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Affinity chromatography in purification of A_1 adenosine receptors

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ABSTRACT

Purification of A_1 adenosine receptor of rat brain membranes was performed using a newly developed affinity gel employing xanthine amine congener (XAC) as an immobilized ligand. The A_1 adenosine receptor was solubilized with digitonin–cholate from brain membranes and then purified by a sequential use of affinity chromatography on XAC–agarose, hydroxyapatite chromatography and reaffinity chromatography on XAC-agarose. The A_1 adenosine receptor was purified *ca.* 45 000-fold with a yield of 5%. The final receptor preparation gave a single broad band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis with a $M_r \approx 34\,000$. This band was also shown to be specifically labelled with an affinity labelling reagent for A_1 adenosine receptors. This purification method was also applicable for the complete purification of A_1 adenosine receptors from rat testis and human brain membranes.

INTRODUCTION

Purification of receptor proteins from plasma membranes is essential to advance the molecular characterization of the receptor and also to provide partial amino acid sequence data for the cloning of receptor molecules. The isolation of sufficient amounts of receptor proteins for such purposes, however, has been difficult and is still a challenging task in spite of progress in recombinant DNA technology. In addition to the low concentration of receptors in the tissues and a low yield of solubilization of receptors from the membranes, the development of an efficient affinity chromatographic system is the most crucial and difficult step for the isolation of receptor proteins.

Because the properties of receptor proteins and also the environment around the receptor in the tissues differ from receptor to receptor, it is difficult to develop a general method for receptor isolation. Therefore, we have to develop a specific purification method including the selection of optimum tissues and detergents for each receptor. However, the basic sequence of the purification method for A_1 adenosine receptors from various sources described in this paper, *i.e.*, solubilization of membranes with mild detergents, affinity chromatography using specific antagonists or agonists as an immobilized ligand, hydroxyapatite chromatography and gel filtration using high-performance columns such as TSK-3000, may be applicable to the purification of other receptors.

Adenosine, which is known to modulate various physiological activities of many tissues and cell types, is considered as an important endogenous modulator in central and peripheral nervous systems [1]. Some of the effects of adenosine are inhibition of neurotransmitter release, sedation of neuronal activity, decrease of heart rate, control of renin release and relaxation or contraction of smooth muscle. Most of these actions are mediated via specific membrane receptors. These receptors were originally classified as P_1 and P_2 purinergic receptors based on the preference for adenosine and adenine nucleotides, respectively [2]. The adenosine-sensitive P_1 purinergic receptors, which are usually called adenosine receptors, are subclassified as A_1 and A_2. It is believed that A_1 adenosine receptor

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mediates an inhibition of adenylate cyclase by coupling with G_i of G_o proteins, and A_2 adenosine receptor mediates a stimulation of adenylate cyclase by coupling with G_s proteins (G-protein = guanine nucleotide binding protein) [3,4]. Therefore, adenosine receptors belong to a large family of the G-protein-coupled receptors such as adrenergic receptors, dopamine receptors and muscarinic acetylcholine receptors. In addition to the affect on adenylate cyclase, the adenosine receptors are now known to be involved in the modulation of K^+ channels, Ca^{2+} channels and phosphoinositide turnover via G-proteins [5]. The structure and the functional mechanism of the adenosine receptors are still largely unknown. Although A_1 adenosine receptors have been characterized pharmacologically and biochemically in various tissues, their purification has been hampered by the low concentration of the receptors in the tissues and also by the lack of an efficient affinity chromatographic system. Recently, two affinity adsorbates were developed for the purification of A_1 adenosine receptors. One employs xanthine amine congener (XAC), an A_1 adenosine receptor antagonist [6], and the other uses N^6-aminobenzyladenosine, an A_1 adenosine receptor agonist, as immobilized ligands [7]. Although both methods gave highly purified A_1 adenosine receptors, the receptor proparation purified by the N^6-aminobenzyladenosine-coupled affinity gel was contaminated with several G-proteins in addition to other unknown peptides [7].

The affinity purification procedure described here using XAC as an immobilized ligand has been used successfully for obtaining more than 200 pmol (*ca.* 7 μg of protein) of completely purified rat brain A_1 adenosine receptor from one series of experiments, and also proved to be useful for the complete purification of rat testicular and human brain A_1 adenosine receptors [8–10].

EXPERIMENTAL

Preparation of XAC–agarose gel

Coupling of XAC (Research Biochemicals, Natick, MA, USA) to agarose via amide linkage was carried out in a non-aqueous solvent because of the low solubility of XAC in aqueous solution. Routinely, 100 ml of Affi-Gel 10 (Bio-Rad Labs., Richmond, CA, USA), N-hydroxysuccinimide esters of a derivatized cross-linked agarose, were washed with *ca.* 300 ml of dimethyl sulfoxide (DMSO) and the moist gel cake was resuspended in 200 ml of DMSO containing 100 mg (230 μmol) of XAC. The coupling was carried out at room temperature for 15 h with continuous rotation. The reaction was stopped by washing the gel extensively with DMSO. The washed gel was incubated with 200 m*M* Tris–acetate buffer (pH 8.0) for 24 h. Finally, after washing with water, the XAC–agarose was stored at 4°C in 0.02% sodium azide solution. The amount of covalently bound XAC was estimated to be *ca.* 1.8 μmol/ml of gel by monitoring the absorbance at 310 nm in 0.01 *M* HCl of the starting XAC solution and the wash filtrate. The presumed structure of the XAC-linked agarose is shown in Fig. 1. The prepared gel was stable for at least 6 months at 4°C and

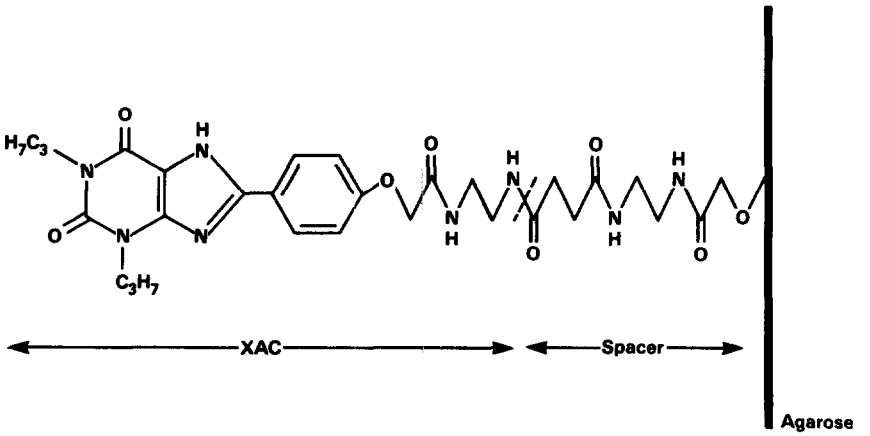

Fig. 1. Presumed structure of XAC–agarose.

could be used repeatedly after regeneration of the gel.

Purification of A_1 adenosine receptors

Rat brains (*ca.* 150 g) were homogenized in three volumes of 50 m*M* Tris–acetate buffer (pH 7.2) containing 1 m*M* EDTA and 1 m*M* phenylmethylsulfonyl fluoride (Sigma, St. Louis, MO, USA) by a Polytron and the homogenate was centrifuged at 39 000 *g* for 20 min. The resulting pellet was washed three times with the same buffer and the final pellet was resuspended in three volumes of the same buffer containing 2 units/ml of adenosine deaminase. The membrane suspension was incubated for 20 min at 30°C to remove endogenous adenosine enzymatically, followed by centrifugation at 39 000 *g* for 20 min. The resulting pellet (150 g wet weight) was homogenized by a Polytron with 1500 ml of 1% digitonin–0.1% sodium cholate in 50 m*M* Tris–acetate buffer (pH 7.2) containing 100 m*M* NaCl, 1 m*M* EDTA, 1 m*M* dithiothreitol and 1 m*M* phenylmethylsulfonyl fluoride. The homogenate was stirred on ice for 1 h and then centrifuged at 46 000 *g* for 1 h at 4°C. The clear supernatant was saved as the solubilized receptor preparation.

The solubilized preparation (1500 ml) was applied to an XAC–agarose column (10 × 5 cm I.D.) at a flow-rate of 70–80 ml/h. After the application, the column was washed with three column volumes of 50 m*M* Tris–acetate buffer (pH 7.2) containing 100 m*M* NaCl, 1 m*M* EDTA and 0.1% digitonin (buffer A) until the absorbance at 280 nm of the effluent was close to the baseline. The A_1 adenosine receptor remaining in the column was eluted with three column volumes of buffer A containing 100 μM 8-cyclopentyltheophylline (CPT) (Research Biochemicals). The active eluate from the column was pooled and applied to a 0.5-ml column of hydroxyapatite (Bio-Rad Labs.). The receptor was eluted from the hydroxyapatite column with 500 m*M* potassium phosphate buffer (pH 7.0) containing 100 m*M* NaCl and 0.1% digitonin. The eluate (3 ml) was diluted two-fold by the addition of buffer A and then applied to an XAC–agarose column (5 × 1 cm I.D.). After washing the column with six column volumes of buffer A, the bound receptor was eluted by the addition of two column volumes of 100 μM CPT in buffer A. In order to assay the receptor preparation from the affinity column, the eluate was first desalted on a Sephadex G-50 column (13.5 × 0.6 cm I.D.) to separate unbound ligand from the receptor. The purified receptor preparation was stored at −85°C.

A_1 adenosine receptors from rat testis and human brain membranes were also solubilized and purified by essentially the same method as described above, except that the gel permeation chromatography using TSK-3000SW (Tosoh, Tokyo, Japan) was added as the last step in order to remove a few contaminating proteins.

Binding assay

Because [^{3}H]DPCPX([^{3}H]-8-cyclopentyl-1,3-dipropylxanthine) (Amersham, Arlington Heights, IL, USA) has reasonably high specificity and high affinity ($K_d \approx 1$ n*M*) for the A_1 adenosine receptor, it was used for the measurement of A_1 adenosine receptor binding activity as described previously [8].

Others

The affinity labeling of purified A_1 adenosine receptors was performed as described previously using *p*-DITC–[^{3}H]XAC as an affinity labeling reagent, where DITC–XAC = 1,3-dipropyl-8-[isothiocyanatophenyl(aminothiocarbonyl{2-aminoethylaminocarbonyl[4-methyloxy(phenyl)]})]xanthine [11]. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli [12]. The protein concentration in the particulate and solubilized preparations was determined by the method of Bradford [13]. The protein content of the eluate from the affinity column or hydroxyapatite column was determined by the Amido Black method [14]. Saturation and displacement curves obtained from the receptor binding assays were analyzed using the computer programs EBDA-LIGAND and GRAPH-PAD, respectively.

RESULTS AND DISCUSSION

The A_1 adenosine receptor was solubilized from rat brain membranes using a mixed detergent system composed of 1% digitonin–0.1% sodium cholate with a yield of *ca.* 30%. A_1 adenosine receptors of rat testis and human brain membranes were also solubilized similarly under the same conditions. The ratio of detergent to membrane proteins is impor-

tant to obtain a maximum and consistent yield of the solubilization. Other detergents, such as 3-[(3-cholamidopropyl)dimethylammonia]-1-propanesulfonate (CHAPS) [7] and sodium cholate [15,16], have been used for the solubilization of A_1 adenosine receptors from rat brain and bovine brain membranes. However, longer ultracentrifugation times (100 000 *g*, 1–2 h) are usually necessary to separate the soluble fractions from the insoluble materials when CHAPS or sodium cholate is used as a solubilizing detergent, and the resulting supernatant is often still turbid (unpublished observation). A clear supernatant can be easily obtained after the centrifugation of the solubilizing mixture (46 000 *g*, 1 h) when digitonin–cholate is employed as a detergent. Recently, an improved condition for the solubilization of A_1 adenosine receptor from pig brain membranes using 0.5% CHAPS–0.5% digitonin was reported [17].

The most effective step in the purification method described here was affinity chromatography using XAC as an immobilized ligand. A typical chromatographic profile of solubilized receptor from rat brain membranes on XAC–agarose is shown in Fig. 2. The same pattern of the affinity chromatography was seen in the purification of A_1 adenosine receptors from either rat testes or human brains. When solubilized receptor preparations were applied on the XAC–agarose column, *ca.* 80% of [^{3}H]DPCPX binding activity remained in the column after the column had been washed with buffer A. The binding activity was specifically eluted by a potent A_1 adenosine receptor antagonist, CPT. This step resulted in a 2500-fold purification over the solubilized preparation with a yield of 40%.

It was shown that the solubilized A_1 adenosine receptor interacts with the XAC–agarose biospecifically because adsorption of the receptor to the affinity column was inhibited by preincubation of the solubilized receptor preparation with A_1 adenosine

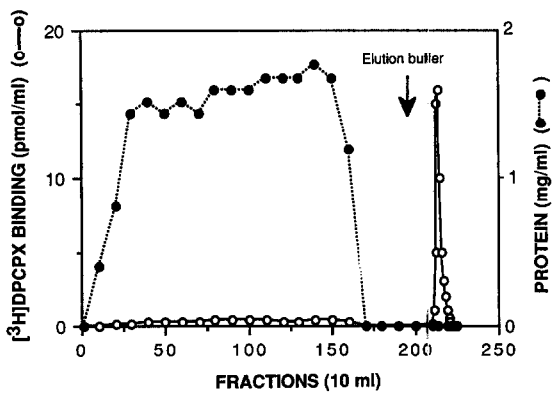

Fig. 2. XAC–agarose chromatography of solubilized rat brain A_1 adenosine receptors. The solubilized preparation (1500 ml) was passed through an XAC–agarose column (10 × 5 cm I.D.) at a flow-rate of 80 ml/h. The column was washed with buffer A [50 m*M* Tris–acetate (pH 7.2)–100 m*M* NaCl–1 m*M* EDTA–0.1% digitonin]. Receptor activity was then eluted biospecifically with 100 μM 8-cyclopentyltheophylline at the position indicated by the arrow.

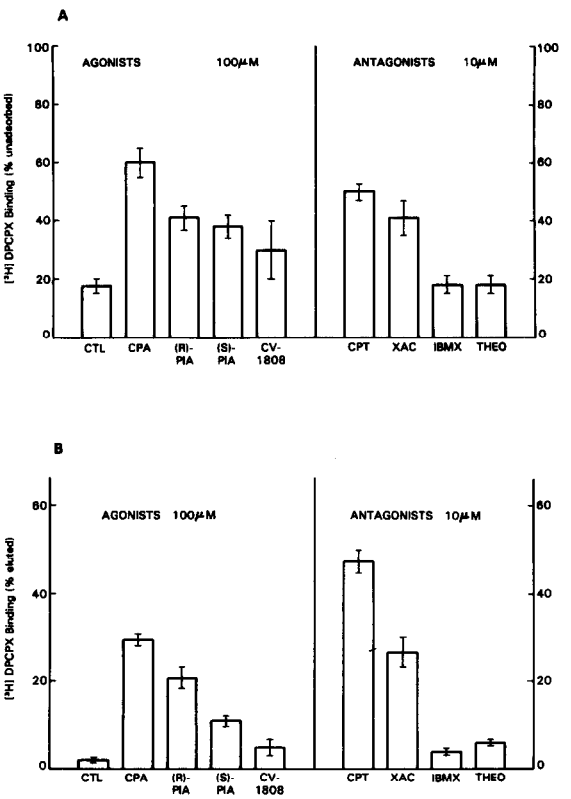

Fig. 3. Biospecific characteristics of XAC–agarose. (A) Biospecificity of adsorption. A solubilized preparation (0.5 ml) from rat brain membranes was incubated with agonists or antagonists and then was applied to a 1-ml column of XAC–agarose. The pass and wash fractions were assayed for [^{3}H]DPCPX binding activity. (B) Biospecificity of elution. A solubilized preparation (0.5 ml) of rat brain membranes was loaded on a 1-ml column of XAC–agarose and washed with buffer A. The column was then eluted with agonists or antagonists solution. The eluates were assayed for [^{3}H]DPCPX binding activity. CPA, N^6-cyclopentyladenosine; PIA, N^6-phenylisopropyladenosine; IBMX, isobutylmethylxanthine; THEO, theophylline.

receptor agonists or antagonists and the specificity of the inhibition was essentially the same as the known specificity of A_1 adenosine receptor (Fig. 3A). The specificity of elution from XAC–agarose was also investigated by the elution of bound A_1 adenosine receptor with various adenosine receptor agonists or antagonists. Again, the specificity of elution was essentially the same as that for inhibition of adsorption described above (Fig. 3B).

Addition of phosphatidylcholine or asolectin to the elution buffer was reported to be essential to increase the recovery of the binding activity from the affinity column when CHAPS was used as a detergent in the purification [7,18]. It appears that a phospholipid–CHAPS suspension provides a milieu that is necessary for the ligand-specific removal of A_1 adenosine receptor from the affinity gel. Such a requirement of phospholipid was not observed in the purification method described here.

The affinity-purified receptor preparation was then applied to a small hydroxyapatite column. Most of the binding activity was adsorbed on this column and eluted with a high concentration of phosphate buffer. The A_1 adenosine receptor antagonist, CPT, which is present in the affinity-purified receptor preparation, was removed from the receptor preparation during the hydroxyapatite chromatography. The final step in the purification was reaffinity chromatography on a small XAC–agarose column.

The results obtained from a typical purification experiment are summarized in Table I together with the results of the purification of A_1 adenosine receptors from rat testis and human membranes. Similar purification profiles were obtained for each source. The maximum specific [^{3}H]DPCPX binding of purified A_1 adenosine receptors was estimated to be 16–26 nmol/mg of proteins from saturation experiments, indicating one binding site per minimum protein component (data not shown).

The final preparations of rat brain, rat testis and human brain A_1 adenosine receptors showed a single broad band on SDS-PAGE at M_r *ca.* 34 000, *ca.* 41 000 and *ca.* 35 000, respectively (Figs. 4 and 5). Affinity labeling with *p*-DITC–[^{3}H]XAC, a specific acylating agent for A_1 adenosine receptors [11], demonstrated that the single band in SDS-PAGE of the purified receptor preparations contained the adenosine-binding sites (data not shown). The band in SDS-PAGE of these purified A_1 adenosine recep-

TABLE I

PURIFICATION OF A_1 ADENOSINE RECEPTORS FROM VARIOUS SOURCES

Binding activities were assessed at 2–5 n*M* [^{3}H]DPCPX. ND = Not determined.

Tissue	Step	Total activity (pmol)	Specific activity (pmol/mg)	Yield (%)	Purification (-fold)
Rat brains	Membranes	4820	0.44	100	1
	Solubilized	1540	0.41	32	0.93
	XAC–agarose	580	1100	12	2500
	Hydroxyapatite	360	4030	7.5	9160
	Re-XAC–agarose	250	19700	5.2	44800
Rat testes	Membranes	122	0.35	100	1
	Solubilized	40.5	0.28	33	0.8
	XAC–agarose	17.8	685	14	1960
	Hydroxyapatite	11.7	1300	9.6	3710
	Re-XAC–agarose	8.5	8500	7.0	24300
	TSK-3000SW	3.0	ND	2.5	ND
Human brains	Membranes	1600	0.99	100	1
	Solubilized	480	1.3	30	1.3
	XAC–agarose	180	30	11	300
	Hydroxyapatite	160	1500	10	1500
	Re-XAC–agarose	53	ND	3.3	ND
	TSK-3000SW	25	13000	1.6	13000

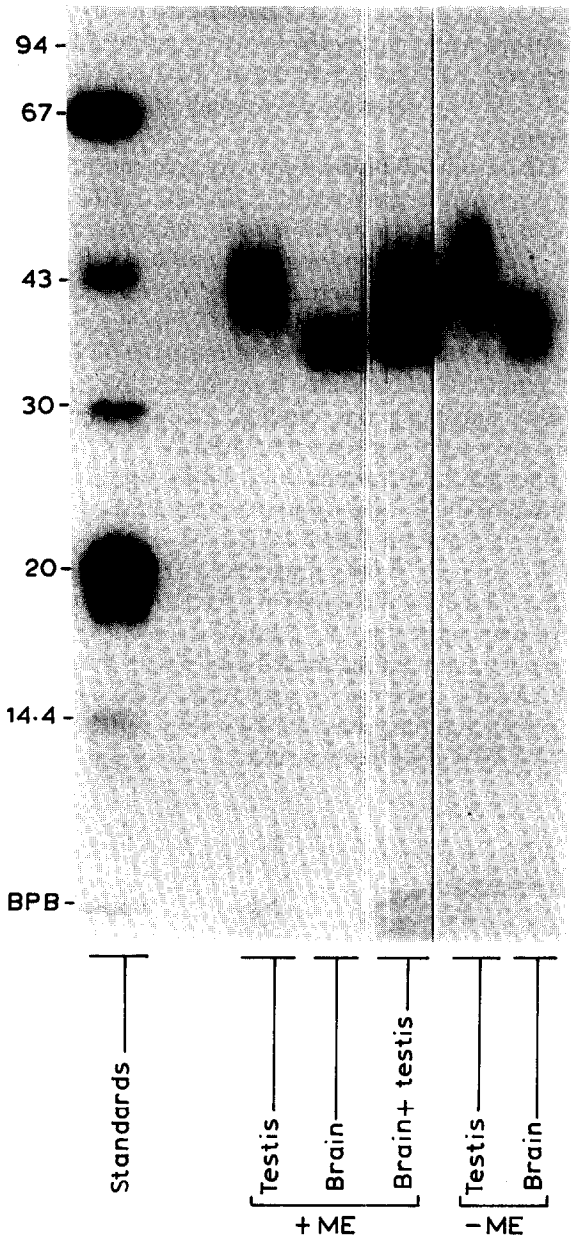

Fig. 4. SDS-PAGE autoradiography of ^{125}I-labelled purified A_1 adenosine receptors from rat brain and rat testis membranes. The purified A_1 adenosine receptor preparations from rat brain rat testis membranes were radioiodinated by the chloramine-T method and subjected to SDS-PAGE. Electrophoresis was performed in the reducing (+ME) or non-reducing (−ME) conditions. ME = 2-Mercaptoethanol; BPB = Bromophenol Blue. Standard molecular mass markers are shown on the left ($M_r \cdot 10^3$).

tors, especially of testicular A_1 adenosine receptor, were always broader than the bands of marker proteins, irrespective of the detection methods. Such a broad band has often been observed in the SDS-PAGE patterns of other membrane-bound receptors such as adrenergic receptors and muscarinic receptors, and it is generally believed to be caused by microheterogeneity in the carbohydrate residues and anomalous binding of SDS due to the presence of tightly bound detergents. In fact, enzymatic deglycosylation with endoglycosidase F of the purified rat brain, rat testis and human brain A_1 adenosine receptors caused a significant reduction in an apparent molecular mass from 34 000 to 30 000, 41 000 to 30 000 and 35 000 to 30 000, respectively (data not shown).

The purified receptors gave a typical ligand binding specificity as A_1 adenosine receptors, *i.e.*, N^6-cyclopentyladenosine > (*R*)-N^6-phenylisopropyladenosine > 5′-N-ethylcarboxamidoadenosine > (*S*)-N^6-phenylisopropyladenosine for agonists and DPCPX > CPT > isobutylmethylxanthine for antagonists (data not shown).

A polyclonal antibody against the rat brain A_1 adenosine receptor was raised by immunizing the purified rat brain A_1 adenosine receptor into a rabbit. By immunoblot experiments, it was shown that the antibody reacted not only with rat brain A_1 adenosine receptor protein, but also with rat testis A_1 adenosine receptor protein as shown in Fig. 6. The immunoreactivity of the purified human brain A_1 adenosine receptor was low compared with that of rat testis A_1 adenosine receptors (Fig. 7). The order of the reactivity of these receptors with the antibody was rat brain > rat testis > human brain.

Previous attempts to purify A_1 adenosine receptors using gel filtration [19] or sucrose density centrifugation [20] did not yield any significant increase in the specific binding activity. Only the report of Ku *et al.* [21] showed about a 40-fold enrichment of the adenosine binding sites by chromatography on adenosine-6-aminocaproyl AH-Sepharose 4B gel. After the first report of partial purification of A_1 adenosine receptor from rat brain membranes using XAC–agarose by our group [6], several others reported partial or complete purification of A_1 adenosine receptors or adenosine-binding proteins from various sources. Munshi and Linden [7] showed that bovine cerebral cortex A_1 adenosine

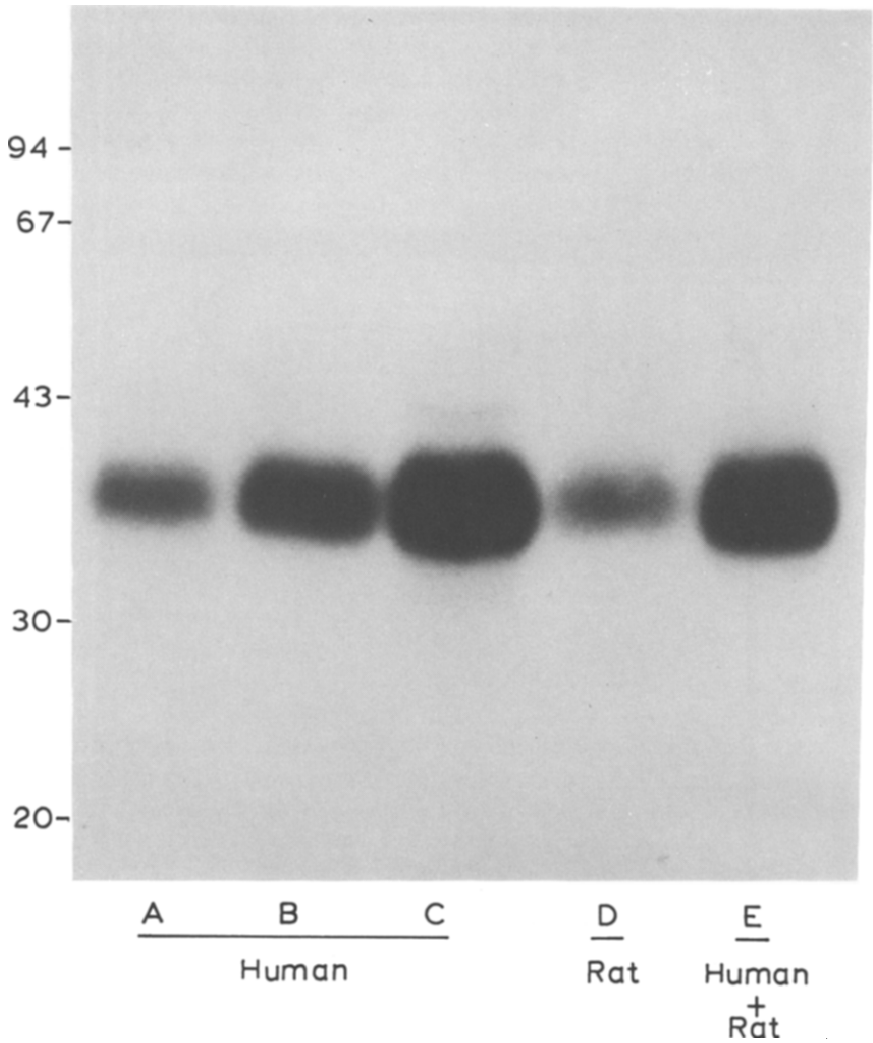

Fig. 5. SDS-PAGE autoradiography of ^{125}I-labelled purified A_1 adenosine receptors from human and rat brain membranes. A_1 adenosine receptors purified from rat brain and human brain membranes were radioiodinated and subjected to SDS-PAGE. Approximately 5000, 10 000, 15 000 and 5000 cpm of the radioiodinated human brain A_1 adenosine receptors were loaded on lanes A, B, C and E, respectively. Approximately 5000 cpm of the radioiodinated A_1 adenosine receptors purified from rat brain membranes were loaded on lanes D and E. Molecular mass indicated on the left ($M_r \cdot 10^3$).

receptor was purified more than 2000-fold by agonist-coupled affinity column using aminobenzyladenosine as an immobilized ligand. In contrast to the affinity chromatographic system described here using antagonist as an immobilized ligand, the A_1 adenosine receptor bound in the agonist-coupled gel column was eluted by addition of GTP or N-ethylmalcimide which uncoupled G-protein–receptor interactions. G-proteins such as G_o and G_i were co-eluted with the receptors from the affinity column. The usefulness of XAC–agarose for the purification of A_1 adenosine receptors from bovine and rat brain membranes was confirmed by other groups [18,22]. Other adenosine binding proteins which are distinct from adenosine receptors have also been isolated from bovine brain and human placental membranes [23–25].

Very recently, molecular cloning of A_1 adenosine

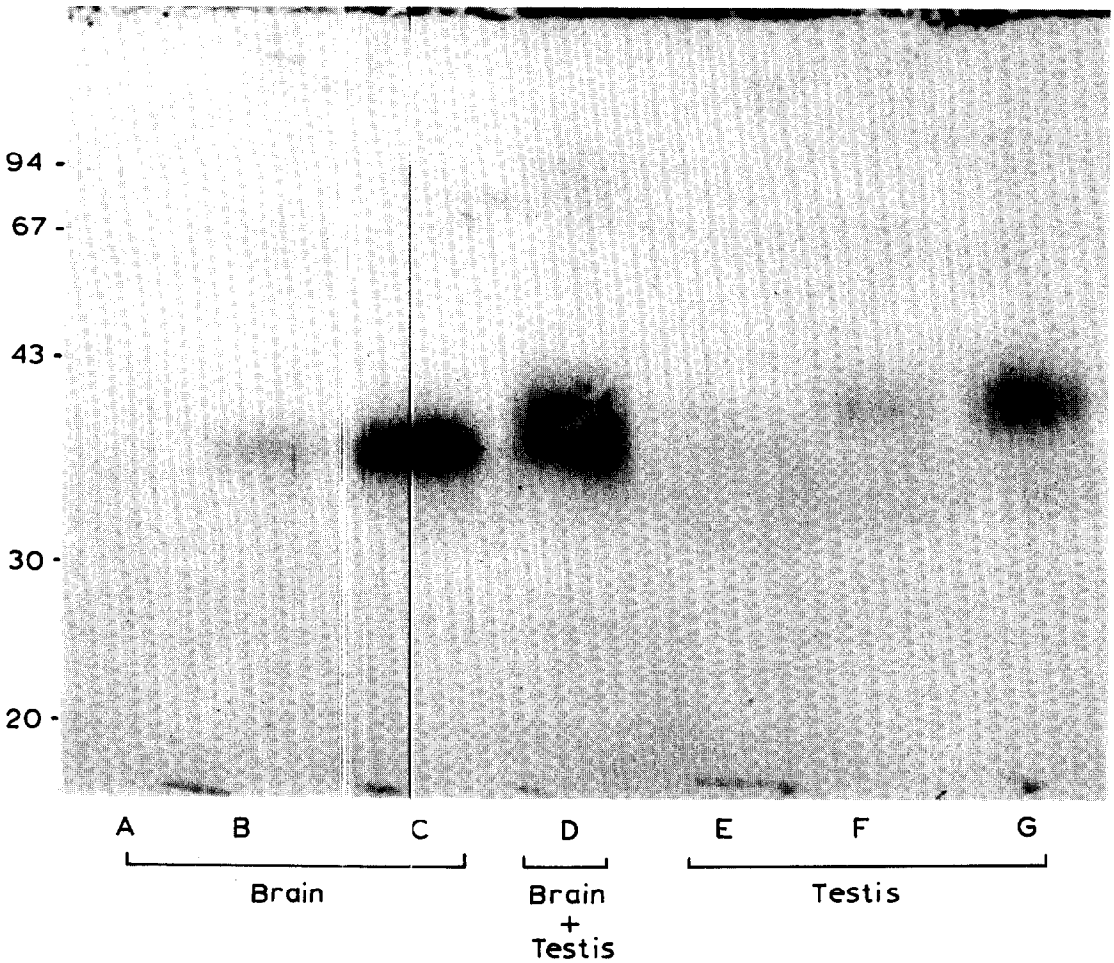

Fig. 6. Immunoblots of purified rat brain and testis A_1 adenosine receptors with anti-rat brain A_1 adenosine receptor antiserum. SDS-PAGE was performed using *ca.* 0.1, 0.9, 1.8 and 1.8 ng of purified rat brain A_1 adenosine receptor proteins in lanes A, B, C and D, respectively. Approximately 4, 0.2, 1.9 and 4 ng of purified rat testis A_1 adenosine receptors were also run in lanes D, E, F and G, respectively. The resolved proteins were then electrophoretically transferred to nitrocellulose membranes. The nitrocellulose blots were incubated with rabbit anti-rat brain A_1 adenosine receptor antiserum (1:2000-fold dilution). Immunoreactivity was revealed by incubating the washed nitrocellulose membranes with horseradish peroxidase-labeled goat anti-rabbit IgG antibodies followed by reaction with 4-chloro-1-naphthol in the presence of hydrogen peroxide. Molecular mass indicated on the left ($M_r \cdot 10^3$).

receptor was reported by two groups [26,27], indicating that the A_1 adenosine receptor has seven transmembrane domains which are homologous with various members of the G-protein-coupled receptor family.

In conclusion, XAC–agarose was found to be a novel affinity resin for the purification of A_1 adenosine receptors from various sources. This progress in the purification and also in the cloning of the A_1 adenosine receptors should facilitate a more detailed molecular characterization of the receptor, including studies on the identification of the ligand binding sites, the interaction of the receptor with various G-proteins and effector systems in reconstitution systems as well as the identification of additional gene members within the broader purine receptor family.

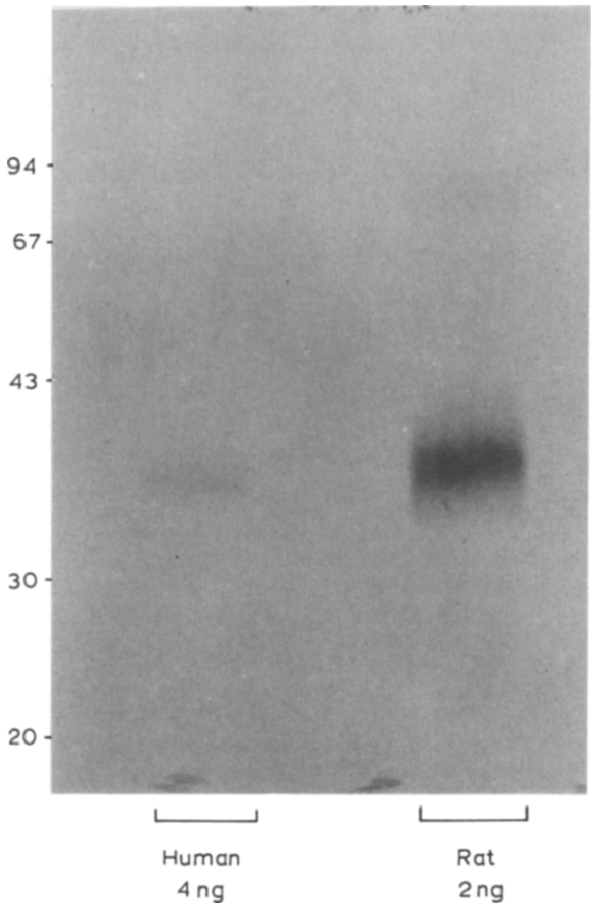

Fig. 7. Immunoblots of purified rat brain and human brain A_1 adenosine receptors with anti-rat brain A_1 adenosine receptor antiserum. SDS-PAGE of *ca.* 2 ng of purified rat brain and 4 ng of purified human brain A_1 adenosine receptor proteins was performed in the lanes shown. After electrophoresis, the resolved proteins were transferred to nitrocellulose membranes and the immunoreactivity of these proteins was determined as described in Fig. 6. Molecular mass indicated on the left ($M_r \cdot 10^3$).

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