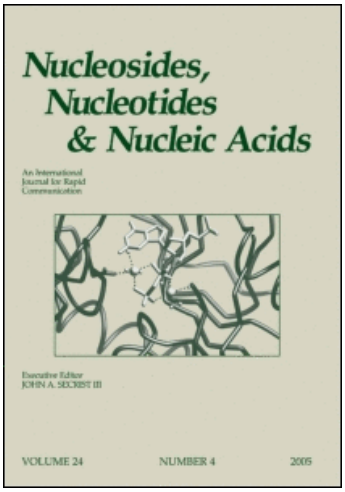

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Recent Progress in the Purification of Adenosine Receptors

Hiroyasu Nakata[a]
[a] Laboratory of Clinical Science, National Institute of Mental Health, Bethesda, MD, USA

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RECENT PROGRESS IN THE PURIFICATION OF ADENOSINE RECEPTORS

Hiroyasu Nakata

Laboratory of Clinical Science, National Institute of Mental Health,
Building 10, Room 3D-48, Bethesda, MD 20892, USA

Abstract A_1 adenosine receptors were purified to an apparent homogeneity from rat brain and testicular membranes by a novel affinity chromatography system using xanthine amine congener (XAC) as an immobilized ligand. This affinity chromatography was also useful for the purification of human brain A_1 adenosine receptor.

It is now well recognized that adenosine affects many physiological functions in various tissues and that most of the effects are mediated via specific membrane receptors. These receptors are originally classified as P_1 and P_2 purinergic receptors based on the preference for adenosine or adenine nucleotides.[1] P_1 purinergic receptors are more responsive to adenosine and P_2 purinergic receptors are more responsive to ATP. The adenosine-sensitive P_1 purinergic receptors, which are usually called adenosine receptors, are subclassified as A_1 adenosine receptor and A_2 adenosine receptor. It is believed A_1 adenosine receptor mediates an inhibition of adenylate cyclase by coupling with G_i (G_o) proteins, and A_2 adenosine receptor mediates a stimulation of adenylate cyclase by coupling with G_s proteins. Therefore, adenosine receptors belong to a family of the G-protein-coupled receptors such as adrenergic receptors, dopamine receptors, etc. Recent progress in the technology of molecular biology, along with the sophistication of the purification techniques, enabled us to obtain the whole DNA sequences encoding some of these G-protein-coupled receptor proteins. However, the structure and the functional mechanism of the adenosine receptors are still largely unknown. Although the A_1 receptor has been better characterized pharmacologically and biochemically in various tissues and species than A_2 adenosine receptor due to the development of A_1 specific ligands. The purification of adenosine receptors, which is the fundamental step toward the detailed characterization of the receptors, has been hampered by the low concentration of the receptors in the tissues and also by the lack of an efficient affinity chromatography method. Recently, A_1 adenosine receptors were highly purified for the first time from rat and bovine brain membranes by an affinity chromatography using N^6-aminobenzyl-adenosine (ABA, A_1 agonist)[2] or xanthine amine congener (XAC, A_1 antagonist)[3] as an immobilized ligand. In this paper, I

describe the application of the novel affinity chromatography system for the complete purification of A_1 adenosine receptors from rat testis membranes, as well as from rat brain and human brain membranes.

Methods

XAC-agarose was prepared as described previously.[3] The presumed structure of XAC-agarose is shown in the inset of FIG. 1. 8-cyclopentyl-1,3-[^{3}H] dipropylxanthine ([^{3}H]DPCPX) binding assays were performed as described.[3] For the assay of the highly purified receptor preparations, "heat-treated pass-through fraction" from the first affinity chromatography was added to each assay tube.[4]

Purification of A_1 adenosine receptor[5]: The receptor was solubilized from membrane preparations of rat testes with 1% digitonin-0.1% sodium cholate solution. The extract (about 75 ml) was applied to an affinity column (XAC-agarose, Vt=15 ml), and the column was extensively washed with 0.1% digitonin-100 mM NaCl-1 mM EDTA-50 mM Tris-acetate buffer (pH 7.2). The receptor was eluted with 8-cyclopentyltheophylline from the affinity column. The pooled active fractions were applied to a small hydroxylapatite column (0.5 ml). After the column was washed, the receptor was eluted with 500 mM phosphate buffer (pH 7.0) in a concentrated and ligand-free form. The eluate was further purified by re-affinity chromatography using a small XAC-agarose column (Vt=1.5 ml) followed by gel permeation HPLC (TSK 3000SW). A_1 adenosine receptors from rat brain membranes[4] and human cerebral cortex membranes were also purified by essentially the same purification methods described above, except that the gel permeation HPLC was omitted for the purification of rat brain A_1 adenosine receptor.

Results and discussion

The most effective step in the purification was affinity chromatography using XAC as an immobilized ligand. A typical chromatographic profile of solubilized receptor from rat testis membranes on XAC--agarose is shown in FIG. 1. The same pattern of the affinity chromatography was obtained in the purification of receptors either from rat brain or human brain. Approximately 50-80% of [^{3}H]DPCPX binding activity applied was absorbed on the column, and more than 30% of the binding activity was specifically eluted by a potent A_1 adenosine receptor antagonist, 8-cyclopentyl theophylline. This step resulted in a 2000-fold purification over the solubilized preparation. It was also shown that A_1 adenosine receptor interactions with XAC-agarose are biospecific because adsorption of the receptor to the affinity column was inhibited by preincubation of the solubilized receptor preparation with A_1 adenosine receptor agonists or antagonists and the specificity of the elution from the XAC-agarose was essentially the same as the known specificity of A_1 adenosine receptor (FIG. 2)

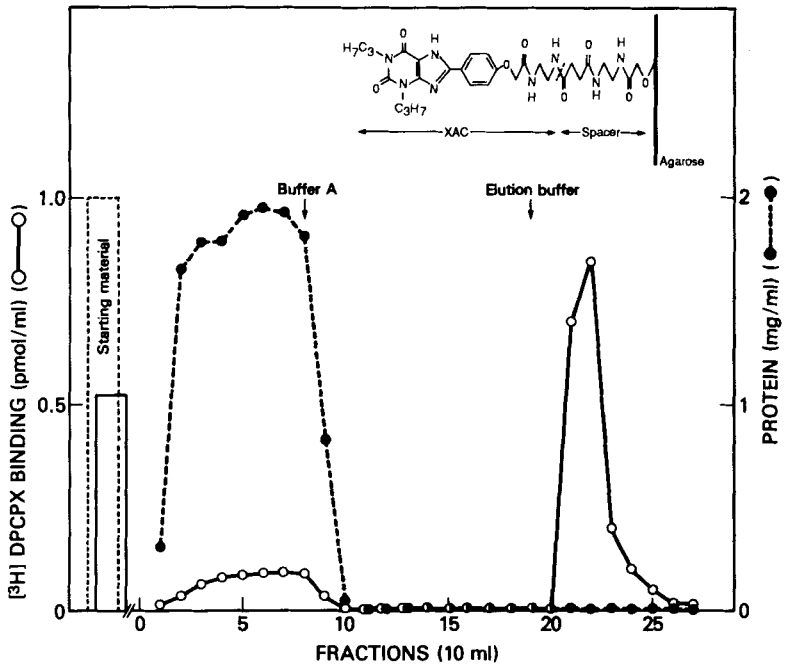

FIG. 1. XAC-agarose chromatography of solubilized rat testis A_1 adenosine receptors. The solubilized preparation (75 ml) was passed through a XAC-agarose column (2 x 4.8 cm) at a flow rate of 15 ml/h. The column was then washed with buffer A (50 mM Tris-acetate pH 7.2, 100 mM NaCl, 1 mM EDTA, 0.1% digitonin). Receptor activity was eluted biospecifically with 0.1 mM 8-cyclopentyltheophylline in buffer A.

The results of a typical purification of rat testicular A_1 adenosine receptor are summarized in TABLE 1 along with those of rat brain and human brain A_1 adenosine receptors. Very similar purification profiles were obtained from each source. The maximum specific [^{3}H]DPCPX binding of these purified A_1 adenosine receptors was estimated to be 16-26 nmol/mg of protein from saturation experiments (data not shown), indicating one binding site per minimum protein component (34,000-41,000 Dalton, See below). The binding activity of [^{3}H]DPCPX to the highly purified receptor preparation was very low when the assays were performed without the addition of the heat-treated pass-through fractions of the first affinity chromatography. The addition of a small amount of the heat-treated pass-through fractions increased the binding activity to 5-fold and a similar but less effect was observed with bovine serum albumin or calmodulin as shown in FIG. 3. It is likely that a small amount of protein prevents the receptors from nonspecific binding to the assay tubes or to the filtration apparatus. Essentially, the same phenomenon was observed with the highly purified rat brain and human brain A_1

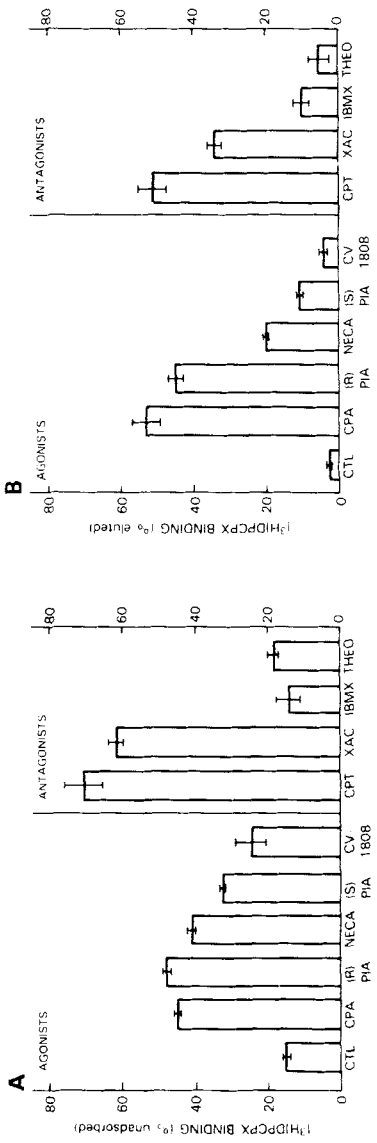

FIG. 2. Biospecific characteristic of XAC-agarose. (A) Biospecificity of adsorption. A solubilized preparation (0.5 ml) from rat testis membranes was incubated with agonists or antagonists and then was applied to a 1-ml column of XAC-agarose. The pass and wash fractions were assayed for [^{3}H]DPCPX binding activity. (B) Biospecificity of elution. A solubilized preparation (0.5 ml) was loaded on a 1-ml column and washed with buffer. The column was then eluted with agonists or antagonists. The eluates were assayed for [^{3}H]DPCPX binding activity.

TABLE 1. Purification of A_1 adenosine receptors

Tissue	Step	Total Activity[a]	Specific Activity	Yield	Purification
		pmol	pmol/mg	%	-fold
	Membranes	122	0.35	100	1
Rat	Solubilized	40.5	0.28	33	0.8
testis	XAC-agarose	17.8	685	14	1960
	Hydroxylapatite	11.7	1300	9.6	3710
	Re-XAC-agarose	8.5	8500	7.0	24300
	TSK-3000	3.0	ND	2.5	ND
	Membranes	3830	0.44	100	1
Rat	Solubilized	1150	0.41	30	0.9
Brain	XAC-agarose	460	1100	12	2500
	Hydroxylapatite	300	10000	7.8	22700
	Re-XAC-agarose	160	21900	4.2	49800
	Membranes	1600	0.99	100	1
	Solubilized	480	1.3	30	1.3
Human	XAC-agarose	180	300	11	300
brain	Hydroxylapatite	160	1500	10	1500
	Re-XAC-agarose	53	ND	3.3	ND
	TSK-3000	25	13000	1.6	13000

[a] Binding activities were assessed at 2-5 nM [^{3}H]DPCPX. ND, not determined.

adenosine receptor preparations. Other groups reported that the addition of phospholipid to the incubation tubes or chromatography buffers is critical for the preservation of some radioligand binding.[2,6].

The final preparation of rat testis A_1 adenosine receptor showed a single broad band on SDS-PAGE at a position of Mr 41,000 which is significantly larger than that of the purified receptor, either from rat brain membranes (FIG. 4) or from human brain membranes (data not shown). The band in SDS-PAGE of the purified A_1 adenosine receptors, especially of testicular receptor, was always broader than the bands of marker proteins irrespective of the detection methods. A similar broadness has been observed for other receptors such as ß-adrenergic receptors and muscarinic receptors. Such broadness is generally caused by microheterogeneity in the

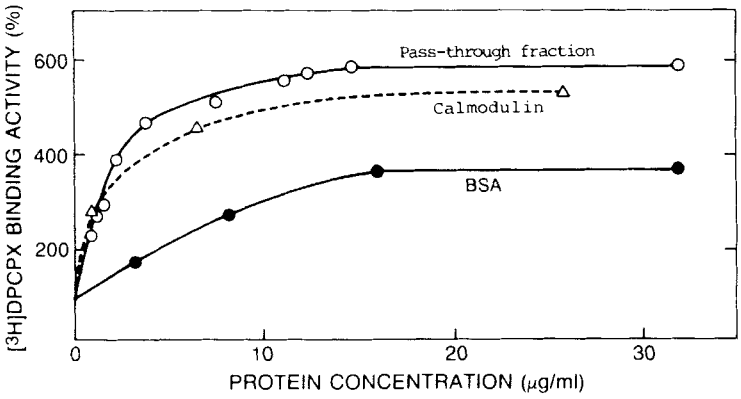

FIG. 3. Activation of [^{3}H]DPCPX binding activity of A_1 adenosine receptor by "heat-treated pass-through fraction." The affinity-purified A_1 adenosine receptor preparation from rat testis membranes was assayed with [^{3}H]DPCPX in the presence of various concentrations of additives indicated.

carbohydrate residues and anomalous binding of SDS due to the presence of tightly bound detergents. In fact, deglycosylation with endoglycosidase F treatment of the purified testis or brain A_1 adenosine receptor caused a reduction in apparent molecular weight from 41,000 to 30,000 in the testicular receptor and from 34,000 to 30,000 in the brain receptor, suggesting that the A_1 adenosine receptor is a glycoprotein (data not shown). Affinity labeling with p-DITC-[^{3}H]XAC, a specific acylating agent for A_1 adenosine receptors,[7] demonstrated that the single band of Mr 41,000 or 34,000 in the SDS-PAGE of the purified receptor preparation contains the adenosine binding sites for the rat testicular receptor and brain receptor, respectively (data not shown).

The purified receptors gave a typical A_1 adenosine receptor ligand-binding specificity which is similar to that of unpurified receptor preparation i.e., CPA>R-PIA>NECA>S-PIA for agonists and DPCPX>CPT>IBMX for antagonists (data not shown). It should be noted that the affinity with agonists was significantly decreased after the purification. The results that the purified receptor preparation showed (1) single protein band in SDS-PAGE, (2) the decrease in the affinity with agonists, (3) no activation of [^{3}H]DPCPX binding by guanine nucleotides (data not shown)[3,5] suggest that the receptor preparation purified through the antagonist affinity chromatography does not contain any G-proteins.

A polyclonal antibody against the purified rat brain A_1 adenosine receptor was recently obtained. By the immunoblots experiments, it was shown that the antibody reacts with not only rat brain A_1 adenosine receptor protein, but also testis A_1 receptor and human brain A_1 receptor proteins (FIG. 5). The order of the reactivity

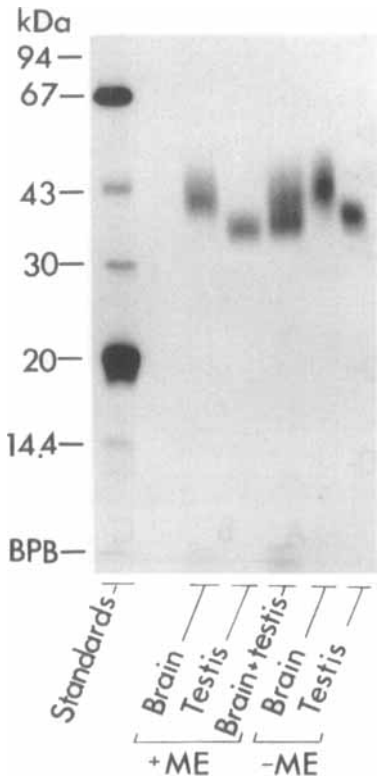

FIG. 4. SDS-PAGE autoradiography of ^{125}I-iodinated purified A_1 adenosine receptors from rat testis and rat brain membranes. SDS-PAGE was performed in 10% polyacrylamide gel under reducing (+ME) or noreducing (-ME) conditions.

of these receptors with the antibody was rat brain>rat testis>human brain. These results, along with the experiment of endoglycosidase F treatment, suggest that the core protein structure among these A_1 adenosine receptors are similar, although the mode or extent of glycosylation may be different.

The purification method described here, including the affinity chromatography using XAC-agarose, was found to be applicable for the purification of A_1 adenosine receptors from three sources i.e., rat brain, rat testis, and human brain membranes. It is likely that this purification method can be useful for the purification or at least for the concentration of A_1 adenosine receptors from other sources where A_1 adenosine receptors exist in a low concentration. After the first report of XAC-affinity chromatography system for the purification of A_1 adenosine receptor from rat brain membranes,[3] at least three groups reported the partial

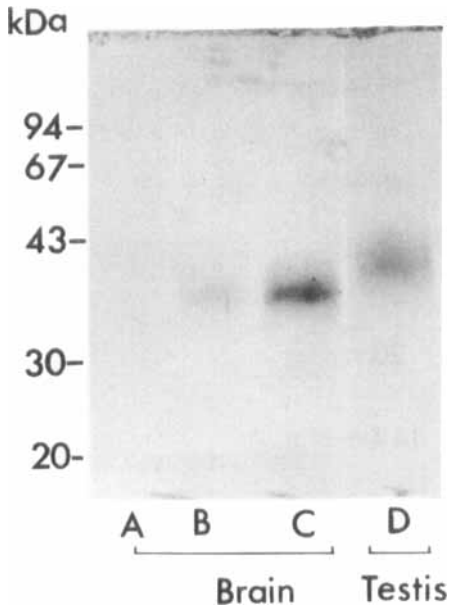

FIG. 5. Immunoblots of rat brain and testis A_1 adenosine receptors with anti-rat brain A_1 adenosine receptor antiserum. Approximately 0.1 ng, 0.9 ng, 1.8 ng and 4 ng of receptor proteins were run in lane A, B, C, D, respectively.

purification of A_1 adenosine receptor from bovine or rat brains by affinity chromatography. Dr. Linden's group showed that bovine cerebral cortex A_1 adenosine receptor was purified more than 2000-fold by agonist affinity chromatography using ABA as a ligand.[2] In contrast to the antagonist-affinity chromatography described in this paper, the highly purified receptor could be eluted from the agonist-affinity column by addition of GTP or NEM which uncouples G-protein-receptor interactions. G-proteins were co-eluted with the receptor from the affinity column. Therefore, this may be a good method to identify the endogenous G-proteins which are originally coupled with adenosine receptors in the membranes. Recently, Dr. Stiles' group[6] and Dr. Schwabe's group[8] confirmed the usefullness of XAC-agarose for the purification of A_1 adenosine receptors from bovine and rat brain membranes.

In summary, more detailed properties of A_1 adenosine receptors can now be studied because of the recent progress in the purification of A_1 adenosine receptors from various tissues. Molecular characterization, such as sequencing of the receptor protein and cloning of the gene, should be facilitated by these findings.

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