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Identification of Specific [^{3}H]Adenine-Binding Sites in Rat Brain Membranes

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We recently reported that adenine acts as a neurotrophic factor independent of adenosine or P2 receptors in cultured Purkinje cells [Watanabe S. *et al.* (2003) *J. Neurosci. Res.* 74, 754–759], suggesting the presence of specific receptors for adenine in the brain. In this study, the characterization of adenine-binding activity in the rat brain was performed to further characterize the receptor-like adenine-binding sites. Specific binding sites for [^{3}H]adenine were detected in membrane fractions prepared from rat brains. The kinetics of [^{3}H]adenine binding to membranes was described by the association and dissociation rate constants, 8.6 × 10^5 M^{-1} min^{-1} and 0.118 ± 0.045 min^{-1}, respectively. A single binding site for [^{3}H]adenine with a K_D of 157.1 ± 20.8 nM and a B_{max} of 16.3 ± 1.1 pmol/mg protein (n = 6) was demonstrated in saturation experiments. A displacement study involving various related compounds showed that the [^{3}H]adenine binding was highly specific for adenine. It was also found that [^{3}H]adenine-binding activity was inhibited by adenosine, although other adenosine receptor ligands were ineffective as to [^{3}H]adenine binding. The brain, especially the cerebellum and spinal cord, showed the highest [^{3}H]adenine-binding activity of the tissues examined. These results are consistent with the presence of a novel adenine receptor in rat brain membranes.

Key words: adenine, binding site, purine, purinergic, rat brain.

Abbreviations: 4APP, 4-aminopyrazolo[3,4-*d*]pyrimidine; CGS-21680, 2-*p*-(2-carboxyethyl)phenethylamino-5′-*N*-ethylcarboxamidoadenosine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; IB-MECA, 1-deoxy-1-(6-[((3-iodophenyl]methyl)amino]-9*H*-purine-9-yl)-*N*-methyl-β-D-ribofuranuronam-de; NBTI, *S*-(4-nitrobenzyl)-6-thioinosine; NECA, 5′-*N*-ethylcarboxamidoadenosine; NGF, nerve growth factor; PMSF, phenylmethylsulfonyl fluoride; PPADS, pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid).

Adenosine and adenine nucleotides act not only as neurotransmitters but also as neuroprotective products in the central nervous system *via* P1 and P2 receptors (*1–3*). Other purine derivatives are also known to have neurotrophic effects, *i.e.*, guanosine and GTP enhance NGF-induced neurite outgrowth (*4–6*), inosine promotes neurite outgrowth in goldfish and rat ganglion cells (*4*), and stimulates axonal regrowth after injury (*7*), and propentofylline has neuroprotective effects in astrocytes and rat pheochromocytoma cells (*8*, *9*).

Recently, we found that adenine has a neuroprotective effect on Purkinje neurons in rat primary cerebellar cultures (*10*). Although other purines improved Purkinje cell survival, adenine was the most effective of the compounds examined. P1 and P2 receptor agonists were ineffective as to Purkinje cell survival. Furthermore, it was confirmed that adenine promoted Purkinje cell survival without conversion to adenosine or ATP. In mammalian organisms, however, the signaling functions of adenine are poorly understood. In non-mammalian organisms, hormonal functions have been demonstrated for adenine derivatives. Starfish oocyte maturation is induced by the follicle cell–derived hormone 1-methyladenine *via* a G protein coupled receptor (*11*). Cytokinins, a group of phytohormones, are adenine derivatives that carry either an isoprene-derived or aromatic side chain at the *N*(6) terminus, and regulate plant cell division and differentiation *via* plasma membrane receptors (*12*).

Our previous study (*10*) suggested the possibility that adenine acts as a signaling molecule in the mammalian brain. As adenine is not a ligand for P1 or P2 purinergic receptors, it is possible that adenine acts on Purkinje neurons *via* a novel purinergic receptor specific to the nucleobase. The presence of specific binding sites for several other nucleosides or nucleotides such as uridine (*13*), guanosine (*14*), and GTP (*15*) has been reported.

In this study, detailed characterization of [^{3}H]adenine-binding activity in rat brain membranes was performed in order to identify a specific adenine binding site that can account for the existence of adenine receptors.

MATERIALS AND METHODS

Preparation of Rat Brain Membranes—Rat brain membranes were prepared from male Wistar rats. The whole brain was homogenized with a Polytron homogenizer in 9 volumes of ice-cold buffer comprizing 50 mM Tris-acetate buffer, pH 7.2, 1 mM EDTA, 100 μM phenylmethylsulfo-

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nyl fluoride (PMSF), and one tablet of Complete/25 ml (protease inhibitors, Roche Diagnostics). The homogenate was centrifuged at 37,000 × *g* for 20 min at 4°C. The pellet was washed by homogenization followed by 3× centrifugation. The resulting pellet was suspended in the same buffer to a final protein concentration of 6 mg/ml and then stored at –80°C until use.

Preparation of a Synaptosome-Rich Fraction—The cerebra from two male Wistar rats (2.3 g) were homogenized in 9 volumes of homogenization buffer (0.32 M sucrose, 10 mM Tris-acetate buffer, pH 7.2, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, and one tablet of Complete protease inhibitor cocktail/25 ml [Roche Diagnostics]) by 10 up-and-down strokes at 1,000 rpm in a Teflon-glass homogenizer. The homogenate was centrifuged at 1,000 × *g* for 10 min and the supernatant was centrifuged at 15,000 × *g* for 30 min. Subfractionation of the pellet (P2 fraction) was performed according to Dunkley *et al.* (*16*). The P2 fraction was suspended in 8 ml of homogenization buffer, layered over a gradient consisting of 8 ml each of 23, 15, 10, and 3% Percoll (v/v) in the homogenization buffer, and then centrifuged for 5 min at 31,500 × *g* in an angle rotor. Ten fractions (4 ml each) were aspirated off. The synaptophysin-rich fractions identified with a specific antibody (Progen Biotechnik Gmbh, Heidelberg, Germany) were taken as the synaptosome fractions.

Binding Assay—Rat brain membranes (50 μg protein) were incubated for 90 min at 37°C with 100 nM [^{3}H]adenine in a total volume of 250 μl of 50 mM Tris-acetate buffer, pH 7.2, containing 1 mM EDTA, and 5 mM $MgCl_2$. The reaction was terminated by filtration through Whatman GF/B glass fiber filters pre-soaked with 0.3% polyethyleneimine under vacuum. The filters were washed three times with 4 ml of cold 50 mM Tris-acetate buffer, pH 7.2, followed by counting in Aquasol. Unlabeled adenine (100 μM) was used to determine non-specific binding. For the [^{3}H]adenine saturation binding experiments, a concentration range of 25–500 nM [^{3}H]adenine was used.

HPLC Analysis—Rat brain membranes (50 μg protein) were incubated with 300 nM [^{3}H]adenine in 250 μl reaction mixtures at 37°C for 90 min. Each reaction mixture was then centrifuged, and the supernatant was collected and diluted to 1:50 in 20 mM ammonium acetate/acetic acid (pH 5.5). The diluted supernatant (10 μl) was injected into a reverse phase analytical column (Inertsil ODS-2 column, 5 μm, 150 × 4.6 mm I.D.; GL Science, Tokyo, Japan) and then eluted using the following program: 0–13 min solvent A, 13–35 min solvent B (solvent A: 20 mM ammonium acetate/acetic acid pH 5.5, solvent B: acetonitrile/solvent A, 5/95). The flow rate was kept constant at 1.0 ml/min. To assess radioactivity, fractions collected every 30 s were subjected to counting with a liquid scintillation counter. To determine the endogenous adenosine concentration in the reaction mixture, 100 μl of supernatant was injected into a reverse phase analytical column (Cosmosil 5C18-PAQ column, 5 μm, 250 × 4.6 mm I.D.; Nacalai Tesque, Kyoto, Japan), and eluted using the following program: 0–18 min solvent A, 18–28 min linear gradient of 0–20% solvent B, and 28–40 min linear gradient of 20–50% solvent B, (solvent A: 20 mM ammonium acetate/acetic acid pH 5.5, solvent B: acetonitrile/solvent A, 50/50). The amount of purine eluted was measured as to the absorbance at 260 nm.

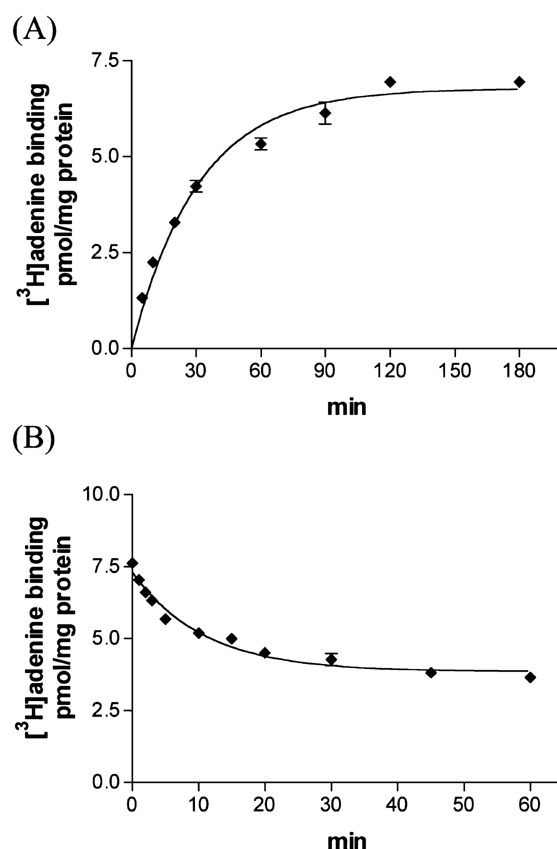

Fig. 1. **Association and dissociation kinetics of [^{3}H]adenine binding to rat brain membranes.** (A) Membranes were incubated with 100 nM [^{3}H]adenine under the standard assay conditions for the various times indicated. (B) Membranes were incubated with 100 nM [^{3}H]adenine for 90 min under the standard conditions. At this time point, 100 μM adenine was added to the mixture to initiate dissociation. The binding was measured at the times indicated. The data (mean ± SEM) are for a representative experiment performed three times. The dissociation rate constant (K_{off}) was 0.093 ± 0.012 min^{-1}. The association rate constant (K_{on}) was 6.1 × 10^5 M^{-1} min^{-1}. An independent estimate of the dissociation constant at equilibrium was (K_D) = K_{off}/K_{on} = 154.7 nM.

Statistical Analysis—The binding data were analyzed by nonlinear regression analysis using the program Prism (GraphPad Software, San Diego, CA, USA).

Drugs and Chemicals—[^{3}H]Adenine (24.2 Ci/mmol) was purchased from PerkinElmer (Boston, MA, USA), and adenine, hypoxanthine, cytosine, CDP, 1-methyladenine, 3-methyladenine, and 9-ethyladenine were purchased from Sigma (St. Louis, MO, USA). Guanine, xanthine, uracil, adenosine, xanthosine, inosine, uridine, cytidine, xanthosine 5′-monophosphate (XMP), inosine 5′-monophosphate (IMP), UMP, CMP, ADP, UDP, ATP, UTP, and allopurinol were purchased from Wako (Osaka, Japan). GDP, GTP, and CTP were purchased from Nacalai Tesque (Kyoto, Japan). Guanosine, GMP, and thymine were purchased from Kohjin (Tokyo, Japan). AMP was purchased from Oriental Yeast (Tokyo, Japan). Adenosine receptor ligands and other compounds were purchased from Sigma (St. Louis, MO, USA).

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Table 1. **Specificity of various ligands as to displacement of [^{3}H]adenine binding to rat brain membranes.**

	K_i	(μM with 95% confidence intervals)
Adenine	0.120	0.0956–0.150
4APP	25.9	19.6–34.2
2,6-Diaminopurine	45.1	34.2–59.5
Adenosine	15.3	11.7–20.1
AMP	8.52	5.68–12.8
ADP	11.1	8.25–15.0
ATP	11.6	8.10–16.6
2′-Deoxyadenosine	21.9	7.04–68.1
5′-Deoxyadenosine	0.823	0.500–1.35
2′,3′-Dideoxyadenosine	13.5	8.69–21.0
2′,5′-Dideoxyadenosine	5.85	2.57–13.4

K_i values were determined with at least 6–8 concentrations of each compound in three experiments. K_i values were calculated according to Cheng and Prusoff (*30*); $K_i = EC_{50}/(1 + [\text{ligand}]/K_D)$.

RESULTS

[^{3}H]Adenine Binding to Rat Brain Membranes—A rat brain homogenate was briefly fractionated into membrane and soluble fractions, and then the [^{3}H]adenine binding activities in the fractions were compared. Approximately 92% (n = 3) of the [^{3}H]adenine-binding activity of the rat brain homogenate was localized in the membrane fractions (data not shown). Furthermore, a synaptosome-rich fraction prepared by cell fractionation was also found to contain a [^{3}H]adenine-binding site. Specific [^{3}H]adenine binding was detected by the assay method described under "METHODS," and optimal binding to rat brain membranes was obtained by incubation at 37°C and pH 7.2, there being decrease at a higher or lower temperature and/or pH (data not shown).

Figure 1A shows the time course of [^{3}H]adenine binding to rat brain membranes. The steady state was reached within 90 min. The observed association constant (K_{obs}) was 0.032 ± 0.005 min^{-1} (mean ± SD, n = 3). The addition of 100 μM unlabeled adenine at the steady state rapidly reduced the amount of [^{3}H]adenine bound, as shown in Fig. 1B. The dissociation constant (K_{off}) was 0.118 ± 0.045 min^{-1} (mean ± SD, n = 3). The calculated association rate constant (K_{on}) was 8.6 × 10^5 M^{-1} min^{-1}. An independent estimate of the dissociation constant at equilibrium was (K_D) = K_{off}/K_{on} = 137.8 nM. This value was in reasonable agreement with the apparent dissociation constant (K_D) calculated in the saturation binding studies described below.

Saturation Studies—Saturation analysis of [^{3}H]adenine binding to rat brain membranes was performed as shown in Fig. 2. The data were analyzed using a one-site model to yield K_D and B_{max} values of 157.1 ± 20.8 nM and 16.3 ± 1.1 pmol/mg, respectively (mean ± SD, n = 6). The synaptosome-rich fraction also contained a [^{3}H]adenine-binding site with K_D and B_{max} values of 206.8 ± 49.3 nM and 83.2 ± 18.1 pmol/mg protein (mean ± SD, n = 3), respectively.

Displacement Studies—The specificity of the [^{3}H]adenine binding was examined by displacement studies involving various related compounds, as shown in Table 1. Only a limited number of compounds, *i.e.*, 4APP, 2,6-diaminopurine and adenosine, showed significant inhibitory activity. Purine at 100 μM also inhibited the [^{3}H]adenine-binding activity (36.6% inhibition). The displacement curves for these compounds are shown in Fig. 3A, indicating a potency order of adenine > adenosine ≥ 4APP ≈ 2,6-diaminopurine. The curves were resolved by nonlinear regression analysis with a one-site model. Adenosine was found to be a partial displacer (approximately 61% maximum inhibition) with a K_i value of 15.3 μM (Fig. 3A). ATP, ADP and AMP were also found to displace the [^{3}H]adenine binding, similar to adenosine. As the inhibitory effect of these adenine nucleotide compounds was blocked when assayed in the presence of adenosine deaminase, it is likely that adenosine produced from adenine nucleotides during the binding assay inhibited the [^{3}H]adenine binding (data not shown). It is also possible that a small amount of endogenous adenosine in brain membranes affects [^{3}H]adenine binding. Because the concentration of endogenous adenosine was determined to be in the ~10^{-7} M range on HPLC analysis, and the K_i value of adenosine was in the 10^{-5} M range (Table 1 and Fig. 3A), the endogenous level of adenosine did not affect the [^{3}H]adenine-binding activity. Indeed, removal of adenosine from the assay system by the addition of adenosine deaminase (2 U/ml) did not affect the K_D and B_{max} values for [^{3}H]adenine binding.

The inhibition of [^{3}H]adenine binding by adenosine is examined further in the next section. Other adenosine receptor ligands (100 μM NECA, 100 μM 2-chloroadenosine, 100 μM caffeine, 100 μM theophylline, 50 μM CGS-21680, 50 μM DPCPX and 20 μM IB-MECA) and P2 receptor ligands (100 μM reactive blue 2, 100 μM PPADS, 100 μM α,β-methyleneATP and 100 μM suramin) showed no significant inhibitory effect on the [^{3}H]adenine binding. Guanine, guanosine, GMP, GDP, GTP, hypoxanthine, inosine, IMP, xanthine, xanthosine, XMP, cytosine, cytidine, CMP, uracil, uridine, UMP, UDP, UTP, thymine and thymidine were not able to inhibit the [^{3}H]adenine binding (at 100 μM, data not shown). In addition, adenine analogs such as 1-methyladenine, 3-methyladenine, 9-ethyladenine, 6-dimethylaminopurine, 2-aminopurine and *trans*-zeatin had no significant inhibitory effect on the [^{3}H]adenine binding either (at 100 μM, data not shown).

To exclude the possibility that [^{3}H]adenine binds to purine transporter systems, we examined the ability of a nucleobase transporter inhibitor, papaverine (*17–19*), and nucleoside transporter inhibitors, NBTI and dipyridamole, to alter [^{3}H]adenine binding. Papaverine, NBTI and dipyridamole (at 100 μM) had no effect on [^{3}H]adenine binding.

The Role of Hydroxyl Groups of Adenosine in the Inhibition of [^{3}H]Adenine Binding—Adenosine has three OH groups on its ribose. We examined the role of the OH groups as to an inhibitory effect on the [^{3}H]adenine binding site by using various deoxyadenosines, as shown in Fig. 3B. The displacement was analyzed using a one-site binding model yielding maximum inhibition values for adenosine, 2′-deoxyadenosine, 2′,5′-dideoxyadenosine, 5′-deoxyadenosine, 2′,3′-dideoxyadenosine and adenine of 60.8%, 58.4%, 58.7%, 55.3%, 83.2% and 88.7%, respectively. Adenosine, 2′-deoxyadenosine, 2′,5′-dideoxyadenosine and 5′-deoxyadenosine did not show inhibition as

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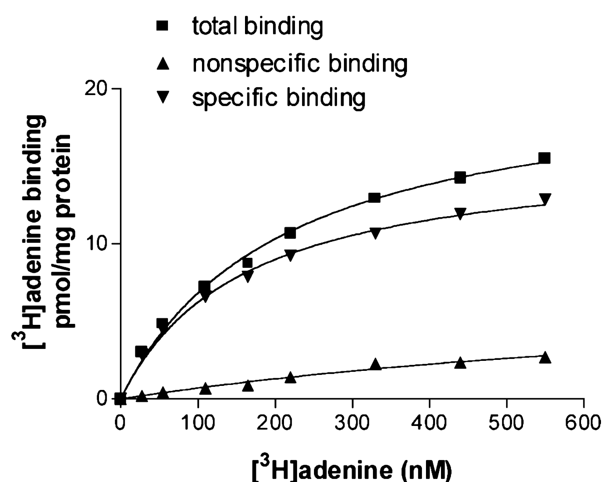

Fig. 2. **Saturation binding of [^{3}H]adenine to rat brain membranes.** Membranes (50 μg protein) were incubated with increasing concentrations of [^{3}H]adenine (25–500 nM) under the standard assay conditions. Non-specific binding was detected with unlabelled adenine (100 μM). The data (mean ± SEM) are for one experiment representative of six. The data were fitted using computerized nonlinear regression analysis and resolved with a one-site model. K_D = 158.9 ± 0.4 nM; B_{max} = 16.3 ± 0.38 pmol/mg protein.

Table 2. **Organ distribution of the [^{3}H]adenine-binding site.**

	K_D (nM) ± SD	B_{max} (pmol/mg protein) ± SD
Cerebellum	180.7 ± 28.6	19.9 ± 3.0
Cerebrum	142.0 ± 35.1	6.8 ± 1.2
Brain stem	211.5 ± 49.2	14.8 ± 1.5
Spinal cord	210.7 ± 52.8	23.6 ± 3.8
Heart	141.8 ± 30.4	1.7 ± 0.2
Kidney	192.1 ± 38.8	3.5 ± 0.5
Testis	164.3 ± 32.3	2.3 ± 0.1

The organs were isolated from rats and homogenized by the same procedure as that for the preparation of rat brain membranes. [^{3}H]Adenine-binding activity was determined by incubating each membrane sample (50 μg protein) with 25–500 nM [^{3}H]adenine at 37°C for 90 min under the standard assay conditions. Non-specific binding was detected with unlabelled adenine (100 μM). The data (mean ± SD) are for three experiments. The data were fitted by computerized nonlinear regression analysis and resolved with a one-site model.

significant as adenine. The most noticeable finding was that the absence of an OH group at both the 2′- and 3′-positions increased the maximum inhibition similar to in the case of adenine. On the other hand, the lack of 5′-OH improved the affinity to the [^{3}H]adenine binding site and the lack of 3′-OH markedly lowered the affinity to the [^{3}H]adenine binding site. The absence of 2′-OH or both 2′ and 5′-OH did not affect the adenosine selectivity.

Stability of [^{3}H]Adenine during the Binding Assay—To ensure that [^{3}H]adenine was not metabolized during the binding assays with rat brain membranes, we performed HPLC analysis. As shown in Fig. 4, [^{3}H]adenine was not substantially metabolized or degraded on 90-min incubation at 37°C with rat brain membranes. Although around 10^{-7} M adenosine was detected after 90-min incubation on HPLC (detected as to the absorbance at 260 nm, data not shown), it should be noted that [^{3}H]adenine binding was not affected in this concentration range (Fig. 3A).

Organ Distribution of [^{3}H]Adenine Binding—The distribution of [^{3}H]adenine-binding activity in isolated membranes from various tissues was determined. As shown in Table 2 significant [^{3}H]adenine-binding activity was detected in every tissue examined except liver and spleen. The K_D values were found to be similar in the tissues. The highest binding activity was found in the brain, particularly the cerebellum and spinal cord.

DISCUSSION

Previously we reported that adenine has a neurotrophic effect on Purkinje neurons, suggesting that it acts as a signaling molecule in the brain, as do adenosine and ATP (*10*). In this study, we showed that rat brain membranes possess a specific adenine binding site exhibiting K_D and B_{max} values of 157.1 ± 20.8 nM and 16.3 ± 1.1 pmol/mg protein, respectively (n = 6). As the [^{3}H]adenine binding was specific, reversible and able to be saturated, it fulfils important criteria as a hormone or drug receptor.

As adenine is a naturally occurring compound, [^{3}H]adenine may be degraded in the purine metabolic pathway under our assay conditions. However, [^{3}H]adenine was not substantially metabolized or degraded to other purines or pyrimidines, as shown in Fig. 4. The degradation of [^{3}H]adenine in our binding experiments does not affect our conclusions regarding the characterization of the [^{3}H]adenine binding site.

It should be stressed that the [^{3}H]adenine binding was specific to the adenine structure. Only a few adenine derivatives showed some affinity to the [^{3}H]adenine-binding site. It is of interest that this [^{3}H]adenine binding-site was partially inhibited by adenosine. Therefore, endogenous adenosine in brain membranes, if present at higher concentrations, may affect [^{3}H]adenine binding. However, the concentration of endogenous adenosine was determined to be in the ~10^{-7} M range on HPLC analysis, *i.e.*, under the level that affects [^{3}H]adenine-binding activity (Fig. 3A). Indeed, removal of adenosine from the assay system by the addition of adenosine deaminase did not affect the K_D and B_{max} values for [^{3}H]adenine binding. These data suggest that endogenous adenosine is not able to control [^{3}H]adenine binding activity.

As adenosine receptor ligands (NECA, 2-chloroadenosine, caffeine, theophylline, CGS-21680, DPCPX and IB-MECA), P2 receptor ligands (reactive blue 2 , PPADS, α,β-methyleneATP and suramin), a nucleobase transporter inhibitor (papaverine), and nucleoside transporter inhibitors (NBTI and dipyridamole) did not show a significant inhibitory effect on the [^{3}H]adenine-binding site, this adenosine-sensitive [^{3}H]adenine-binding site is likely to be distinct from purinergic receptors or related proteins. Guanine, xanthine and hypoxanthine-based purines, uracil, and cytosine and thymine-based pyrimidines showed no effects on [^{3}H]adenine binding.

In addition, 2-aminopurine was not able to inhibit [^{3}H]adenine binding, and 2,6-diaminopurine and purine were able to displace the [^{3}H]adenine-binding site with low affinity. It is considered that the 6-amino group of adenine is important for adenine binding to the membrane and the 2-amino group inhibits purine binding to the [^{3}H]adenine-binding site. Adenine derivatives such

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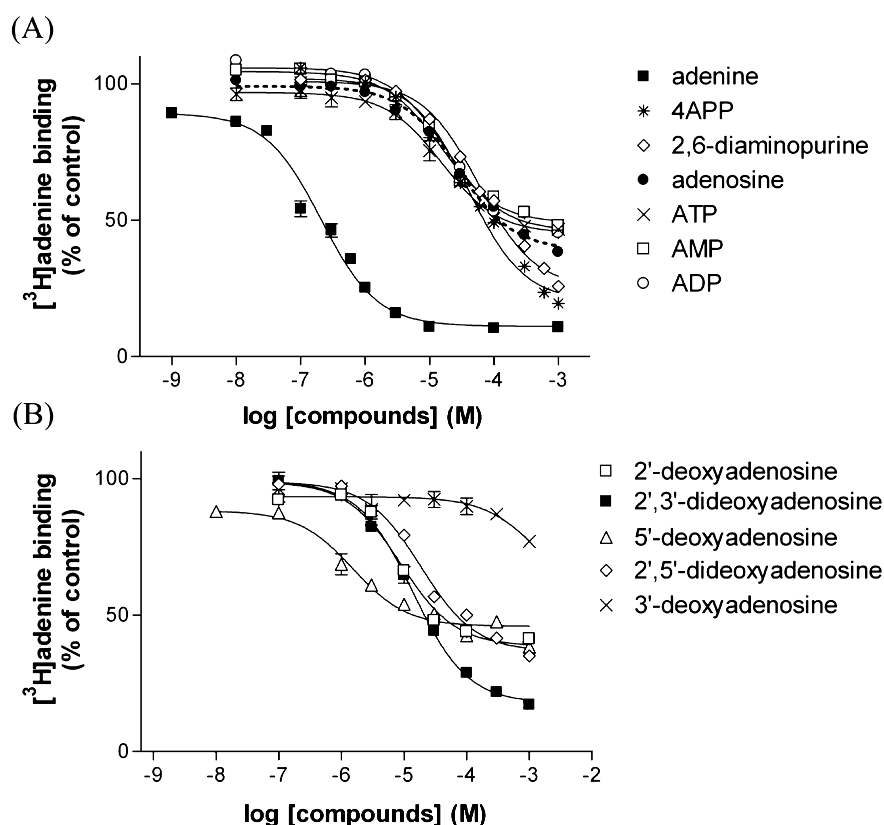

Fig. 3. **Displacement of [3H]adenine binding by purines.** The binding activity of 100 nM [3H]adenine binding to rat brain membranes was determined in the presence of various purines at the indicated concentrations. The data (mean ± SEM) are for one experiment representative of three. The data were fitted to a one-site model by non-linear regression analysis. The displacement curve for adenosine is presented as a dotted line. (A) Displacement of [3H]adenine binding by purine bases, adenosine and adenine nucleotides. The [3H]adenine binding site was partially inhibited by adenosine. (B) Displacement of [3H]adenine binding by various deoxyadenosines. The displacing ability depends on the OH group on the ribose of adenosine.

as 1-methyladenine, 3-methyladenine, 9-ethyladenine and 6-dimethylaminopurine failed to inhibit the [3H]adenine binding. This indicated that the adenine structure with a non-alkyl purine ring is important for [3H]adenine binding. Bender *et al.* (*20*) reported recently that [3H]adenine binding to an adenine-specific G protein coupled receptor in dorsal root ganglia was displaced by 1-methyladenine but not by adenosine. In contrast, the [3H]adenine-binding site in the brain was displaced by adenosine but not by 1-methyladenine in our study. Therefore, the [3H]adenine-binding site in the brain studied here is likely to be distinct from the adenine-specific G protein coupled receptor reported by Bender *et al.* (*20*).

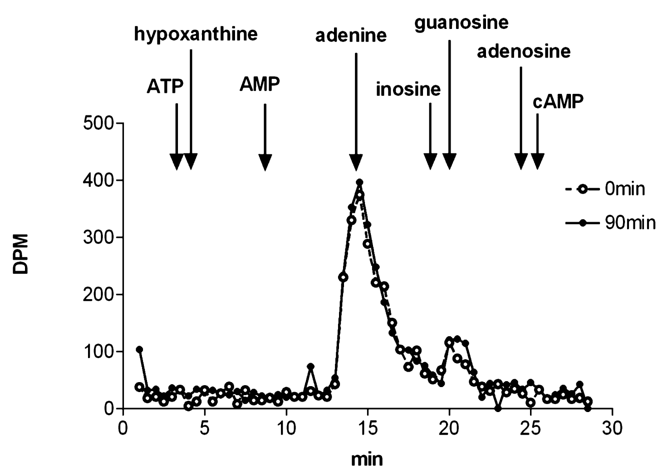

Fig. 4. **The fate of [3H]adenine during incubation with rat brain membranes.** HPLC analysis of mixtures of [3H]adenine (300 nM) and rat brain membranes under the same conditions as for the binding assay was performed as described under "METHODS." The reaction mixture was incubated for 0 min (dotted line) or 90 min (solid line). Fractions (0.5 ml) were collected every 30 s (0.5 ml), and then radioactivity was counted. Adenine was eluted at 15.1 ± 0.4 min.

It is known that nucleoside transporters can also bind nucleobases or nucleosides. However, a nucleobase transporter inhibitor, papaverine (*17–19*), and nucleoside transport inhibitors NBTI and dipyridamole showed no effect on [3H]adenine binding. Although many nucleobase transporters interact with not only adenine but also other purine bases (*19*, *21–24*), the [3H]adenine-binding site studied here was specific to adenine and adenosine. Recently, ENT2 (equilibrative nucleoside transporter 2) was shown to transport nucleobases (*25*), but ENT2 transports not only adenine but also other nucleobases. Furthermore, the K_m and K_i values of adenine with these transporters were in the 10^{-6}–10^{-3} M range (*19*, *21–23*, *26*), but the K_D value of [3H]adenine-binding sites in this study was approximately 10^{-7} M. Therefore, a nucleobase or nucleoside transporter does not seem to be related with the [3H]adenine-binding site studied here, although more studies are necessary to clarify this issue.

It has been reported that adenosine and adenosine analogs with ribose ring modifications inhibit adenylate cyclase activity (*27–29*). Adenosine and adenosine analogs with ribose ring modifications such as 2′-deoxyadenosine, 5′-deoxyadenosine, 2′,5′-dideoxyadenosine and 2′,3′-dideoxyadenosine also inhibited the [3H]adenine binding, as shown in Fig. 3B. However, [3H]adenine binding was not displaced by 3′-deoxyadenosine (Fig. 3B), which has also been reported to inhibit adenylate cyclase activity (*28*, *29*). These results suggest that the [3H]ade-

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nine-binding activity did not originate from adenylate cyclase. As described above, our results suggested that the [^{3}H]adenine-binding site is not a purinergic receptor, transporter or adenylate cyclase.

As shown in Fig. 3A, the [^{3}H]adenine binding site was composed of adenosine-sensitive and -insensitive components. The effect of adenosine on the [^{3}H]adenine binding site was found to be dependent on the position of OH groups on the adenosine ribose ring (Fig. 3B). Since the displacement of [^{3}H]adenine binding by adenosine analogs lacking 2′ and/or 5′-OH was also found to be partial, these adenosine analogs were thought to bind to an adenosine-sensitive site. Furthermore, 2′,3′-dideoxyadenosine inhibited [^{3}H]adenine binding as much as adenine. These results suggested that the 2′-OH and 3′-OH groups of adenosine inhibit adenosine binding to an adenosine-insensitive binding site.

The [^{3}H]adenine-binding site was highly localized in the brain, particularly in the cerebellum and spinal cord (Table 2). Furthermore, the synaptosome-rich fraction was demonstrated to contain [^{3}H]adenine binding sites similar in K_D value to those in brain membranes. These results suggest that adenine acts as a neurotransmitter or another factor that influences nerve functions. Adenine acts as a neurotrophic factor in Purkinje neurons, which are the principal relay neuronal cells in the cerebellar cortex, without being metabolized to other purines such as adenosine or ATP (*10*). It is possible that the high [^{3}H]adenine-binding activity in the cerebellum is associated with Purkinje neurons because it is expected that adenine-specific receptors are present in Purkinje neurons, which maintain Purkinje-neuron survival (*10*). Since adenosine is not able to improve the survival of Purkinje neurons, adenine may affect Purkinje neurons *via* an adenosine-insensitive [^{3}H]adenine-binding site.

In summary, this study suggested the presence of a novel adenine receptor in the rat brain. Because hormonal functions have been shown for adenine derivatives in several species (*11*, *12*, *20*), it is possible that adenine acts as an extracellular signaling molecule in the rat brain. Future studies will focus on molecular determination of the adenine-binding sites and the related signal transduction mechanism.

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