Solubilization and partial characterization of adenosine binding sites from rat brainstem

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Binding sites for adenosine were solubilized from rat brainstem membranes with either sodium cholate, sodium deoxycholate or 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate. About 30% of the binding activity were released by these detergents as assayed by [^{3}H]phenylisopropyladenosine (PIA) binding. Specific [^{3}H]PIA binding to the solubilized fraction was saturable and was found to be a monophasic saturation profile. In contrast, [^{3}H]PIA binding to the brainstem membranes exhibited a biphasic profile suggesting the presence of two binding sites. By gel filtration on a Sepharose CL-6B column, the adenosine binding site–detergent complex was estimated to have app. M_r 280000 and r_s = 5.4 nm.

Adenosine *Adenosine receptor* *Solubilization* *Soluble receptor* *Rat brain membrane* *N^6-Phenylisopropyladenosine*

1. INTRODUCTION

Pharmacological and biochemical studies suggest that adenosine may perform significant functions in central nervous system via adenosine-binding sites; i.e., adenosine receptor [1–4]. Elucidation of the molecular mechanisms of these functions requires detailed knowledge of the structural components of the receptor system. Progress in purifying the receptor, however, has been retarded by the lack of an efficient technique for solubilizing adenosine receptor in adequate quantities without changing their affinities for ligands. Here, we have tried to separate adenosine-binding sites in active form from the membranes of rat brainstem by several detergents. Of the solubilizing agents, tested, sodium cholate, sodium deoxycholate and CHAPS were effective in solubilizing membrane-bound adenosine-binding sites. Gel filtration of the solubilized binding site on a Sepharose CL-6B column gave an M_r estimate of ~ 280000 for the binding site–detergent complex. A similar solubilization procedure of adenosine-binding sites from bovine forebrain was reported in [5].

Abbreviations: PIA, N^6-phenylisopropyladenosine; CHA, N^6-cyclohexyladenosine; NECA, 5′-*N*-ethylcarboxamideadenosine; CHAPS, 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate

2. MATERIALS AND METHODS

2.1 *Materials*

[^{3}H]PIA (20 Ci/mmol) and [^{3}H]NECA (34 Ci/mmol) were obtained from Amersham. [^{3}H]CHA (13.5 Ci/mmol) was purchased from New England Nuclear. PIA and adenosine deaminase were from Boehringer and sodium cholate, sodium deoxycholate, Lubrol PX, digitonin, Tween 80 and Nonidet P-40 were from Nakarai Co. CHAPS was a gift of Dr. L.H. Hjelmeland and subsequently purchased from Sigma.

2.2 *Membranes*

Fresh brainstems of Wistar rats were homogenized in 3 vol. of 50 mM Tris–acetate buf-

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fer (pH 7.6) containing 2 mM dithiothreitol and 1 mM phenylmethylsulfonylfluoride with a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 39000 × *g* for 30 min at 4°C and the resulting pellet was washed 3 times in 5 vol. same buffer, ice-cold. The washed pellet was suspended in the same buffer and incubated with 2 U/ml adenosine deaminase at 30°C for 30 min, followed by centrifugation at 39000 × *g* for 30 min at 4°C. The final pellet was suspended in 5 vol. 50 mM Tris–acetate buffer (pH 7.6).

2.3 *Solubilization*

To the membrane suspension (8 mg/ml) was added leupetin, chymostatin, antipain and pepstatin A to a final concentration of 10 μg/ml each. After the addition of sodium cholate and dithiothreitol to final concentrations of 1% and 1 mM, respectively, the mixture was stirred for 30 min at 0°C, then centrifuged at 106800 × *g* for 60 min at 4°C. The pellet was discarded and the supernatant was stored at −80°C until used. Although the following experiments were performed within a month, storage of the solubilized preparation thus obtained for several weeks led to ~50% loss of the binding activity.

2.4. *Binding assays*

The binding assay of membrane fractions was performed as follows unless otherwise indicated. [^{3}H]PIA (5 nM) and brainstem membranes (~400 μg/assay) were incubated at 37°C for 20 min in a 0.5 ml total vol. of 50 mM Tris–acetate buffer (pH 7.4) in the absence or presence of 80 μM PIA. The reaction was terminated by vacuum filtration through Whatman GF/C filter, the sample tube and the filter were washed 3 times with 5 ml ice-cold Tris–acetate buffer (pH 7.4) the filter was dried and the radioactivity was determined by a liquid scintilation spectrometer. For the assay of solubilized fractions, incubations were carried out at 30°C for 90 min and separation of the bound ligand from the free was accomplished as in [6]. After incubation, bovine γ-globulin and polyethylene glycol 6000 were added to the incubation mixture to final concentrations of 0.8 mg/ml and 15%, respectively. After further incubating for 20 min at 0°C, the solution was filtered through Whatman GF/B filters and the filters were washed 3 times with 5 ml 15% polyethylene glycol 6000, dried, and analyzed for radioactivity. Specific binding was defined as radioactivity bound after subtraction non-specific binding assayed in the presence of 80 μM PIA.

2.5. *Protein assay*

Protein was determined as in [7] with bovine serum albumin as a standard.

3. RESULTS AND DISCUSSION

Of the solubilizing agents tested, sodium cholate, sodium deoxycholate and CHAPS were effective in releasing [^{3}H]PIA-binding activity

Table 1

Solubilization of [^{3}H]PIA-binding sites of rat brainstem membranes by various detergents

Treatment (%)	% Protein solubilized	% Binding sites solubilized
Cholate (1)	37	30
Deoxycholate (0.2)	20	15
Triton X-100 (1)	30	0
Digitonin (1)	17	0
Nonidet P-40 (1)	33	0
Lubrol PX (5)	50	4
Brij 35 (1%)	9	1
Tween 80 (1)	15	0
CHAPS (1.5)	34	32

The membrane fraction of rat brainstem (8 mg/ml) was incubated in 50 mM Tris–acetate buffer (pH 7.4) containing 1 mM dithiothreitol, protease inhibitors and the indicated detergents for 30 min at 0°C, and then centrifuged at 106800 × *g* for 60 min at 4°C. The supernatant was used for the binding assay

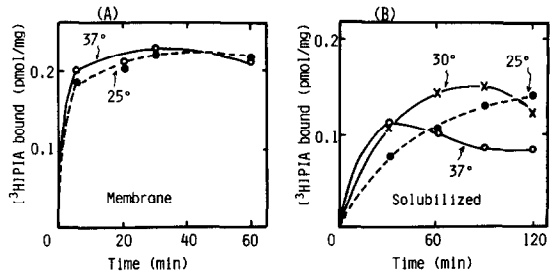

Fig. 1. Time course of PIA-binding activity of rat brainstem membrane (A) and the membrane solubilized with cholate (B). Incubations were done as in section 2 but for the indicated times at the indicated temperatures.

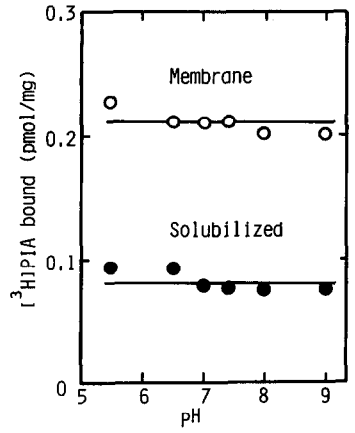

Fig.2.

Fig. 2. Effect of pH on PIA-binding activity of rat brainstem membrane (○) and the membrane solubilized with cholate (●). Incubations were performed for 20 min at 37°C for assay of membrane preparation and for 30 min for assay of solubilized preparation in 40 mM potassium phosphate buffer (pH 5.5–9).

from rat brainstem membranes (table 1). Although Triton X-100, Nonidet P-40 and Lubrol PX were effective in solubilizing the membrane proteins but ineffective in solubilizing active PIA-binding activity. These results suggest that the molecular structure of cholic acid in the detergents may be

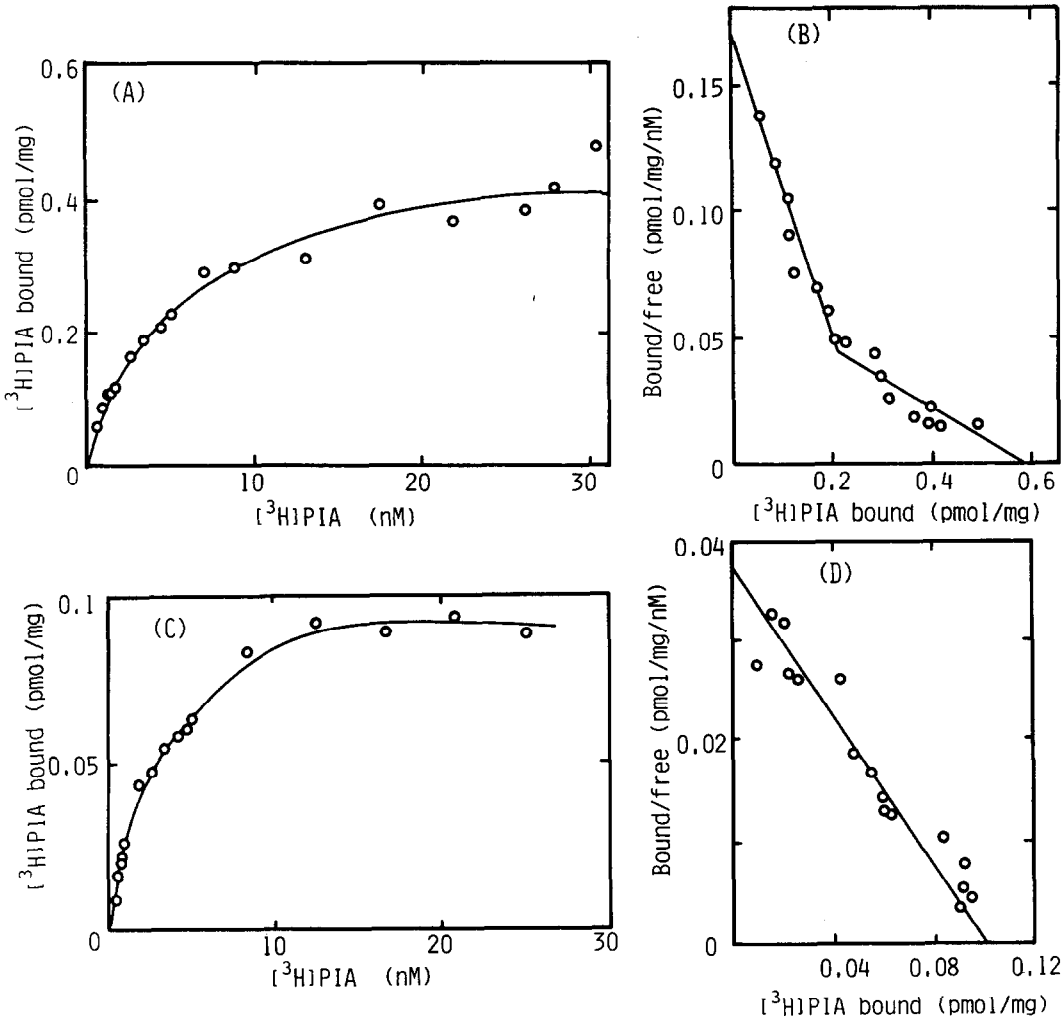

Fig. 3. Saturation analysis of [^{3}H]PIA binding to rat brainstem membrane (A, B) and the membrane solubilized with cholate (C, D). Incubations were performed as in section 2, except that the concentration of [^{3}H]PIA was varied as indicated. Scatchard plots (B) and (D) were obtained from the data shown in (A) and (C), respectively.

necessary for the solubilization of adenosine-binding activity.

Time course of PIA binding to the membrane preparation from rat brainstem and the preparation solubilized with cholate are shown in fig. 1. The initial rates of PIA binding increased with an increase in temperature in both preparations, but the temperature dependence was much greater in the solubilized preparation than in the membrane preparation. The maximal extent of PIA binding to the membrane preparation at 37°C were the same as at 25°C and prolonged incubation after reaching a maximal level resulted in little or no loss of activity (fig. 1A). In contrast, the maximal extent of PIA binding to the solubilized preparation was decreased as the incubation temperature increased (1B). Thus, the PIA-binding sites appeared to become unstable upon solubilization. The PIA-binding activity of both the membrane and solubilized preparations were not significantly affected over pH 6–9 (fig. 2).

Scatchard analysis of [^{3}H]PIA binding to rat brainstem membranes showed the existence of 2 distinct specific binding sites are shown in fig. 3B. The dissociation constants were determined to be 1.7 and 9 nM from the slopes of the plots. The maximal numbers of the high and low affinity binding sites were estimated to be 0.28 and 0.58 pmol/mg protein, respectively, from the intercept on the abscissa. Similar kinetic analysis of [^{3}H]CHA and [^{3}H]NECA binding also indicated the presence of two distinct binding sites (not shown). In contrast, Scatchard analysis of [^{3}H]PIA binding to the preparation solubilized with cholate showed a linear relationship indicating the only one species of the binding site as shown in fig. 3D. The dissociation constant was determined to be 2.7 nM, which was identical to that of the high affinity binding site of the membranes. Scatchard plots of [^{3}H]CHA and [^{3}H]NECA binding to the preparation solubilized with cholate also showed linear relationships.

The studies of various adenosine derivatives on adenylate cyclase activity suggested the existence of at least two distinct adenosine receptors: A_1 receptor mediating a decrease in adenylate cyclase activity and A_2 receptor mediating an increase [8, 9]. In [9–11] PIA and CHA at nM-levels were preferentially effective on A_1 receptor and NECA to A_2 receptor. However, the kinetic behavior of

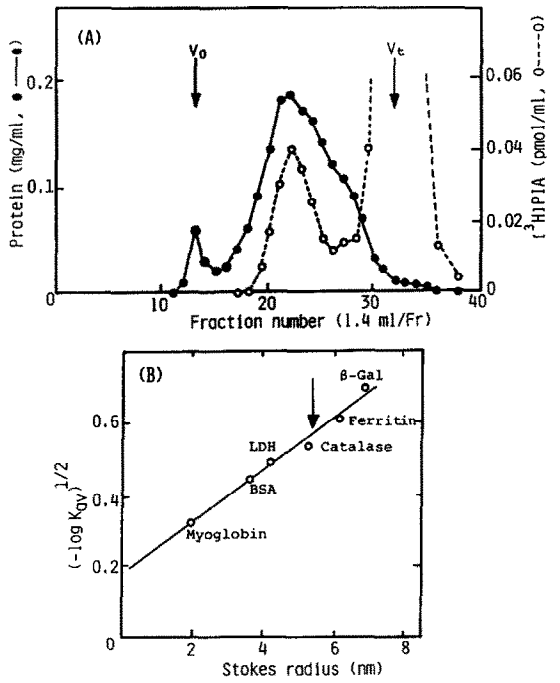

Fig. 4. Gel filtration of solubilized [^{3}H]PIA-binding sites on Sepharose CL-6B. [^{3}H]PIA (8 nM) was incubated for 15 min at 37°C with brainstem membrane (8 mg/ml) in 50 mM Tris–acetate buffer (pH 7.4) in the presence or in the absence of 80 μM PIA. After the incubation, sodium cholate, dithiothreitol and protease inhibitors (leupeptin, chymostatin, antipain and pepstatin A) were added to final concentrations of 1%, 1 mM and 10 μg/ml, respectively, followed by further incubation for 30 min at 0°C with stirring. The mixture was centrifuged at 106800 × g for 60 min at 4°C and the resulting supernatant (1 ml) was immediately applied to a Sepharose CL-6B column (1.4 × 33 cm) equilibrated in 50 mM Tris–acetate buffer (pH 7.4) containing 0.1% Triton X-100, 1 mM dithiothreitol and 0.15 M NaCl. When incubation was performed in the presence of unlabelled PIA, the radioactive peak around fraction 22 was not observed, indicating that it may be derived from [^{3}H]PIA-adenosine binding site complex. Estimation of the Stokes radius of the adenosine-binding site by comparison with the marker proteins. The arrow indicates the position of [^{3}H]PIA-binding site complex.

Abbreviations: BSA, bovine serum albumin; LDH, lactate dehydrogenase; β-Gal, *E. coli* β-galactosidase

[^{3}H]NECA binding to rat brainstem membranes and the solubilized preparation were essentially similar to those of [^{3}H]PIA and [^{3}H]CHA binding under our experimental conditions.

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REFERENCES

[1] Daly, J.W., Bruns, R.F. and Snyder, S.H. (1981) Life Sci. 28, 2083–2097.
[2] Snyder, S.H. (1981) Trends Neurosci. 4, 242–244.
[3] Schwabe, U. (1981) Trends Pharmacol. Sci. 2, 299–303.
[4] Daly, J.W. (1982) J. Med. Chem. 25, 197–207.
[5] Gavish, M., Goodman, R.R. and Snyder, S.H. (1982) Science 215, 1633–1635.
[6] Cuatrecasas, P. (1972) Proc. Natl. Acad. Sci. USA 69, 318–322.
[7] Lowry, O.H., Rosebrough, M., Farr, L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[8] Van Calker, D., Muller, M. and Hamprecht, B. (1979) J. Neurochem. 33, 999–1005.
[9] Londos, C., Cooper, D.M.F. and Wolff, J. (1980) Proc. Natl. Acad. Sci. USA 77, 2551–2554.
[10] Bruns, R.F., Daly, J.W. and Snyder, S.H. (1980) Proc. Natl. Acad. Sci. USA 77, 5547–5551.
[11] Schwabe, U. and Trost, T. (1980) Naunyn-Schmiedeberg's Arch. Pharmacol. 313, 179–187.