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 \([3H]\)nitrobenzylthioinosine (\([3H]\)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 \(B_{	ext{max}}\) of guinea pig extracts are 0.13 ± 0.02 nM 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 \([3H]\)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 \([3H]\)cyclohexyladenosine (\([3H]\)CHA) to the solubilized brain extract was also studied and compared with that of \([3H]\)NBI. In contrast to the \([3H]\)NBI binding site \([3H]\)CHA binds to two apparent populations of adenosine receptor, a high-affinity site with a \(K_d\) of 0.32 ± 0.06 nM and a \(B_{	ext{max}}\) of 105 ± 30 fmol/mg protein and a lower-affinity site with a \(K_d\) of 5.50 ± 0.52 nM and \(B_{	ext{max}}\) of 300 ± 55 fmol/mg protein. The pharmacology of the \([3H]\)CHA binding site is consistent with that of the adenosine receptor and quite distinct from that of the uptake (\([3H]\)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., 1982a). 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, 1983a). 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, 1983a).

Recent investigations examining adenosine uptake sites directly have employed binding studies using \([3H]\)nitrobenzylthioinosine (\([3H]\)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, phenylmethylsulfonyl fluoride.
into both nervous (Barberis et al., 1981) and non-
nervous tissue (Pickard and Paterson, 1972; Bra-
jeswar et al., 1975). [3H]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 (Ham-
mond et al., 1982) and guinea pig cortical tissue
(Hammond and Clanachan, 1982, 1983), have shown
that displacement of [3H]NBI binding by a test com-
pound was indicative of that compound’s ability to
block adenosine uptake into these tissues. Thus, the
measurement of site-specific binding of [3H]NBI
seems to offer a convenient probe for studying mol-
ecular interactions at adenosine uptake sites just as
the stable radioactive adenosine analogs do for
adenosine receptors.

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

MATERIALS AND METHODS

Membrane preparation

Frozen rat or guinea pig whole brains were suspended in
10 volumes of cold 50 mM Tris-HCl buffer (pH 7.5)
containing 10 mM EDTA, 100 mM phenylmethylsulfonyl
fluoride (PMSF), and 1 mM 1,10-phenanthroline 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 mM Tris-HCl (pH
7.5) and spun again as above. The following pellets were
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 μM NBI (Calbio-
chem) in the assay and routinely represented 10% or less
of the total binding at 0.7 nM [3H]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
[3H]NBI concentrations ranging from 0.05 to 25.0 nM.
Inhibition studies were done by incubating the extracts in
the presence of six different concentrations of inhibitor
and 0.7 nM [3H]NBI. All binding data are expressed as
specific binding and all assays were performed in triplic-
te. Kd values were calculated using the relationship

\[
K_d = \frac{I_{cp}}{\left(1 + [L]/K_p\right)}
\]

[3H]CHA binding assay

[3H]CHA binding to soluble extracts was measured in
a manner similar to that described above for [3H]NBI
binding, except that the incubation was performed at 22°C
for 2 h. Nonspecific binding was determined by incor-
porating 50 μM CHA (Calbiochem). Scatchard analysis
was performed by incubating extracts with [3H]CHA (25
Ci/mmol) concentrations ranging from 0.40 to 25.0 nM.
Inhibition studies were done using 5 nM [3H]CHA.
For calculating Kd values the Kd value for [3H]CHA
binding was taken as 1 nM rather than using the component
high- and low-affinity Kd 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 uti-
lizing nimodipine, procedures were performed in dark-
cened rooms. Diltazep 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 [3H]NBI to soluble extractions from guinea pig and rat brains was pro-
portional to the protein concentration in the assay
over the range of 0.04 to 0.6 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 [3H]NBI binding to guinea pig brain solubilized extractions is shown in Fig. 3a. The specific binding of 0.45 nM [3H]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_{diss}), obtained from the slope of the line in Fig. 3c was 0.046 min⁻¹. K_D, as determined using the equation $K_D = (k_{obs} - k_{diss}) / ([3H]NBI)$ (Williams et al., 1976), was 2.2 × 10⁸ M⁻¹. An estimate of the equilibrium dissociation constant obtained as the ratio $k_{diss} / k_{obs}$ was 0.21 nM.

The saturation isotherm and Scatchard analysis of [3H]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 nM and $B_{max}$ value of 150 ± 35 fmol/mg protein. Similar plots were obtained in examining [3H]NBI binding to soluble extracts from rat brain with a $K_D$ value of 0.12 ± 0.03 nM and $B_{max}$ of 133 ± 18 fmol/mg protein. All Scatchard plots performed for [3H]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 [3H]CHA to soluble guinea pig brain extracts are shown in Fig. 5. Saturable, specific binding of [3H]CHA to [3H]NBI binding, revealed biphasic Scatchard plot.
FIG. 3. Reversibility of [3H]NBI binding to soluble guinea pig extracts. a: Solubilized extracts were incubated with [3H]NBI (0.45 nM) 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 [3H]NBI binding. c: Dissociation of [3H]NBI binding. The dissociation constant (kₐ) was calculated from the slope of the line in c as 0.046 min⁻¹. The rate of association (kₐ) was calculated from the slope of the line in b as 0.143 min⁻¹ and kₐ was determined using the equation Kₑq = (kₐ)⁻¹/(kₐ)NBI as 2.2 × 10⁸ 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 [3H]CHA. Apparent Kᵦ values for the two [3H]CHA binding sites were 0.32 ± 0.06 nM and 5.50 ± 0.52 nM, and Bₘₐₓ 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 [3H]NBI binding constants whereas [3H]CHA binding showed a strict dependence on this procedure (data not shown).

FIG. 4. Saturation isotherm and Scatchard plot for the binding of [3H]NBI to soluble guinea pig brain sites. a: Soluble extracts (0.4 mg/mg protein) were incubated with graded concentrations of [3H]NBI at 22°C in the absence and presence (O) of 5 µM NBI. Specific binding (A) 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 Kᵦ of 0.13 nM ± 0.06 and a Bₘₐₓ of 150 ± 35 fmol/mg protein.
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 also 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 nM, 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 \(\mu\)M whereas caffeine was a very poor inhibitor in both species with \(K_i\) values well above 400 \(\mu\)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 \(\mu\)M. The other uptake inhibitors tested showed much higher micromolar \(K_i\) values as did the adenosine antagonist caffeine (73 \(\mu\)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
and Clanachan, 1982, 1983) show [3H]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 [3H]NBI as a probe. These sites, solubilized from guinea pig and rat brains, retain characteristics similar to those reported for [3H]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 [3H]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 [3H]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 [3H]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 [3H]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 [3H]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., 1982b; 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 \(K_d\) value of 7.4 nM (Mohler and Okada, 1978) showed a much lower affinity for the \(^{3}H\)NBI binding sites extracted from rat and guinea pig brains \(K_d\) values of 6 \(\mu M\) and 9 \(\mu M\), 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 \(K_d = 5-6 \mu M\) than diazepam, this and other dihydropyridines have been shown to have nanomolar \(K_d\) 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., 1982a; 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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