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PURIFICATION AND BIOCHEMICAL CHARACTERIZATION OF α_2-ADRENERGIC RECEPTOR FROM THE RAT ADRENOCORTICAL CARCINOMA[1]

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The α_2-adrenergic receptor was purified from rat adrenocortical carcinoma 494 by an affinity chromatographic step using a novel para-aminoclonidine-sepharose resin followed by a gel-permeation high performance liquid chromatographic step. The iodinated receptor protein was homogeneous as evidenced by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and by high performance liquid chromatography. Both SDS-PAGE and high performance liquid chromatographic studies revealed that Mr of the protein was 64,000, suggesting the monomeric nature of the receptor protein. The purified protein showed the typical binding characteristics of α_2-adrenergic receptor.

Rat adrenocortical 494 tumor cells (1,2) possess α_2-adrenergic receptors that have been extensively characterized in both their membrane-bound (3,4) and solubilized forms (4). Because of the homogeneity of the cell type and the relative ease of obtaining the tumor tissue in large quantities, we have elected to attempt purification of the α_2-receptor from this tissue with the ultimate purpose of obtaining sufficient material for structure/function analysis and eventually for cloning the α_2-receptor gene.

Here we report a relatively simple and straightforward procedure for purifying the receptor in two steps involving affinity chromatography and gel-permeation chromatography on HPLC. The purified material showed a single-band by SDS-PAGE with a Mr of 64,000 and has the necessary specific binding characteristics to suggest that it represents the α_2-adrenergic receptor.

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Abbreviations: PAC, para-aminoclonidine; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography.

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MATERIALS AND METHODS

Yohimbine hydrochloride, epinephrine and norepinephrine were purchased from Sigma; [^{3}H]yohimbine (80.2 Ci/mmol), [^{3}H]PAC[2] (40 Ci/mmol), [^{3}H]azidoclonidine (31 Ci/mmol) and carrier free Na^{125}I from New England Nuclear; all of the reagents for SDS-PAGE were obtained from Bio Rad; AH-Sepharose 4B and SDS-PAGE molecular weight standards were from Pharmacia. Digitonin was purchased from Fisher Scientific Company. All other reagents were analytical grade from commercial sources.

Rat adrenocortical carcinoma 494, a spontaneously occurring tumor discovered in (1) and maintained in our laboratory (2) was used for the membrane preparation and binding studies.

Preparation of Sepharose-PAC Affinity Resin - Succinic anhydride solution (1 mmol/ml gel) was added to the AH-Sepharose-4B (1 g) suspension in 4 ml water, the pH was adjusted to 6.0 by the addition of 20% NaOH and the mixture was shaken at 4° for 12 hrs. The gel was filtered, washed successively with 0.1 M NaOH (250 ml) and water (1 L), suspended in 2 ml deionized water (pH 4.5) and 2 ml of an aqueous solution of PAC (0.1 mmol/ml gel) was added. The pH was adjusted to 4.5 - 5.0. To the reaction mixture 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide hydrochloride was added to a final concentration of 0.1 M and the mixture shaken for 72 hrs. The coupled gel was washed alternatively with 0.1 M acetate buffer (pH 4.0) in 0.5 M NaCl and with 0.1 M bicarbonate buffer (pH 8.0) in 0.5 M NaCl and then extensively with deionized water.

Membrane Preparation - Adrenocortical carcinoma membranes were prepared as in (4) except that 10 μg/ml soyabean trypsin inhibitor, 200 μM benzamidine hydrochloride and 30 μM phenylmethylsulfonylfluoride were also present in buffer.

Solubilization of α_2-Adrenergic Receptors - To the freshly prepared pellet, 1% digitonin solution in 50 mM Tris-HCl buffer pH 7.5 containing protease inhibitors was added. "Gentle" homogenization using a hand-turned glass-homogenizer to a homogeneous suspension was followed by "gentle" stirring at 4° for 60 minutes with a magnetic stirring bar. The suspension was centrifuged at 105,000 x g for 30 min. The freshly prepared supernatant (solubilized receptors) was immediately used for the binding studies or for further purification of receptors.

Binding Assays - The binding assays were performed as described earlier (3,4) unless otherwise stated. Briefly, [^{3}H]PAC (10 nM) and membranes (200-300 μg) were incubated at 25° for 40 min in a total volume of 500 μl incubation buffer (10 mM $MgCl_2$, 50 mM Tris-HCl [pH 7.5]) with or without competing agonists or antagonists. Incubation was terminated by diluting the incubation mixture with 5 ml buffer followed by immediate filtration through Whatman GF/C glass fiber filter which was then washed with cold 3 x 5 ml incubation buffer. In the case of solubilized membranes, the reaction was stopped by adding 4 ml cold buffer. The unbound ligand was removed by immediate filtration through Whatman GF/B filters pretreated with 0.6% polyethyleneamine (5). The filter was washed four times with 5 ml cold buffer, dried and counted for radioactivity in Omnifluor/toluene scintillation mixture.

HPLC - Zorbax GF-250 (DuPont), a gel-filtration column, was used to purify the receptor protein. Mobile phase containing detergent was prepared by boiling 0.2% digitonin solution in deionized water. The solution was kept at room temperature for 24 hrs and any insoluble material was removed by filtration through Whatman No. 1 filter paper. The solution was then kept in a refrigerator for 3 days and again any insoluble material was removed by filtration. The solution was finally filtered through a millipore membrane

(0.45 μ) just before use. This "digitonin solution" was used to prepare 50 mM Tris SO_4 (pH 7.0) solution containing protease inhibitors (10 μg/ml soyabean trypsin inhibitors 200 μM Benzamidine hydrochloride and 30 μM phenylmethyl sulfonyl fluoride) and was used for the HPLC analysis of the receptors. Amicon concentrated receptor samples (0.2 ml) were injected and chromatographed at a flow rate of 1 ml/min on a IBM LC9533 Ternary Gradient liquid chromatograph. Fractions (200-μl) were collected and assayed for binding activity and the active fractions were pooled and concentrated.

Photoaffinity Labeling - Affinity purified receptors (2 ml) were incubated with [^{3}H]para-azidoclonidine (16 nM; 0.06 μCi/pmol) with or without non-radioactive para-aminoclonidine (1 x 10^{-4}M) in a aluminum wrapped beaker for 16 hours at 4°; 0.5 ml of cold buffer comprised of 50 mM Tris-HCl containing 0.1% digitonin was added and the reaction mixture was irradiated for 15 min with ultraviolet light (Mineralite, UVSL-25). The unbound ligand was separated by passage of the sample through a PD-10 column; a 3.5 ml fraction was collected after discarding the initial 2.5 ml effluent. After concentration, the photoaffinity labeled receptors were analyzed by HPLC and SDS-PAGE for determination of the molecular weight and subunit composition.

Radioiodination of α_2-Adrenergic Receptor - HPLC purified receptor after concentration by Amicon, was iodinated by chloramine T according to the method of Hunter and Greenwood (6). ^{125}I-receptor and unreacted Na^{125}I were separated by filtration on a Sephadex G-25 column presaturated with 1% bovine serum albumin in 0.05 M sodium phosphate buffer, pH 7.5.

Polyacrylamide Gel Electrophoresis - The iodinated- or photoaffinity labeled receptors were analyzed by SDS-PAGE using gels with the composition of 10% acrylamide containing 0.1% SDS and 0.3% bis acrylamide (7,8). The nonradioactive receptors were stained with 0.05% Coomassie brilliant blue and destained with 7.5% acetic acid.

The gel containing the iodinated sample was analyzed by autoradiography (8). The gel containing the photoaffinity labeled sample was cut into 2 mm slices, dissolved in 2.0 ml of 30% hydrogen peroxide at 65°, and mixed with 7 ml Scintiverse. Radioactivity was determined by liquid scintillation spectrometry.

Protein Determination - Protein was determined by the method of Bradford (9) using the Bio-Rad reagent and bovine serum albumin as a standard. In the samples where the protein concentration was too low to be measured by the Bradford method (9), it was approximated by the HPLC detection system (254 nm) using bovine serum albumin (monomer) as a standard.

RESULTS AND DISCUSSION

Solubilized receptors (300-350 mg protein) were applied to the top of the affinity column (4 ml of packed gel). Most of the protein (> 99.5%) did not bind to the resin and was in the "flow through" fraction. The column was extensively washed with detergent containing buffer (50 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 5 mM NaCl, 0.2% digitonin and protease inhibitors as described earlier) at 4° until the absorbance at 280 nm returned to the baseline. Subsequently, the gel was eluted with the above detergent buffer containing 100 μM phentolamine; 3 ml fractions were collected. The receptor in most cases was eluted in the first two fractions. Phentolamine was removed from the receptor by either chromatography on a PD-10 column or by extensive

dialysis against the detergent buffer containing 0.02% of digitonin instead of 0.2%. Appropriate aliquots (100- to 200-µl) were assayed for α_2-adrenergic binding affinity using [^{3}H]PAC. Those fractions possessing α_2-binding characteristics were pooled and concentrated 15-20-fold by Amicon ultrafiltration through a PM-30 membrane.

The affinity purified receptors showed typical binding characteristics of α_2-adrenergic receptors. Fig. 1 shows the displacement of [^{3}H]para-aminoclonidine binding from the receptor by various adrenergic agonist and antagonists. Among the antagonists phentolamine was most potent in displacing the para-aminoclonidine (Fig. 1). There was no binding affinity for the α_1-antagonist prazosin (10) or for WB4101 (11) (data not shown).

HPLC was employed as a final purification procedure. A single symmetrical protein peak was observed (Fig. 2A). When the HPLC-purified receptor protein was radioiodinated and subjected to SDS-PAGE, a single band corresponding to Mr = 64,000 was obtained (Fig. 3). These results demonstrated that Mr 64,000 α_2-receptor protein is homogeneous.

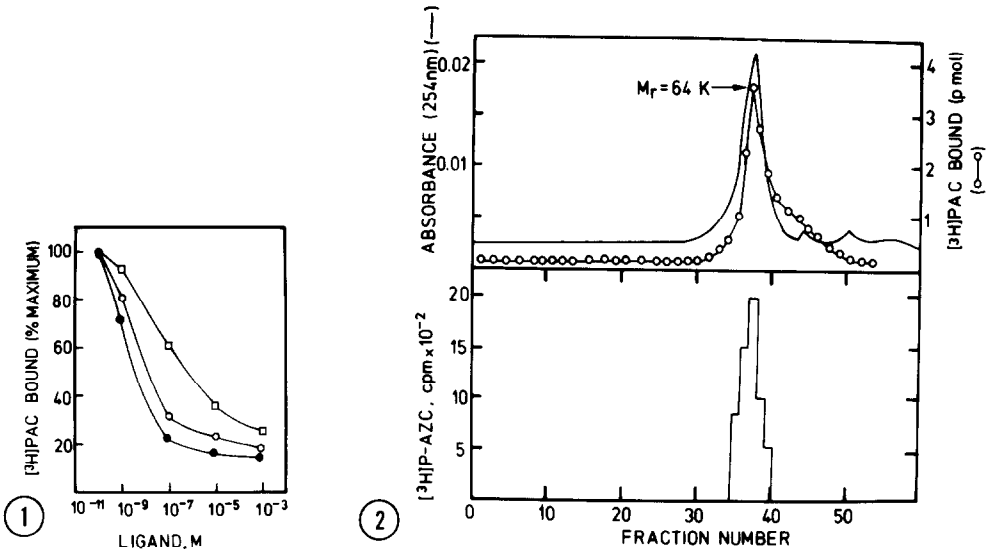

Fig. 1: Displacement of bound [^{3}H]PAC (10 nM; 0.04 Ci/pmol) from the affinity purified α_2-adrenergic receptor with phentolamine, yohimbine and (-) epinephrine. Experiments were done in triplicate as described in "Materials and Methods" and were repeated at least three times. The results are representative of one experiment. (●), Phentolamine; O, Yohimbine; (□) epinephrine.

Fig. 2: (A, top) The HPLC elution profile of the affinity- purified α_2-adrenergic receptor. The concentrated eluate of a Sepharose-PAC affinity gel was chromatographed on a Zorbax GF-250 gel filtration column as described in Materials and Methods". (B, bottom) The α_2-adrenergic receptor was labeled with 16 nM [^{3}H]para-azidoclonidine in the presence or absence of 1 x 10^{-4}M PAC as described under "Materials and Methods." The samples were analyzed by HPLC using a Zorbax GF-250 gel filtration column as described above. The 200-µl fractions were collected and the peak of [^{3}H]para-azidoclonidine binding activity was measured by counting in a scintillation counter. The samples incubated with PAC did not have any significant radioactivity above back ground.

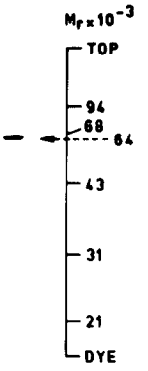

Fig. 3: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of ^{125}I-α_2-adrenergic receptor. The iodinated α_2-adrenergic receptor was subjected to SDS/PAGE on 10% gels. The gel was autoradiographed as described under "Materials and Methods."

Since the protein concentration, as assessed by the Bradford method (9), was below the detection limit, it was approximated by HPLC using bovine serum albumin (monomer) was a standard. The specific receptor activity by this method was in excess of 3875 pmol/mg, representing 23,500-fold purification of the α_2-adrenergic receptor as compared with the specific receptor activity of the solubilized membranes (Table 1). It is noteworthy that this specific activity is 5-fold lower than theoretically possible if one assumes

Table 1

Summary of Purification of α_2-Adrenergic Receptor of Adrenocortical Carcinoma Membrane

Step	Activity pmol	Overall Yield %	Specific Activity pmol/mg	Purification
Digitonin Extract	58	100	0.165	1
Eluate of PAC Affinity	22	38	150	901
HPLC	11	19	3875	23500

Solubilized receptors (300-350 mg protein) were applied on 4.0 ml of the affinity resin. The resin was washed with 0.2% digitonin containing 5 mM NaCl, 50 mM Tris-HCl (pH 7.5), 10 µg/ml soyabean trypsin inhibitor, benzamidine HCl (200 µM) and phenylmethylsulfonylfluoride (30 µM), and the receptors were eluted in the same buffer containing 0.1 mM phentolamine at 4°. Eluated fractions containing receptor activity were pooled, concentrated, and chromatographed on PD-10 columns to remove receptor-bound phentolamine. The receptor activity was assayed by [^3H]para-aminoclonidine binding.

that one mole of ligand binds one mole of receptor. Probably, this deficit in the level of purification reflects the gross inaccuracy of the protein determinations in the final product. In addition, it is possible that we were unable to remove the residual bound phentolamine from the affinity purified receptors, resulting in the gross underestimation of the receptor-bound ligand.

Due to the limited size of the sample, we could not carry out the detailed binding kinetics of the homogeneous receptors. Therefore, selective experiments were performed to scrutinize the α_2-characteristics of the affinity purified receptors.

The HPLC purified receptors were photoaffinity labeled with [^{3}H]para-azidoclonidine, a specific α_2-photoaffinity ligand (12), and then analyzed by high performance liquid chromatography. The radioactive peak corresponded to the Mr = 64,000 protein (Fig 2B). In the presence of nonradioactive PAC (1×10^{-4}M), no radioactivity was observed in the Mr 64,000 protein, indicating the α_2-specificity of the receptors. When the photoaffinity labeled receptor was subjected to SDS-PAGE, the radioactive peak again corresponded with Mr = 64,000 protein (Fig. 4); there was no radioactivity in the protein sample which was photolyzed in the presence of nonradioactive PAC (Fig 4). All these results demonstrated the authenticity of Mr 64,000 protein as α_2-adrenergic receptor protein.

Purification steps of the α_2-adrenergic receptor involved the introduction of protease inhibitors in all the buffers. This was done to overcome any possible proteolysis of the protein.

Regan et al., recently reported (14) the partial purification of α_2-adrenergic receptor protein from human platelets. Using [^{3}H]phenoxybenzamine as a labeling probe for the α_2-receptor, they demonstrated by SDS-PAGE that the radioactivity was specifically bound to a protein having Mr = 61,000, indicating that it was the receptor protein.

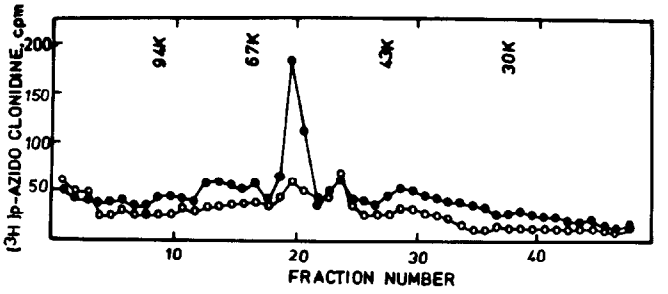

Fig. 4: SDS-PAGE of affinity purified α_2-adrenergic receptor from adrenocortical carcinoma membranes labeled with [^{3}H]para-azidoclonidine. Affinity purified receptor (0.2 pmol) was incubated with [^{3}H]para-azidoclonidine with (○) or without (●) 0.1 mM PAC. The gel was sliced in 2 mM-thick slices and dissolved in 2 ml 30% H_2O and counted as described under "Materials and Methods."

Although many tissues such as nerve terminals (15,16), rat heart membranes (17), rat brain (18), hamster adipocytes (19), and human platelets (20-23) contain α_2-adrenergic receptors, the biological function of these receptors in cellular regulation is not clearly defined. It appears that these receptors are negatively coupled (21,22) with adenylate cyclase and positively (23) with the adrenocortical carcinoma guanylate cyclase. We anticipate that the simplified purification method of α_2-adrenergic receptors described herein will allow one to isolate on a large scale quantities of the pure α_2-receptor that could be used for reconstitution, antibody production, and eventually molecular cloning of the receptor gene.

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