Published in final edited form as:

Arch Biochem Biophys. 1990 December ; 283(2): 440–446.

Purification and Characterization of Bovine Cerebral Cortex A₁ Adenosine Receptor¹

Mark E. Olah², Kenneth A. Jacobson^{*}, and Gary L. Stiles

Departments of Medicine (Cardiology) and Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

^{*}Laboratory of Chemistry, National Institute of Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892

Abstract

A₁ adenosine receptors (A₁AR) acting via the inhibitory guanine nucleotide binding protein inhibit adenylate cyclase activity in brain, cardiac, and adipose tissue. We now report the purification of the A₁AR from bovine cerebral cortex. This A₁AR is distinct from other A₁ARs in that it displays an agonist potency series of N⁶-R- phenylisopropyladenosine (*R*-PIA) > N⁶-*S*-phenylisopropyladenosine > (*S*-PIA) > 5'-*N*-ethylcarboxamidoadenosine (NECA) compared to the traditional potency series of *R*-PIA > NECA > *S*-PIA.

The A₁AR was solubilized in 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (Chaps) and then purified by chromatography on an antagonist [xanthine amine congener (XAC)]-coupled Affi-Gel 10 followed by hydroxylapatite chromatography. Following purification, sodium dodecyl sulfate–polyacrylamide gel electrophoresis revealed a single protein of *M*_r 36,000 by silver staining, Na¹²⁵I iodination with chloramine T and photoaffinity labeling with [¹²⁵I]8-[4-[[[2-(4-aminophenyl acetyl amino) ethyl] carbonyl] methyl] oxy] - phenyl]-1,3-dipropylxanthine. This single protein displayed all the characteristics of the A₁AR, including binding an antagonist radioligand ([³H]XAC) with high affinity (*K*_d = 0.7 nM) and in a saturable manner (*B*_{max} > 4500 pmol/mg). Agonist competition curves demonstrated the expected bovine brain A₁AR pharmacology: *R*-PIA > *S*-PIA > NECA. The overall yield from soluble preparation was 7%.

The glycoprotein nature of the purified A₁AR was determined with endo- and exoglycosidases. Deglycosylation with endoglycosidase F increased the mobility of the A₁AR from *M*_r 36,000 to *M*_r 32,000 in a single step. The A₁AR was sensitive to neuraminidase but resistant to α-mannosidase, suggesting the single carbohydrate chain was of the complex type. This makes the bovine brain A₁AR similar to rat brain and fat A₁AR in terms of its carbohydrate chains yet the purified A₁AR retains its unique agonist potency series observed in membranes.

¹M.E.O. is supported by a NIH Postdoctoral Fellowship (1F32-GM-13713-01) from the National Institute of General Medical Sciences. G.L.S. is an Established Investigator of the American Heart Association and supported in part by NHLBI (Grant RO1-HL-35134 and Supplement) and Grant-in-Aid 880662 from the American Heart Association and 3M Riker.

© 1990 Academic Press, Inc.

²To whom correspondence should be addressed at Duke University Medical Center, Box 3444, Durham, NC 27710.

The activation of the A₁ adenosine receptor (A₁AR)³ by adenosine produces a variety of effects in several systems including the cardiovascular and central nervous systems (1). The A₁AR is a cell surface glycoprotein with an *M_r* of approximately 38,000 (2,3). The A₁AR has been distinguished from the A₂ adenosine receptor subtype based on the potency series of adenosine analogs and by the fact that the A₁AR is inhibitory and the A₂AR is stimulatory in terms of adenylate cyclase activity. Additionally, the A₁AR may be coupled to the activation of potassium channels (4), activation of guanylate cyclase (5), and alterations in phosphoinositol metabolism (6). The A₁AR is coupled to the inhibition of adenylate cyclase via the G protein, G_i, but unlike other receptor–G protein systems the A₁AR–G_i association appears to be very “tight” (7, 8).

The A₁AR of bovine brain differs from the A₁AR of adipocytes and cerebral cortex from other species in that it binds antagonists, such as XAC, with subnanomolar affinities and has a unique agonist potency series of *R*-PIA > *S*-PIA > NECA (2). The features of the A₁AR adenylate cyclase signaling system have been elucidated via studies with membrane and soluble receptor preparations (9). To obtain the primary sequence of the A₁AR as well as to completely characterize at the molecular level its interaction with ligands, G proteins and effector systems, the receptor must be purified. We have recently reported (10) a partial purification of the bovine cerebral cortex A₁AR via chromatography on an affinity column consisting of the A₁ adenosine receptor selective antagonist XAC coupled to an activated agarose support matrix.

In this paper, we describe the purification to near apparent homogeneity of the bovine brain A₁AR by chromatography on the XAC affinity gel followed by hydroxylapatite chromatography. The isolated receptor displays several functional and structural characteristics expected of the A₁AR and appears to be completely dissociated from G_i. Recently, the purification of the A₁AR from rat brain (11) has been reported. In this report, we present a detailed characterization of the purified bovine brain A₁AR and demonstrate how the unique properties of this receptor are maintained in the purified state.

MATERIALS AND METHODS

Materials

Bovine brain was obtained from a local abattoir. XAC was synthesized by one of the authors (K.A.J.). [³H]XAC was from New England Nuclear. Affi-Gel 10, hydroxylapatite (Bio-Gel HT), and silver stain reagent were from Bio-Rad. *R*-PIA, *S*-PIA, NECA, adenosine deaminase, Chaps, and endoglycosidase F were all from Boehringer- Mannheim. L- α -phosphatidylcholine, theophylline, α -mannosidase, and neuraminidase were obtained from Sigma.

Synthesis of XAC Affi-Gel

Affi-Gel 10 (25 ml) was washed with 250 ml of DMSO over 15 min and excess DMSO was removed. To the gel slurry was added 20 ml of a 75% DMSO/25% ethanol solution containing 12.5 mg of XAC. The pH of the XAC and gel mixture was kept at pH 8 by titration with 2 M Hepes (pH 9.5). The slurry was mixed batchwise for 20 h at 22°C. The gel was washed extensively with DMSO to remove uncoupled XAC and the wash solution was

³Abbreviations used: XAC, xanthine amine congener; *R*-PIA, N⁶-*R*-phenylisopropyladenosine; *S*-PIA, N⁶-*S*-phenylisopropyladenosine; NECA, 5'-*N*-ethylcarboxamidoadenosine; Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DMSO, dimethyl sulfoxide; A₁AR, A₁ adenosine receptor; PAPAXAC, 8-[4-[[[2-(4-aminophenyl)acetylaminomethyl]carbonyl]-methyl]oxy]phenyl]-1,3-dipropylxanthine; PMSF, phenylmethylsulfonyl fluoride; SANPAH, N-succinimidyl 6-(4'-azido-2'-nitrophenylamine); Gpp(NH)p, guanylyl-5-yl-(β , γ -imido)diphosphate.

gradually increased to 100% H₂O. Finally, the gel was washed with 0.5 M Tris for 24 h at 5°C to block any remaining free ester groups.

Membrane preparation and solubilization

Bovine brain membranes, prepared as previously described (8), were suspended at a detergent/protein ratio of 2.5/1 in 1% Chaps, 20 mM Tris, 5 mM EDTA, and 100 mM NaCl, pH 7.4, at 5°C. The mixture was dounce homogenized and stirred on ice for 30 min followed by centrifugation at 105,000*g* for 40 min. The supernatant was taken as the soluble fraction and diluted with an equal volume of 50 mM Tris, 1 mM EDTA, and 125 mM NaCl, pH 7.4, at 5°C containing 20 μ M Gpp(NH)p.

Chromatography

Typically, 350–400 ml of Gpp(NH)p-treated solubilized preparation was pumped onto a 12- to 15-ml XAC Affi-Gel column at 35 ml/h for 14 h at 5°C. The gel was then washed batchwise with 15 ml of 0.33% Chaps in 50 mM Tris, 1 mM EDTA, 125 mM NaCl, pH 7.4, at 5°C for 10 min. The above buffer was then pumped through the gel at 35 ml/h for 75 min at 5°C. The column was brought to room temperature and washed with a buffer consisting of 0.05% L- α -phosphatidylcholine suspended via sonication in 30 mM Hepes, 5 mM MgCl₂, and 100 mM NaCl diluted with an equal volume of 0.66% Chaps, 50 mM Tris, 10 mM MgCl₂, and 250 mM NaCl (Buffer A), pH 7.4, and pumped at a rate of 30 ml/h for 45 min. Elution of A₁AR from the gel was also performed at room temperature with 5 mM theophylline in Buffer A, pH 6.8, at a rate of 10 ml/h for 2.5 h. The eluate was collected on ice and desalted on Sephadex G50 columns preequilibrated with 0.1% Chaps in 50 mM Tris, 1 mM EDTA, 125 mM NaCl, pH 7.4, at 5°C (Buffer B). The desalted eluate was then applied to a 0.4 ml hydroxylapatite column at a rate of 15 ml/h. The column was washed with 3 ml of Buffer B followed by 2 ml of 75 mM potassium phosphate in Buffer B and then 2 ml of 200 mM potassium phosphate in Buffer B. The majority of A₁AR activity was eluted from the hydroxylapatite column by successive washings with 2 ml each of 300, 400, and 500 mM potassium phosphate in Buffer B. These three washings were combined to give the total elution fraction. All hydroxylapatite chromatography procedures were performed at 5°C.

Radioligand binding assays

Binding assays in soluble preparation were performed as previously described (8). Saturation binding assays consisted of either 50 μ l of affinity chromatography-purified or 25 μ l of hydroxylapatite-purified material, 50 μ l of [³H]XAC (0.2–6.0 nM), 25 μ l of H₂O or 25 mM theophylline, 25 μ l of the heat-inactivated void volume fraction of the XAC Affi-Gel column, and the volume of the assay was made 250 μ l with 0.1% Chaps in 50 mM Tris, 1 mM EDTA, 125 mM NaCl, pH 7.4, at 22°C (Buffer C). The heat-inactivated preparation did not by itself demonstrate specific [³H]XAC binding but its addition enhanced [³H]XAC binding of the purified preparations approximately 10–15%. Binding may be enhanced due to the presence of phospholipids in this heat-inactivated fraction. It appears that phospholipids are necessary to demonstrate ligand binding as indicated by their required presence in the affinity gel elution buffer (see Results). In competition binding experiments, the assay consisted of 50 μ l (XAC Affi-Gel) or 25 μ l (hydroxylapatite) of purified receptor, 50 μ l of 0.5 nM [³H]XAC, 25 μ l of competitor, and 25 μ l of heat-inactivated protein and the assay volume was made 250 μ l by the addition of Buffer C. All incubations were for 2 h at 22°C and terminated by washing with three 3-ml aliquots of 0.01% Chaps in 50 mM Tris, 10 mM MgCl₂, and 1 mM EDTA with rapid vacuum filtration over polyethylenimine-treated glass fiber filters. Saturation and competition curves were analyzed using a nonlinear least-squares fitting technique with statistical analysis as previously described (12).

Protein quantification and determination

All protein concentrations were determined by the method of Bradford (13), or by the Amidoschwarz method for purified preparations (14). For radioiodination, typically 0.20 ml of either XAC Affi-Gel or hydroxylapatite eluate was iodinated with 0.1 mCi of Na¹²⁵I as previously described (15) except that bovine serum albumin was omitted from the reaction mixture. Samples were desalted on Sephadex G50 columns into 0.2% SDS in 10 mM Tris, pH 6.8, prior to lyophilization. SDS–PAGE gels were silver stained according to the manufacturer's protocol (Bio-Rad).

Photoaffinity labeling

[¹²⁵I]PAPAXAC–SANPAH was synthesized as described (3). Hydroxylapatite-purified receptor (0.3–2 ml) was incubated alone or in the presence of 5 mM theophylline with 0.8 nM [¹²⁵I]PAPAXAC–SANPAH in subdued lighting for 1 h at 22°C. The sample was exposed to UV light for 4 min from a Model UVCG-25 mineral lamp and then desalted on a Sephadex G50 column into Buffer C to remove unincorporated ligand. Samples were either frozen in liquid nitrogen and lyophilized prior to gel electrophoresis or prepared for glycosidase treatment.

Glycosidase treatments

Aliquots (0.25 ml) of hydroxylapatite-purified receptor labeled with [¹²⁵I]PAPAXAC and treated with 0.1 mM PMSF were used for exo- and endoglycosidase studies. For neuraminidase treatment, the sample was made pH 5 and incubated with 1.25 U of neuraminidase for 16 h at 5°C. For α -mannosidase treatment, samples were made pH 4.5 and incubated with 2.5 U of α -mannosidase for 16 h at 5°C. Endoglycosidase F (0.4 U) treatment was performed for 4 h at 22°C with samples at pH 6.5. At the end of the incubation period, samples were placed in liquid nitrogen, lyophilized, and then resuspended in SDS–PAGE sample buffer prior to electrophoresis.

SDS–PAGE

Electrophoresis was performed on homogenous 12–16% acrylamide slab gels (16). Following lyophilization samples were resuspended in a buffer consisting of 10% SDS, 10% glycerol, 25 mM Tris, and 6% β -mercaptoethanol, pH 6.8, at 22°C. After drying, gels were exposed to Kodak XAR5 film with dual intensifying screens at –80°C.

RESULTS

The method described for membrane solubilization was optimized for both the most efficient solubilization of the A₁AR and receptor stability. In the absence of MgCl₂ and the presence of 10 μ M Gpp(NH)p, [³H]XAC bound to solubilized A₁AR with a $B_{\max}$ of 1.24 ± 0.08 pmol/mg and a K_D of 0.32 ± 0.05 nM ($n = 10$). Under these conditions, approximately 50–60% of the membrane receptor was solubilized and [³H]XAC binding remained stable for 24 h when the soluble A₁AR preparation was stored at 5°C.

Following application of 350–400 ml of soluble preparation to the XAC Affi-Gel column, the void volume fraction displayed an approximate 25% decrease in [³H]XAC binding. Approximately 5% of the applied protein was retained by the gel. Following extensive washing, A₁AR activity was eluted from the column with 5 mM theophylline. The eluate of the column bound [³H]XAC with a $B_{\max}$ of 3400 ± 538 pmol/mg and a K_D of 0.79 ± 0.22 nM ($n = 8$). Approximately 70% of the bound A₁AR could be recovered from the gel resulting in an overall yield of $16.1 \pm 1.0\%$ on the basis of the activity of the starting solubilized material. Inclusion of phospholipids in the elution buffer is required for A₁AR activity as assessed by [³H]XAC binding. The interaction of the receptor with XAC Affi-Gel

is biospecific as no [^3H]XAC binding activity can be recovered if theophylline is omitted from the elution buffer or if the solubilized preparation is applied to Affi-Gel 10 without XAC coupled to it.

Further purification of the A₁AR was obtained via a second chromatography procedure using a hydroxylapatite column. Virtually all of the applied A₁AR activity and approximately 80% of the total protein was bound by the 0.4-ml hydroxylapatite column. Typically 20–30% of the A₁AR activity and the majority of the contaminating proteins were removed from the hydroxylapatite with the 75 and 200 mM potassium phosphate washes. Peak A₁AR activity and yield was obtained by the combined elution procedure described under Materials and Methods as judged by the ability of this fraction to bind [^3H]XAC saturably and with high affinity (Fig. 1A). In five experiments, the material eluted from the hydroxylapatite column bound [^3H]XAC with a K_D of 0.73 ± 0.12 nM and had a B_{max} of 4542 ± 461 pmol/mg. Approximately 50% of the applied receptor was recovered in the eluate. As compared to the starting solubilized material, this specific activity represents a minimum of a 3700-fold purification of the A₁AR with the overall receptor yield being a minimum of $6.6 \pm 0.4\%$. In that the autoradiograph of the iodinated hydroxylapatite eluate following SDS–PAGE (see below) indicates the absence of significant quantities of protein other than the A₁AR, the calculated degree of purification based on [^3H]XAC binding likely underestimates the actual homogeneity of the final preparation. This apparent disparity between specific activity and purity likely relates to the critical requirement for the appropriate lipid milieu for the receptor to bind ligands. This will be elaborated upon in the discussion.

Iodination [^{125}I] of the eluted material by the chloramine T method illustrates the purification obtained by the successive chromatography procedures and shows the homogeneity of the material eluted from the hydroxylapatite column (Fig. 2A). The predominant protein in the eluate obtained in both chromatography procedures migrates as a broad band and has an M_r of $36,200 \pm 700$ ($n = 5$), which closely corresponds to that previously reported for the A₁AR (2,3). Several protein bands present in the eluate of the XAC Affi-Gel do not appear in the hydroxylapatite eluate, thus resulting in negligible amounts of contaminating proteins of higher molecular weights in the final preparation. Proteins were also visualized following SDS–PAGE by silver staining of the gel. A sample of hydroxylapatite eluate (~ 3 μg protein) was electrophoresed on a 16% acrylamide gel to better resolve proteins of lower molecular weight which may be present. Again, a predominant band of M_r 36,000 was observed with no appreciable amounts of contaminating protein present (Fig. 2B).

We next sought to characterize the major product of the hydroxylapatite chromatographic step and to confirm that this protein is the authentic A₁AR. In addition to the binding parameters for [^3H]XAC, which correspond to those obtained for soluble and membrane preparations (2, 10), competition binding assays show that the hydroxylapatite fraction displays the pharmacology expected of the A₁AR (Fig. 1B). The IC₅₀ values for the inhibition of [^3H]XAC binding in two experiments for *R*-PIA, *S*-PIA, and NECA were 0.71 ± 0.05 μM , 35.0 ± 0.8 μM , and 675.5 ± 97 μM , respectively. This is the potency order observed for the bovine brain A₁AR in membrane and soluble preparations (2, 10). The IC₅₀ values for these agonists are similar to those obtained for the XAC Affi- Gel-purified receptor (data not shown).

Photoaffinity labeling of the hydroxylapatite-purified preparation with [^{125}I]PAPAXAC-SANPAH also indicates that the A₁AR binding subunit has been isolated. The photoaffinity probe specifically labels a single protein of M_r $37,000 \pm 300$ ($n = 5$), which is consistent with the molecular weight of the A₁AR reported for various tissues (Fig. 3). The major

protein labeled with Na¹²⁵I by the chloramine T method comigrated on 12% acrylamide gels exactly as the [¹²⁵I]PAPAXAC-SANPAH-labeled species (Fig. 3).

To study the structure of the hydroxylapatite-purified A₁AR and compare it to the membrane form of the receptor, the partially purified receptor was photoaffinity labeled with [¹²⁵I]PAPAXAC-SANPAH and then subjected to treatment with glycosidases. Results of a representative experiment are shown in Fig. 4. In this experiment, the untreated receptor migrated as a protein of M_r 37,000. Following treatment with endoglycosidase F, which cleaves N-linked high mannose and complex-type carbohydrate chains from proteins (17), the mobility of the protein is increased and it now migrates as a M_r 32,000. This occurs as a single-step reaction, making it similar to what we have previously shown for adipocyte and rat brain membrane A₁AR. The receptor is insensitive to α -mannosidase, an enzyme which removes terminal mannose residues, as no change in protein mobility is observed following incubation with the enzyme. This is again consistent with results from rat adipocyte A₁AR. Finally, incubation with neuraminidase, which removes terminal sialic acid residues, resulted in a protein with increased mobility to M_r 35,000. The results are similar to those obtained in two other experiments and are in agreement with those reported for the A₁AR of rat fat and brain (17).

DISCUSSION

This paper describes the purification of the bovine brain A₁AR via affinity chromatography followed by chromatography on a hydroxylapatite column. These methods result in a homogenous preparation of a protein of approximately M_r 36,000, which is characterized to be the functional A₁AR. The first step is the chromatography of solubilized bovine cerebral cortex membranes on an affinity column in which XAC, a high affinity, relatively selective A₁AR antagonist is used as the immobilized ligand. A substantial receptor purification of approximately 2750-fold based on [³H]XAC binding was obtained with this procedure. Interestingly, the omission of phosphatidylcholine in the theophylline elution buffer resulted in very little [³H]XAC binding activity in the eluate. Addition of phospholipids after a phosphatidylcholine-free elution did not appreciably increase [³H]XAC binding. Thus, it appears that a phospholipid/Chaps suspension provides a milieu that is necessary for the ligand-specific removal of A₁AR from the XAC Affi-Gel. The presence of phospholipids was also required for the demonstration of significant ligand binding in a purified preparation of the D₂ dopamine receptor (18).

The XAC Affi-Gel-purified receptor was chromatographed on a hydroxylapatite column resulting in a minimum 3700-fold purification of the A₁AR with nearly a 7% yield. On the basis of a M_r of 36,000 for the A₁AR and one binding site per molecule, the theoretical specific activity of the receptor purified to homogeneity is approximately 27,700 pmol/mg. The hydroxylapatite eluate with a specific activity of 4500 pmol/mg is, therefore, calculated to represent nearly a 20% pure preparation. However, both iodination and silver staining of the final material display a predominant A₁AR band at M_r 36,000 and negligible quantities of a few contaminating proteins. The visualization of protein content by these methods indicates that the A₁AR accounts for well over 70% of the total protein present in the final fraction. Since protein determination by two different methods, i.e., iodination and silver staining, gave similar results it is unlikely that additional proteins are undetected. Thus, it appears that the calculated specific activity of 4500 pmol/mg of the hydroxylapatite eluate underestimates the actual purity of the receptor. This is likely the result of the presence of appreciable quantities of A₁AR which no longer recognize [³H]XAC in radioligand binding assays yet are detected during protein quantitation. Such a loss in activity may result during purification as the receptor is progressively removed from an environment and conditions that are conducive to ligand binding. Alternatively, the A₁AR may undergo conformational

changes during purification. Isolation of significant quantities of the A₁AR with a higher specific activity following the hydroxylapatite step could not be accomplished by several procedures including chromatography on wheat-germ agglutinin, heparin, and DEAE Sephacel gels as well as rechromatography on XAC Affi-Gel.

Characterization of the material eluted from the hydroxylapatite column indicates that the major protein present is the functional A₁AR. Both antagonist and agonist radioligand binding display the expected parameters. [³H]XAC binding is specific, saturable, and of high affinity ($K_D = 0.7$ nM), which is similar to that observed in membrane and soluble A₁AR preparations (2, 10). Slight decreases in antagonist affinity have also been observed following purification of the D₁ dopamine (19) and muscarinic (20) receptors. Agonist binding displays the potency series of *R*-PIA > *S*-PIA > NECA, which is that expected of the bovine brain A₁AR (2). The affinities of these agonists for the hydroxylapatite-purified A₁AR, as judged by their IC₅₀ values obtained in competition assays versus [³H]XAC, are significantly increased (10- to 50- fold) as compared to solubilized receptor. This decline in agonist affinity indicates an uncoupling of the A₁AR from its G protein, G_i. Such a dissociation of the A₁AR from G_i appears to have occurred during the affinity chromatography procedure as the decreased agonist affinity is observed in XAC Affi-Gel-purified material. Furthermore, pertussis toxin labeling experiments demonstrate that G_i is not present in the eluate of the XAC Affi-Gel (data not shown). The manner in which the membrane A₁AR was solubilized, i.e., in the absence of MgCl₂ and the presence of Gpp(NH)p, may promote the dissociation of A₁AR from G_i. This technique does not, however, by itself completely disrupt the tight coupling of A₁AR and G_i (8). The total disruption of A₁AR-G_i coupling probably involves both the solubilization conditions and the subsequent affinity chromatography. Munshi and Linden have recently reported on the partial copurification of A₁AR with a G protein from an agonist affinity column provided that *N*-ethylmaleimide or Mg²⁺-GTP was included in the elution buffer in addition to the receptor counterligand, 8-*p*-sulfophenyltheophylline (21). They did not include guanine nucleotides in their solubilization procedure. The agonist column likely stabilizes the A₁AR-G_i complex and permits them to obtain the complex in the eluate.

The findings obtained following SDS-PAGE of the hydroxylapatite eluate also indicate that the purified protein is the A₁AR. This predominant protein migrates on a 12% acrylamide gel as a broad band of M_r approximately 36,000. This molecular weight is in agreement with that previously reported for the A₁AR (2, 3). This protein is confirmed to be the A₁AR by comparing its identical migration on gels to that of the unique protein labeled by the photoaffinity probe, [¹²⁵I]PAPAXAC. Additionally, incorporation of [¹²⁵I]PAPAXAC into this protein is completely abolished by 5 mM theophylline. On the basis of the functionality of the isolated protein and the absence of protein bands of molecular weight lower than M_r 36,000, it appears that the purified protein is the intact A₁AR and that little if any degradation of the receptor occurs during the chromatographic procedure.

Glycosidase treatment of the purified preparation confirms the glycoprotein nature of the A₁AR that has been reported for the receptor obtained from rat fat and brain (17). The A₁AR contains carbohydrate chains with a terminal sialic acid as evidenced by its susceptibility to neuraminidase. Carbohydrate chains with a terminal mannose apparently are not present on the A₁AR as α -mannosidase did not change receptor mobility on SDS-PAGE. The increase in mobility of the A₁AR following endoglycosidase F treatment likely indicates the presence of a single N-linked complex-type carbohydrate chain. This makes the carbohydrate nature of the A₁AR from bovine brain very similar to that reported for the rat brain and adipocyte A₁AR (17). This suggests that the carbohydrate chain likely does not contribute to the unique agonist potency series displayed by this receptor. Since the purified A₁AR retains these unique binding characteristics in spite of the fact that it has been

removed from its normal environment, the primary protein structure, i.e., amino acid sequence, may well be responsible for these differences. Comparisons of the primary structure of different A₁ARs will have to await their cloning.

In conclusion, the procedures described allow for the purification of the A₁AR to the degree of homogeneity and in the yields necessary to permit the studies required for a more complete understanding of the receptor at a molecular level. Specifically, strategies exist which should permit the partial sequencing of the A₁AR and subsequent isolation of its cDNA as well as the study of the interaction of the A₁AR with various G proteins and effector systems in reconstitution systems.

References

1. Ramkumar, V.; Pierson, G.; Stiles, G. Progress in Drug Research. Vol. 32. Birkhauser; Cambridge, MA: 1988. p. 195-247.
2. Stiles GL, Jacobson KA. Mol Pharmacol. 1987; 32:184–188. [PubMed: 3614192]
3. Barrington WW, Jacobson KA, Stiles GL. J Biol Chem. 1989; 264:13,157–13,164.
4. Bailey, J.; Ravdon, D. Basic and Clinical Aspects. A. R. Liss, Inc; New York: 1987. Clinical Electrophysiology and Pharmacology of Adenosine and ATP; p. 119-133.
5. Kurtz A. J Biol Chem. 1987; 262:6296–6300. [PubMed: 2883183]
6. Delahunty TM, Cronin MJ, Linden J. Biochem J. 1988; 255:69–77. [PubMed: 2848512]
7. Stiles GL. J Biol Chem. 1985; 260:6728–6732. [PubMed: 2987229]
8. Stiles GL. J Neurochem. 1988; 51:1592–1598. [PubMed: 3139838]
9. Stiles GL. Clin Res. 1990; 38:10–18. [PubMed: 2293954]
10. Olah ME, Jacobson KA, Stiles GL. FEBS Lett. 1989; 257:292–296. [PubMed: 2583275]
11. Nakata H. J Biol Chem. 1989; 264:16,545–16,551.
12. DeLean A, Hancock AA, Lefkowitz RJ. Mol Pharmacol. 1982; 21:5–16.
13. Bradford MM. Anal Biochem. 1976; 72:248–254. [PubMed: 942051]
14. Schaffner W, Weissman C. Anal Biochem. 1973; 56:502–514. [PubMed: 4128882]
15. Greenwood FC, Hunter WM. Biochem J. 1963; 89:114–123. [PubMed: 14097352]
16. Laemmli UK. Nature (London. 1970; 227:680–685. [PubMed: 5432063]
17. Stiles GL. J Biol Chem. 1986; 261:10,839–10,843.
18. Senogles SE, Amlaiky N, Falardeau P, Caron MG. J Biol Chem. 1988; 263:18,996–19,002.
19. Gingrich JA, Amlaiky N, Senogles SE, Chang WK, McQuade RD, Berger JG, Caron MG. Biochemistry. 1988; 27:3907–3912. [PubMed: 3415962]
20. Haga K, Haga T. J Biol Chem. 1985; 260:7927–7935. [PubMed: 4008482]
21. Munshi R, Linden J. J Biol Chem. 1989; 264:14,853–14,859. [PubMed: 2909511]

FIG. 1.

Radioligand binding characteristics of the purified A₁AR. (A) [³H]XAC saturation curves following hydroxylapatite chromatography. [³H]XAC was added at the concentrations shown on the abscissa and the amount bound is on the ordinate. Binding assays were performed as described under Materials and Methods. The assay contained 25 μ l of hydroxylapatite eluate (~50 ng protein). Nonspecific binding was determined with 25 mM theophylline. This experiment is representative of five similar experiments. (B) Agonist competition curve analysis versus [³H]XAC in the hydroxylapatite-purified A₁AR. The assay contained ~0.5 nM [³H]XAC and 25 μ l of hydroxylapatite eluate (~50 ng protein). Competing ligand was added to give the concentration shown on the abscissa and the amount of [³H]XAC bound is on the ordinate. This experiment was performed twice.

FIG. 2.

SDS-PAGE analysis of receptor fractions following chromatography. (A) Specific fractions following affinity chromatography and hydroxylapatite chromatography were subjected to radioiodination with chloramine T and Na¹²⁵I and then subjected to SDS-PAGE/autoradiography on a 12% acrylamide gel as described under Materials and Methods. The A₁AR migrates as a M_r 36,000 protein. Molecular weight markers are shown to the left. This experiment is representative of five similar experiments. (B) Silver staining of gel containing purified receptor following hydroxylapatite chromatography. This sample was run on a 16% acrylamide gel and the mobility of molecular weight markers is shown to the left. The A₁AR migrates as a M_r 36,000 protein. The interface between the stacking and the separating gels is visible at the top of the lane.

FIG. 3.

Photoaffinity labeling of the purified A_1AR . Following hydroxylapatite chromatography, an aliquot of eluted material was photoaffinity labeled with [^{125}I]PAPAXAC-SANPAH in the presence and absence (Control) of 5 mM theophylline as described under Materials and Methods. The sample was then subjected to SDS-PAGE/autoradiography. A single M_r 37,000 protein is specifically labeled. For comparison, a lane containing a $Na^{125}I$ radioiodinated receptor is included. Receptors labeled by the two separate techniques comigrate on SDS-PAGE. Positions of molecular weight markers are shown to the left. This experiment was repeated three times.

FIG. 4.

Glycoprotein nature of the purified A₁AR. The purified A₁AR was photoaffinity labeled with [¹²⁵I]PAPAXAC-SANPAH in the presence of 0.1 mM PMSF and then subjected to either endoglycosidase F 0.4 U for 4 h, α-mannosidase 2.5 U for 16 h or neuraminidase 1.25 U for 16 h. Control indicates no treatment. The samples were then prepared and subjected to SDS-PAGE on a 12% acrylamide gel followed by autoradiography. Positions of molecular weight markers are shown to the left. This experiment was performed three times.