all powerful inhibitors of [^{3}H]NBI binding in guinea pig preparations with low nanomolar K_I values. Of the three uptake inhibitors only dilazep was a potent displacer of binding in both rat and guinea pig extracts. Hexobendine and dipyridamole had a much lower affinity for the soluble rat sites showing K_I values of 312.5 and 1,084 n*M*, respectively. The order of potency of these compounds, however, was similar in both species (dilazep, hexobendine, dipyridamole).

Adenosine was a much poorer displacer of [^{3}H]NBI binding in both species with K_I values in the high micromolar range. The adenosine receptor agonists CHA and L-PIA displayed K_I values of about 1 μ*M* whereas caffeine was a very poor inhibitor in both species with K_I values well above 400 μ*M*. Diazepam, a benzodiazepine, had low micromolar K_I values in both species as did the dihydropyridine Ca^{2+} antagonist nimodipine. Verapamil, a nondihydropyridine Ca^{2+} antagonist, had a much lower affinity for [^{3}H]NBI sites than nimodipine.

In contrast to the [^{3}H]NBI binding site, the [^{3}H]CHA binding site in guinea pig preparations showed a high affinity for the adenosine analogs CHA and L-PIA with low nanomolar K_I values for these compounds. Of the adenosine uptake inhibitors, NBI was the strongest displacer of [^{3}H]CHA binding with a K_I value of 1.4 μ*M*. The other uptake inhibitors tested showed much higher micromolar K_I values as did the adenosine antagonist caffeine (73 μ*M*).

DISCUSSION

It is becoming quite clear that adenosine serves as a major modulator of physiologic function in the cardiovascular system (Berne, 1980) and in both the peripheral (Burnstock, 1975) and central nervous system (Phillis and Wu, 1981). The ubiquitous presence of this purine, and its role in many metabolic processes has, however, made it difficult to characterize the mechanism of adenosine action in brain.

[^{3}H]NBI binding has been a useful probe for adenosine uptake sites in erythrocytes and HeLa cells because occupation of these sites by [^{3}H]NBI correlates well with inhibition of nucleoside transport and because this high-affinity binding is competitively displaced by other potent nucleoside transport inhibitors (Cass et al., 1974; Lauzon and Paterson, 1977; Jarvis and Young, 1980). Similar studies using rat brain tissue (Marangos et al., 1982) and guinea pig cerebral cortical tissue (Hammond

TABLE 1. *Pharmacology of [^{3}H]NBI and [^{3}H]CHA binding to soluble rat and guinea pig brain extracts*

Inhibitor	[^{3}H]NBI (K_I)		[^{3}H]CHA (nM)
	Rat	Guinea pig	Guinea pig
Adenosine uptake inhibitors			
NBI	0.59 ± 0.33	1.86 ± 0.44	1,369 ± 155
Dilazep	37.5 ± 5.6	11.46 ± 3.61	52,885 ± 6,799
Hexobendine	312.5 ± 103.2	31.30 ± 4.62	105,769 ± 13,598
Dipyridamole	1,084 ± 110	36.12 ± 7.56	51,923 ± 8,159
Adenosine and analogs			
Adenosine	92,000 ± 1,500	87,900 ± 1,700	
CHA	739.6 ± 77.34	726.56 ± 77.34	5.00 ± 1.02
L-PIA	968.8 ± 31.3	950 ± 17.5	6.41 ± 1.11
Caffeine	400,000	400,000	73,100 ± 21,000
Benzodiazepines			
Diazepam	6,250 ± 563	9,200 ± 1,350	
Ca^{2+} Antagonists			
Nimodipine	5,078 ± 391	6,075 ± 550	
Verapamil	46,875 ± 15,600	39,500 ± 4,700	

Inhibitory potency of various agents on the binding of [^{3}H]NBI and [^{3}H]CHA to solubilized brain sites. Inhibition of [^{3}H]NBI binding was performed in both rat and guinea pig preparations and that for [^{3}H]CHA in guinea pig alone. Six concentrations of each agent were tested for their effect on specific (total − nonspecific) binding of [^{3}H]NBI and [^{3}H]CHA. IC_{50} values were determined from semilog plots and the K_I values derived as described in Materials and Methods. Each experiment was repeated three times with values representing means ± SD.

and Clanachan, 1982, 1983) show [^{3}H]NBI to be a good probe for central adenosine uptake sites as well.

This study demonstrates that methods used to solubilize brain adenosine receptors (Bruns et al., 1983) can also be used to solubilize adenosine uptake sites from brain using [^{3}H]NBI as a probe. These sites, solubilized from guinea pig and rat brains, retain characteristics similar to those reported for [^{3}H]NBI binding sites in insoluble brain membrane preparations from these respective species (Hammond and Clanachan, 1982, 1983; Marangos et al., 1982).

Using the procedure described by Bruns et al. we obtained good yields of solubilized adenosine receptors and uptake sites. However, this procedure gave cloudy preparations due to the presence of glycerol in the solubilization buffer, and brought into question the solubility of the preparation. Even though our preparations were centrifuged at 145,000 g for 1 h, the density of glycerol in our samples affected the centrifugation process. Thus, it was uncertain whether the binding sites were solubilized. Removing glycerol from the solubilizing buffer gave us a clear preparation that still contained active binding sites but lost its activity rapidly. By extracting the sites without any glycerol and then reconstituting the supernatant with glycerol after the extraction, we were able to obtain clear, soluble preparations that were also stable. To ensure solubility, all extractions were passed through a 0.22 μM filter before assaying. The use of glycerol in this manner was necessary for stability and is thus recommended for such extractions.

In the present study, we found that [^{3}H]NBI binding sites solubilized from rat and guinea pig brains had similar kinetic binding parameters. The binding was rapid in both cases and the K_D value for [^{3}H]NBI binding to rat brain extracts as determined by Scatchard mass law analysis (K_D = 0.12 nM) was similar to K_D values for guinea pig soluble sites as determined separately by Scatchard (K_D = 0.13 nM) and kinetic analyses (K_D = 0.21 nM). The total number of binding sites was similar in both cases (rat, B_{max} = 133 fmol/mg protein; guinea pig, B_{max} = 150 fmol/mg protein) and linear Scatchard plots indicated the presence of only one class of binding site in both species.

In binding displacement studies NBI was found to be the most potent inhibitor of [^{3}H]NBI binding to soluble extracts from both species. In guinea pig preparations the coronary vasodilators dilazep, hexobendine, and dipyridamole were all very potent, competitive inhibitors of [^{3}H]NBI binding. These compounds produce their dilatory effect via an inhibition of adenosine uptake leading to the accumulation of endogenous adenosine (Sano, 1974). The K_I values reported here for soluble guinea pig sites are similar to those reported for inhibition of [^{3}H]NBI binding in human erythrocytes (Clanachan et al., 1981), HeLa cells (Paterson et al., 1980), and dog heart and brain membrane preparations (Marangos et al., 1984). In rat brain soluble extracts, however, of the three adenosine uptake inhibitors,

only dilazep was a strong displacer of [^{3}H]NBI binding. Hexobendine and dipyridamole maintained the low affinity for soluble rat [^{3}H]NBI binding sites that they display in rat insoluble brain membrane preparations (Marangos et al., 1982; Patel et al., 1982*b*; Wu and Phillis, 1982). Although the reason for their markedly lower potency in rat brain is at present unclear, it is important to note that the order of potency of these uptake inhibitors for displacing [^{3}H]NBI binding (dilazep > hexobendine > dipyridamole) was the same in extractions from both species.

It has been suggested that part of the anxiolytic actions of the benzodiazepines may involve inhibition of adenosine uptake (Wu et al., 1981). In our soluble preparations diazepam, which has a very high affinity for benzodiazepine binding sites (K_I value of 7.4 n*M*) (Mohler and Okada, 1978) showed a much lower affinity for the [^{3}H]NBI binding sites extracted from rat and guinea pig brains (K_I values of 6 μ*M* and 9 μ*M*, respectively). These data suggest that if diazepam does indeed inhibit adenosine uptake, it probably does not do so by interacting at the adenosine uptake site that is labeled by NBI.

Conversely, in light of the discrepancies described in rat, compounds inhibiting [^{3}H]NBI binding should be examined for their effects on [^{3}H]adenosine uptake as well. An opportunity to examine such a correlation is provided by dihydropyridine calcium antagonist drugs such as nimodipine. Although in our solubilized rat and guinea pig brain extractions nimodipine was only slightly better as an inhibitor of [^{3}H]NBI binding (K_I = 5–6 μ*M*) than diazepam, this and other dihydropyridines have been shown to have nanomolar K_I values for [^{3}H]NBI sites in dog heart and brain (Marangos et al., 1984) and in human brain as well (Verma and Marangos, 1985). It will be interesting to examine the effect of these calcium antagonists on the uptake of [^{3}H]adenosine as well as their ability to modulate adenosine action. Verapamil, a nondihydropyridine calcium antagonist was a far poorer inhibitor of [^{3}H]NBI binding.

NBI does not interact with the adenosine receptor (Marangos et al., 1982, 1983) and the adenosine analogs CHA and L-PIA showed poor affinity for soluble [^{3}H]NBI binding. In addition, when the binding of the adenosine agonist [^{3}H]CHA to soluble guinea pig brain extracts was examined, a much different kinetic profile was obtained than that for [^{3}H]NBI binding. [^{3}H]CHA is known to label adenosine A_1 receptor sites, and the biphasic Scatchard plot as well as the two K_D values determined for the low- and high-affinity soluble [^{3}H]CHA binding sites in our study are similar to those reported previously for insoluble rat brain preparations (Patel et al., 1982*a*; Marangos et al., 1983). L-PIA and CHA, in contrast to their poor action at the [^{3}H]NBI site, were found to be highly potent inhibitors of [^{3}H]CHA binding to soluble extractions. NBI and the other adenosine uptake inhibitors, on the other hand, were very poor displacers of [^{3}H]CHA binding. These data indicate that [^{3}H]CHA is a rather specific probe for the solubilized adenosine receptor, just as [^{3}H]NBI appears to be a very specific probe for solubilized adenosine uptake sites.

The availability of such distinct probes for adenosine receptors and uptake sites and the ability to use separate and simple assays to study both sites should greatly enhance the understanding of the adenosine system in brain as well as aid in the development of new drugs to affect this system. Solubilization of both sites as described in this study will help in the isolation and further characterization of these two sites.

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