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Characterization of Nitrobenzylthioinosine Binding to Nucleoside Transport Sites Selective for Adenosine in Rat Brain[1]

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Abstract

Nucleoside transport sites in rat brain membrane preparations were labeled with [^{3}H]nitrobenzylthioinosine ([^{3}H]NBI), a potent inhibitor of nucleoside transport systems. The membranes contained a single class of very high affinity binding sites with K_D and B_{max} values of 0.06 nM and 147 fmol/mg of protein, respectively. The displacement of [^{3}H]NBI binding by various nucleosides, adenosine receptor agonists and antagonists, and known nucleoside transport inhibitors was examined. The K_i values (micromolar concentration) of [^{3}H]NBI displacement by the nucleosides tested were: adenosine, 3.0; inosine, 160; thymidine, 240; uridine, 390; guanosine, 460; and cytidine, 1000. These nucleosides displayed parallel displacement curves indicating their interaction with a common site labeled by [^{3}H]NBI. The nucleobases, hypoxanthine and adenine, exhibited K_i values of 220 and 3640 μM, respectively. Adenosine receptor agonists exhibited moderate affinities for the [^{3}H]NBI site, whereas the adenosine receptor antagonists, caffeine, theophylline, and enprofylline, were ineffective displacers. The K_i values for cyclohexyladenosine, (+)- and (−)-phenylisopropyladenosine, 2-chloroadenosine, and adenosine 5′-ethylcarboxamide were 0.8, 0.9, 2.6, 12, and 54 μM, respectively. These affinities and the rank order of potencies indicate that [^{3}H]NBI does not label any known class of adenosine receptors (i.e., A_1, A_2, and P). The K_i values of other nucleoside transport inhibitors were: nitrobenzylthioguanosine, 0.05 nM; dipyridamole, 16 nM; papaverine, 3 μM; and 2′-deoxyadenosine, 22 μM. These results indicate that [^{3}H]NBI binds to a nucleoside transporter in brain which specifically recognizes adenosine as its preferred endogenous substrate. This ligand may aid in the identification of CNS neural systems that selectively accumulate adenosine and thereby control "adenosinergic" function.

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The biochemical characterization and cellular location of sites involved in transporting biologically active substances across cytoplasmic membranes have been greatly facilitated by the use of radioligands which bind to these sites. Ligands which label neurotransmitter uptake sites have been especially useful for identifying neuronal systems releasing particular transmitters. The identification of neuronal uptake sites that contribute to terminating the transmitter actions of, for example, norepinephrine, serotonin, and glutamic acid has been achieved using [^{3}H]desipramine (Lee et al., 1982), [^{3}H]imipramine (Langer et al., 1980), and [^{3}H]glutamic acid (Fonnum, 1984), respectively. Recently, the binding of [^{3}H]nitrobenzylthioinosine ([^{3}H]NBI), a very potent nucleoside transport inhibitor to guinea pig (Hammond and Clanachan, 1983) and rat (Marangos et al., 1982b; Wu and Phillis, 1982) brain membrane preparations, was examined and it was proposed that [^{3}H]NBI labels adenosine uptake sites (Marangos et al., 1982b). The presence of these sites in brain tissue could be of considerable significance with respect to the putative CNS transmitter role of adenosine (Phillis and Wu, 1981), and [^{3}H]NBI may be a valuable marker for determining this role. Other indicators of the functional role of adenosine in the CNS include the association of specific high affinity adenosine receptors in brain and spinal cord with specific neural systems (Goodman and Snyder, 1982; Goodman et al., 1983; Wojcik and Neff, 1983; Geiger et al., 1984a, b); the existence of high affinity neuronal uptake mechanisms for adenosine (Bender et al., 1980, 1981; Thampy and Barnes, 1983a); depolarization-induced, calcium-dependent adenosine release (Stone, 1981); the selective distribution of 5′-nucleotidase, the enzyme responsible for formation of adenosine from 5′-adenosine monophosphate (Schubert et al., 1979); and the discrete localization of neurons in the CNS containing adenosine deaminase, a major degradative enzyme for adenosine (Nagy et al., 1984).

The utility of [^{3}H]NBI as a marker for adenosine uptake sites where adenosine may have its relevant actions depends, of course, on its selectivity. That [^{3}H]NBI labels functional nucleoside transport systems in the periphery appears well established (Plagemann and Wohlhueter, 1980; Paterson et al., 1981; Young and Jarvis, 1983). As a rule, [^{3}H]NBI binding has been identified only on cells that have functional nucleoside transport sites. Cass et al. (1974) found that occupancy of transport sites by NBI correlated with the decreased transport of nucleosides and that the density of functional transport sites correlates on a sites per cell basis with the transport system's maximal velocity (V_{max}). This has been shown for erythrocytes from sheep (Jarvis et al., 1982), fetal lambs (Jarvis and Young, 1982), and humans (Cass et al., 1974); guinea pig and rat heart preparations (Williams et al., 1984), HeLa cells (Dahlig-Harley et al., 1981); and an adenosine-resistant clone (AE_1) of S49 lymphoma cells (Cass et al., 1981).

The nature of the nucleoside transport system labeled by [^{3}H]NBI in brain, however, is less clear. For example, the concentration of NBI necessary to inhibit adenosine uptake (transport plus metabolism) into rat and guinea pig cortical synaptosomes was found to be

approximately 3 to 4 orders of magnitude greater than the K_i value for displacing [^{3}H]NBI from synaptosomal membranes (Wu and Phillis, 1982; Phillis and Wu, 1983). Moreover, displacement studies using the adenosine transport inhibitors, dipyridamole and dilazep, yielded biphasic curves, indicating an interaction with more than one class of binding sites (Hammond and Clanachan, 1984). As there appear to be some important and potentially significant differences between the CNS and peripheral nucleoside transporters labeled by [^{3}H]NBI (Marangos et al., 1982b; Wu and Phillis. 1982), we re-examined the binding characteristics of this ligand to rat brain membranes. Here, we confirm and extend the observations that [^{3}H]NBI labels a nucleoside transport system in rat brain that specifically and selectively recognizes adenosine as the preferred endogenous substrate. These results have implications for the role of rapid reuptake mechanisms for terminating the actions of adenosine.

Materials and Methods

Tissue preparation. Fresh whole brains, excluding the brainstem and cerebellum, were obtained from adult male Sprague-Dawley rats sacrificed by decapitation. The brains were quickly excised, weighed, and placed into 10 vol of ice-cold Tris-HCl buffer (50 mM, pH 7.4). The tissue was homogenized using a Polytron set at medium speed for 15 sec, centrifuged for 10 min, at 35,000 × *g*, resuspended in Tris buffer, and centrifuged as above. This final pellet was resuspended in Tris buffer to a final concentration of 1.5 to 2.0 mg of protein/ml. Protein was determined in aliquots of this final suspension by the method of Lowry et al. (1951) using bovine serum albumin as standard.

[^{3}H]NBI binding assay. [^{3}H]NBI binding site saturation studies were conducted in a total volume of 1 ml of Tris buffer containing 0.025 to 2.5 nM [^{3}H]NBI (16.1 Ci/mmol), 150 to 200 μg of membrane protein in the absence or presence of 5 μM unlabeled NBI. Specific displaceable [^{3}H]NBI binding was determined by subtracting nonspecific binding (presence of NBI) from total [^{3}H]NBI binding (absence of NBI) values obtained from assays performed in duplicate. In a typical experiment using 0.6 nM [^{3}H]NBI, the values for total and nonspecific binding were 2,188 and 375 cpm/mg of protein, respectively. Similar values for nonspecific binding were obtained using 5 μM of either nitrobenzylthioguanosine (NBG) or dipyridamole. The potencies for various compounds displacing [^{3}H]NBI (0.6 nM) were calculated from data plotted on semi-log graphs. The displacing concentrations used for NBI, NBG, and dipyridamole ranged from 0.05 to 10,000 nM, and those for all other compounds tested ranged from 1.0 to 10,000 μM. The samples were incubated for 15 min at 24°C and the reactions were terminated by rapid filtration through Whatman GF/B filters under reduced pressure. The filters were subsequently washed quickly with 3 ml, followed by a single 10-ml wash, of ice-cold Tris buffer. The filters were placed in scintillation vials, and 10 ml of ACS scintillation fluid (Amersham) were added; following at least a 6-hr interval, the filters were counted in a Beckman LS8100 scintillation counter at 38% efficiency. The nonlinear, multipurpose curve-fitting program LIGAND (Munson and Rodbard, 1980) was used to determine dissociation constant (K_D) and maximal number of binding sites (B_{max}) values from the saturation data. The IC_{50} values were calculated as that amount of displacer necessary to inhibit the specific displaceable binding of [^{3}H]NBI by 50%. K_i values were subsequently determined according to the Cheng and Prusoff (1973) equation, $K_i = IC_{50}/(1 + L/K_D)$, where K_D is the dissociation constant for [^{3}H]NBI binding. L represents the concentration of [^{3}H]NBI used in the displacement study, and IC_{50} is the calculated value for the particular displacer compound.

Materials. The following chemicals were obtained from Research Biochemicals Inc. (Wayland, MA): N^6-cyclohexyladenosine, and (−)- and (+)-N^6-phenylisopropyladenosine. 2′-Deoxyadenosine was purchased from P-L Biochemicals (Milwaukee, WI), and [^{3}H]nitrobenzyl-6-thioinosine was from Moravek Biochemicals Inc. (Brea, CA). 2′-Deoxycoformycin (Pentostatin), adenosine 5′-ethylcarboxamide, and enprofylline were kindly supplied by Dr. L. Klienman, National Cancer Institute (Silver Spring, MD), Dr. E. L. Waroch, Abbott Laboratories (Chicago, IL), and Dr. C. G. A. Persson, AB Draco (Lund, Sweden), respectively. All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Results

A representative [^{3}H]NBI saturation binding study using freshly prepared brain membrane preparations is illustrated in Figure 1. The isotherm indicates that the binding sites were saturated under the conditions employed. Iterative nonlinear analysis of the data (*inset* to Fig. 1) demonstrated a single class of very high affinity sites with a K_D of 0.06 nM and a B_{max} of 147 fmol/mg of protein. The same values were obtained whether the incubations were at 4°C (45 min), 24°C (15 min), or 37°C (10 min).

The maximum binding of [^{3}H]NBI to membranes from rat brain was reached within the first 2 min of incubation at 24°C. However, our assays were routinely incubated for 15 min to ensure the attainment of equilibrium and optimal levels of binding. The rapidity of the binding and reports in the literature on the use of NBI as a photoaffinity probe (Marangos et al., 1982a; Shi et al., 1984) led us to examine, as a precautionary measure for subsequent displacement studies, the effects of light on the binding process. When NBI (0.05 to 10,000 nM) was used to displace 0.6 nM [^{3}H]NBI from rat brain membrane preparations incubated either in the dark or exposed to 65 lux fluorescent lighting, the displacement curves were identical, giving K_i values of 0.05 nM (data not shown). [^{3}H]NBI binding was similarly unaffected by changing the pH of the incubation media from 6.0 to 9.0. The effects of endogenous adenosine depletion on [^{3}H]NBI binding was assessed by preincubating the tissue with 1.0 international unit of adenosine deaminase (Sigma, type VII) for 30 min at 37°C, centrifuging the membrane preparation, resuspending the pellet in Tris buffer, and conducting NBI displacement studies. This procedure has previously been shown to eliminate the influence of endogenous adenosine on the binding of radioligands to adenosine receptors. No differences in the K_i values for NBI were observed between the two different tissue preparations (data not included).

The very high affinity of this ligand for the transport sites necessitated calculation of both IC_{50} and K_i values. With a K_D of 0.06 nM and the need to use 0.6 nM [^{3}H]NBI for the displacement studies to give an adequate signal, the IC_{50} values for unlabeled competitors would be shifted approximately one order of magnitude to the right on a semi-log plot of the data. For example, the IC_{50} value for NBI displacing 0.6 nM [^{3}H]NBI was approximately 0.6 nM, whereas this IC_{50} value, recalculated in terms of K_i (0.05 nM), was similar to the K_D (0.06 nM) of [^{3}H]NBI binding.

The results from displacement studies for the nucleosides and nucleobases tested are illustrated in Figure 2, and the IC_{50} and K_i values are listed in Table I. Adenosine, which inhibited the binding of [^{3}H]NBI with a K_i of 3.0 μM, was 55 times more potent than inosine, the nucleoside next in potency. Thymidine, uridine, guanosine, and cytidine were, respectively, 82, 130, 155, and 333 times less potent than adenosine. The nucleobases, hypoxanthine and adenine, also displaced [^{3}H]NBI. However, although hypoxanthine exhibited a displacing potency comparable to some nucleosides, the competition curves for both nucleobases were non-parallel with that of adenosine.

To determine the extent of [^{3}H]NBI interaction with adenosine receptors, various adenosine receptor agonists and antagonists were tested; the displacement curves are shown in Figure 3. The adenosine receptor agonists cyclohexyladenosine (CHA), (+)- and (−)-phenylisopropyladenosine (PIA), 2-chloroadenosine, and adenosine 5′-ethylcarboxamide (NECA) all inhibited the binding of [^{3}H]NBI with parallel displacement curves. At a concentration of 1 mM, the adenosine antagonists caffeine and enprofylline did not affect [^{3}H]NBI binding, whereas theophylline reduced specific binding by only 12%. At similar concentrations various adenine and guanine nucleotides failed to compete for [^{3}H]NBI binding sites. The nucleoside transport inhibitors NBI and NBG were equipotent, whereas dipyridamole and the opiate derivative, papaverine, were less potent in competing for [^{3}H]NBI binding sites (Table I).

Discussion

The present investigation was designed to address the issue of whether [^{3}H]NBI binds to nucleoside transport sites having as broad a nucleoside substrate specificity in rat brain as that reported for peripheral tissues (Cass et al., 1981; Dahlig-Harley et al., 1981; Williams et al., 1984), or labels adenosine uptake sites as suggested

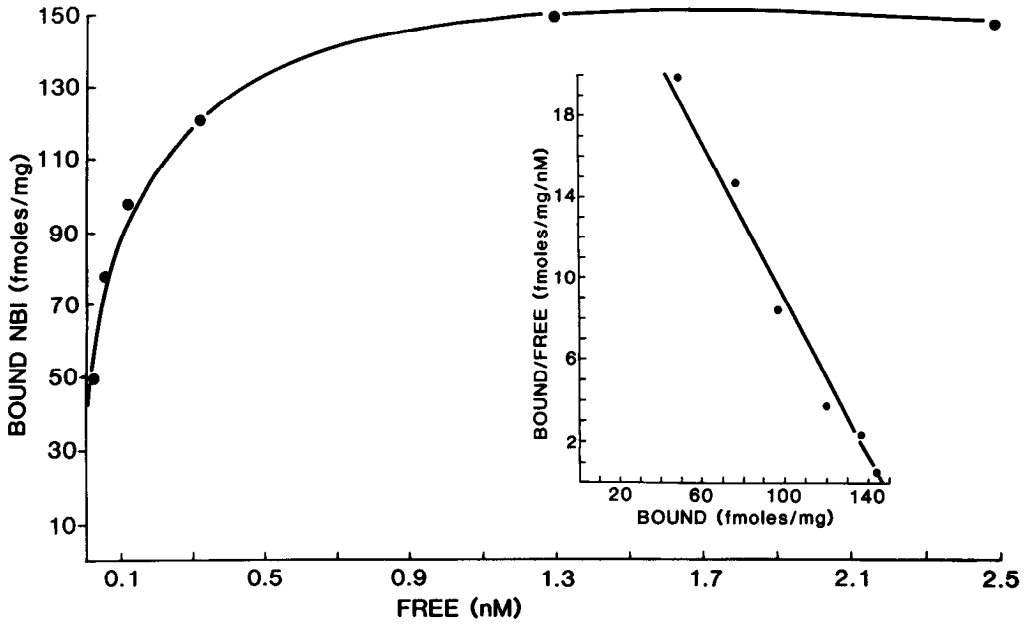

Figure 1. Saturation analysis of [^{3}H]NBI binding to membrane preparations of rat brain. Specific [^{3}H]NBI binding to membranes from whole rat brain (minus the cerebellum and brainstem) is illustrated as the saturation isotherm and Scatchard plot (*inset*). [^{3}H]NBI binding in the presence of 5 μM NBI (nonspecific binding) was subtracted from total binding values (absence of unlabeled NBI) to determine levels of specific displaceable binding. All assays were performed in duplicate, and this figure is representative of similar experiments conducted five times. The K_D and B_{max} values were derived from computer analysis of the data according to the program LIGAND (Munson and Rodbard, 1980). A single class of high affinity binding sites was observed with values for K_D and B_{max} of 0.06 nM and 147 fmol/mg of protein, respectively.

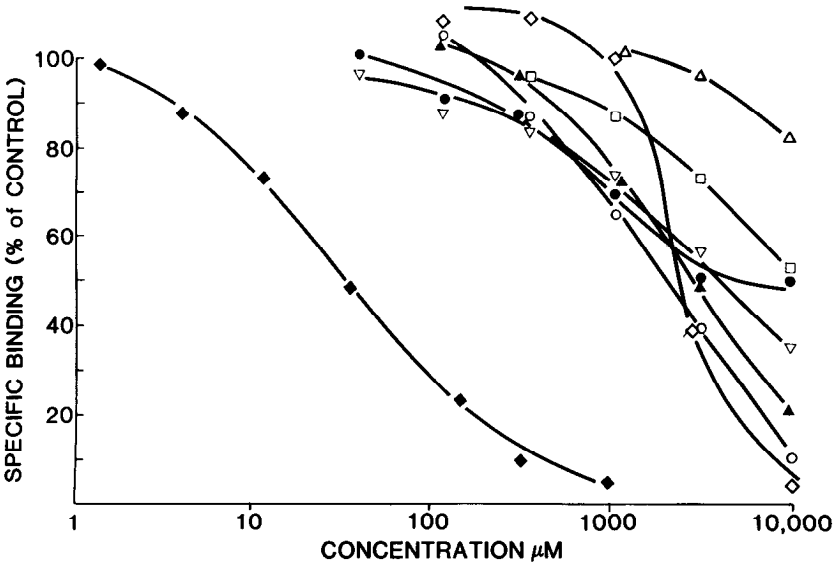

Figure 2. Displacement of [^{3}H]NBI binding to membranes from rat brain by various nucleosides and nucleobases. IC_{50} values for adenosine (◆), inosine (○), thymidine (▲), uridine (▽), guanosine (●), cytidine (□), hypoxanthine (◇), and adenine (△) were determined using 0.6 nM [^{3}H]NBI and 8 to 10 concentrations of the displacers. Control binding values represent the amount of [^{3}H]NBI binding displaceable by 5 μM NBI. These results are representative of experiments performed in duplicate and conducted at least twice with similar results. Note the parallel nature of the displacement curves for the nucleosides and not the nucleobases.

by Marangos et al. (1982b). This issue requires a close examination of information available on nucleoside transporters in peripheral and CNS tissues. Nucleoside transport is a facilitated diffusion system, which, at least in cultured cells, can transport a variety of nucleosides. However, certain methodological problems have been encountered in attempts to fully characterize this apparently simple carrier-mediated transport system. These include the high turnover rate of the nucleoside transporter; the non-concentrative nature of the system which limits the "measurable signal;" the confounding of results by intracellular metabolism, since the K_m values of, for example, adenosine transport and adenosine kinase are similar, and the "awkward" mathematical modelling of the system (Wohlhueter et al., 1979). Bender et al. (1981), for example, found that, within 1 min, adenosine metabolites represented approximately 50% of the [^{3}H]adenosine accumulated by rat brain synaptosomes. Under more controlled conditions, Thampy and Barnes (1983a), using ATP-depleted primary cultures of neurons derived from chick embryo brain, found that incubation times of 5 to 25 sec were required to limit metabolism of newly accumulated [^{3}H]adenosine to less than 1%. Thus, it is not surprising that the measurement of nucleoside transport in CNS tissue has been difficult and the available data subject to different interpretations. This, then, provides the impetus for defining a radioligand binding system which can be used in identifying and characterizing such systems.

NBI reportedly binds to functional nucleoside transport sites in erythrocytes from various species (Cass et al., 1974; Jarvis and Young, 1982; Jarvis et al., 1982), cultured cells (Cass et al., 1981; Dahlig-Harley et al., 1981), and heart preparations (Williams et al., 1984). That NBI interacts with nucleoside transporters is further supported by the close correlation between K_i values for compounds that inhibit [^{3}H]NBI binding and transport of nucleosides. Furthermore, NBI decreases the transport of uridine in intact human erythrocytes as a direct consequence of binding site occupation (Cass et al., 1974). Thus, it is clear from the literature that [^{3}H]NBI binding sites are only found in tissues which possess functional nucleoside transporters. However, it is uncertain whether NBI binds to the substrate recognition site, a closely related site (an allosteric modifier) (Young and Jarvis, 1983), or whether more than one type of nucleoside transporter exists (Paterson et al., 1981). These uncertainties may be particularly important when considering differences between central and peripheral nucleoside transport systems.

In the present study, [^{3}H]NBI bound with very high affinity to a single class of binding sites in rat brain membrane preparations with K_D and B_{max} values of 0.06 nM and 147 fmol/mg of protein, respec-

TABLE I

IC_{50} and K_i values of various compounds for displacement of [^{3}H]NBI binding to rat brain preparations

Values represent means of experiments performed in duplicate (graphically represented in Figs. 2 and 3) and replicated with similar results. The K_i values were calculated using the Cheng and Prusoff (1973) equation ($K_i = IC_{50}/1 + L/K_D$) where L represents the concentration of [^{3}H]NBI used (0.6 nM) and K_D is the equilibrium dissociation constant for [^{3}H]NBI binding to rat brain membrane preparations (0.06 nM). The following compounds at up to 1 mM had no effect on [^{3}H]NBI binding: GMP, GDP, GTP, AMP, ADP, ATP, cAMP, dibutyryl cAMP, 2-deoxycoformycin, caffeine, and enprofylline. Binding of [^{3}H]NBI was inhibited 12% by 1 mM theophylline.

Displacer	IC_{50} Values	K_i Values
Nucleosides and Nucleobases (μM)		
Adenosine	33	3.0
Inosine	1,800	160
Thymidine	2,700	240
Uridine	4,300	390
Guanosine	5,100	460
Cytidine	11,000	1,000
Hypoxanthine	2,400	218
Adenine	40,000	3,640
Adenosine Analogues (μM)		
CHA	8.8	0.8
(+)-PIA	10.2	0.9
(−)-PIA	29	2.6
2-Chloroadenosine	135	12.0
NECA	590	54
Other Inhibitors (nM)		
NBI	0.56	0.05
NBG	0.56	0.05
Dipyridamole	175	16.0
Papaverine	33,000	3,000
2′-Deoxyadenosine	245,000	22,000

tively. These values are very similar to the K_D (0.05 nM) and B_{max} (113 fmol/mg of protein) values reported by Wu and Phillis (1982). However, these workers also observed a lower affinity site with K_D and B_{max} values of approximately 190 μM and 1 nmol/mg of protein, respectively. We did not observe a second class of [^{3}H]NBI binding sites in any of our studies. A single class of [^{3}H]NBI binding sites has been reported in brain membrane preparations from the rat with a K_D of 0.18 nM and B_{max} of 135 fmol/mg of protein (Marangos et al., 1982b) and from the guinea pig with K_D values of 0.15 to 0.38 nM and B_{max} values of 201 to 644 fmol/mg of protein (Hammond and Clanachan, 1983).

In the present displacement studies, parallel inhibition curves were found for all compounds represented in Figures 1 and 2 except for the nucleobases, adenine and hypoxanthine. These nonparallel interactions indicate reactivity with sites labeled by [^{3}H]NBI but are dissimilar to those which recognize the other nucleosides, adenosine analogues and transport inhibitors. Interestingly, Cornford and Oldendorf (1975) reported the presence of two independent transport systems in tissues associated with the blood-brain barrier in rat: one for the nucleobases adenine and hypoxanthine, and the second for the nucleosides adenosine, guanosine, inosine, and uridine. We confirmed that NBI and NBG were the most potent inhibitors of [^{3}H]NBI binding (see Table I). The structurally dissimilar, yet effective, nucleoside transport inhibitors, dipyridamole and papaverine, were also reasonably potent in displacing [^{3}H]NBI binding. In comparison with our K_i values (Table I), the K_i values (nanomolar concentration) recalculated from IC_{50} values given by Marangos et al. (1982b) for NBI, NBG, dipyridamole, and adenosine were: 0.23, 1.3, 283, and 33,333, respectively. The latter three compounds were approximately 10 to 30 times less potent than in our study. The reasons for these discrepancies are unclear.

Measurements of both nucleoside transport and [^{3}H]NBI binding indicate that certain important differences may exist between neuronal and non-neuronal nucleoside uptake systems. The reported K_m values for adenosine uptake (transport plus metabolism) into various tissues were as follows: cultured astrocytes, 3.4 μM (Hertz, 1978); cholinergic synaptosomes from *Torpedo* electric organ, 2.4 μM (Meunier and Morel, 1978); and rat brain synaptosomes, 1.0 μM (Bender et al., 1980, 1981). In the uptake studies by Thampy and Barnes (1983a, b), which largely excluded metabolism secondary to transport, the K_m value for adenosine uptake into primary cultures of neurons from chick embryo brain was 13 μM and that in primary cultures of glial cells of similar origin was found to be of much lower affinity, 370 μM. Neuronal nucleoside transporters not only have higher affinity for adenosine but, when compared to other cell types, exhibit greater selectivity for adenosine relative to other nucleosides. The K_i values for the inhibition of adenosine transport into neuronal cell cultures was 23 μM for adenosine and was in the range of 300 to 1000 μM for inosine, thymidine, and guanosine (Thampy and Barnes, 1983a). Corresponding values for inhibition of adenosine uptake into rat brain synaptosomes were 1.0 μM for adenosine and

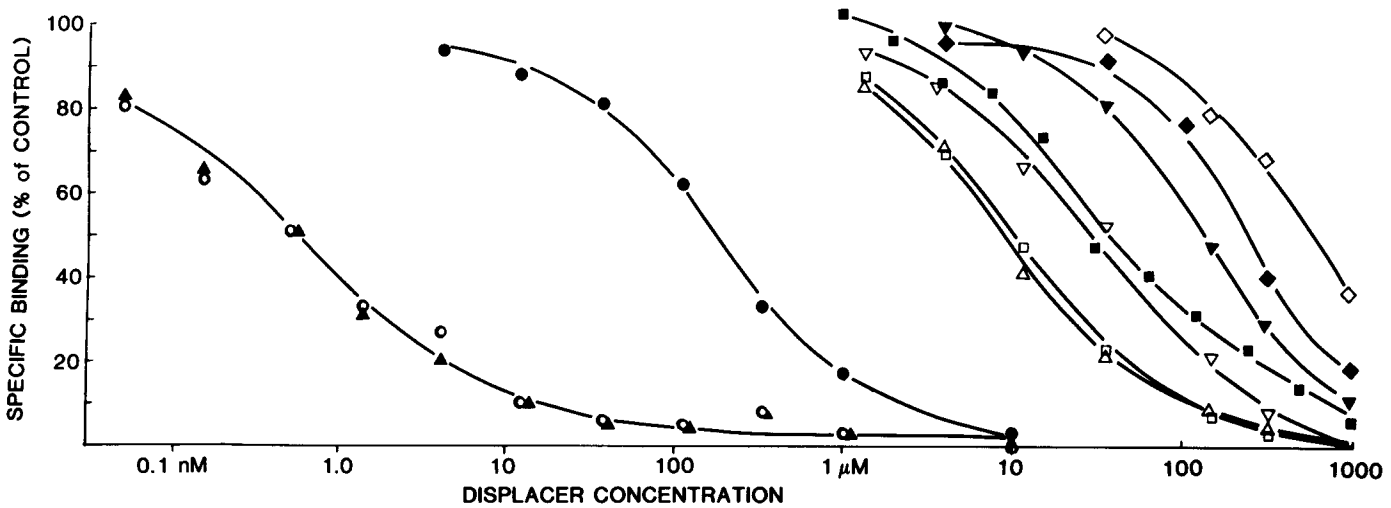

Figure 3. Displacement of [^{3}H]NBI binding to membranes from rat brain by adenosine analogues and adenosine uptake inhibitors. IC_{50} values for NBI (▲), NBG (○), dipyridamole (●), CHA (△), (+)-PIA (□), (−)-PIA (▽), papaverine (■), 2-chloroadenosine (▼), 2′-deoxyadenosine (◆) and NECA (◇) were determined using 0.6 nM [^{3}H]NBI and 8 to 10 concentrations of the displacers. Control binding represents that amount of [^{3}H]NBI binding displaceable by 5 μM NBI. These results are representative of experiments performed in duplicate and conducted at least twice with similar results.

287 to 340 μM for uridine, cytidine, guanosine, and inosine (Bender et al., 1981). These findings in neuronal systems contrast with those reported for other tissues. In cultured Novikoff and P388 cells, for example, the K_m values for adenosine transport were 103 and 123 μM, respectively, whereas those for inosine, thymidine, and uridine ranged from 125 to 250 μM (see Plagemann and Wohlhueter, 1980). Thus, it may be concluded that a high affinity (1 to 10 μM) adenosine transporter exists on neurons, whereas a lower affinity (100 to 1000 μM) system having broader substrate specificity is associated with glial cells (Thampy and Barnes, 1983a) and peripheral tissues.

The present results support the contention that [^{3}H]NBI binds to transport sites in brain which do not have a broad substrate specificity and are active at concentrations of adenosine (about 2.0 μM) normally found in brain (Winn et al., 1979; Zetterstrom et al., 1982). There are two possible explanations for the apparent differences in nucleoside specificity between neuronal and non-neuronal tissues. The neuronal sites that bind [^{3}H]NBI may be fundamentally different from those found on other cell types. Alternatively, at least two populations of nucleoside transport sites may exist in both the periphery and the brain with high affinity [^{3}H]NBI sites in the brain being more abundant and having higher affinity and selectivity for adenosine. In either case, the neuronal adenosine uptake site labeled by [^{3}H]NBI may have a critical role in controlling extracellular adenosine levels, particularly at adenosine release sites. Thus, [^{3}H]NBI may be a useful ligand for localizing neural systems in the CNS where adenosine exerts its physiological actions.

The degree of interaction of various adenosine receptor agonists and antagonists with [^{3}H]NBI binding sites was examined. The affinities of the adenosine receptor agonists CHA, (+)- and (−)-PIA, 2-chloroadenosine, and NECA (Table I) for the [^{3}H]NBI binding site were approximately three orders of magnitude lower than their affinities for adenosine receptors in brain and spinal cord membrane preparations (Daly, 1982; Geiger et al., 1984b). Additionally, the rank order of potencies of these agonists for the [^{3}H]NBI site does not correspond with their affinities for the A_1-receptor where (−)-PIA > CHA > 2-chloroadenosine > NECA > (+)-PIA. The interaction of [^{3}H]NBI with A_2 adenosine receptors is also unlikely since, at this receptor, (+)- and (−)-PIA exhibit little, if any, stereoselectivity; in the present study (+)-PIA was found to have 3-fold greater affinity than (−)-PIA for the [^{3}H]NBI site. The weak potency of NECA for displacing [^{3}H]NBI also tended to rule out an interaction with A_2 sites since NECA is reported to have nanomolar affinity for the A_2-receptor (Daly, 1982). The adenosine receptor antagonists caffeine, theophylline, and enprofylline, as well as adenine and guanine nucleotides, were all virtually without effect on [^{3}H]NBI binding at concentrations up to 1 mM. The intracellular adenosine receptor, classified as the P site, is known to have greater affinity for 2′-deoxyadenosine than for 2-chloroadenosine. However, 2-chloroadenosine was more potent than 2′-deoxyadenosine in displacing [^{3}H]NBI, a finding inconsistent with [^{3}H]NBI interacting with this intracellular site.

As shown in Figure 3, adenosine analogues gave parallel displacement curves of [^{3}H]NBI binding, indicating their competition for similar sites on the membranes. These findings can be interpreted on the basis of structure-activity relationships. The potent inhibitory actions of NBI and related compounds have been attributed to the 6-substituted nitrobenzyl and 9-ribosyl moieties extending from the purine ring (Paterson and Oliver, 1971). CHA and (+)- and (−)-PIA are similar to NBI in that both have the 9-ribosyl group as well as hydrophobic ring structures at the N^6-position. 2-Chloroadenosine and NECA were less potent in displacing [^{3}H]NBI, possibly because of the absence of the N^6 group. Therefore, when considering the biological actions of these adenosine receptor agonists, inhibition of endogenous adenosine uptake may be a factor in contributing to their behavioral and electrophysiological effects.

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