Vol. 109, No. 1, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 16, 1982 Pages 21-29

THE SIZE OF THE MAMMALIAN LUNG β_2-ADRENERGIC RECEPTOR AS DETERMINED BY TARGET SIZE ANALYSIS AND IMMUNOAFFINITY CHROMATOGRAPHY

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Received September 20, 1982

The subunit molecular weight of mammalian lung β_2-adrenergic receptors has been determined to be 59,000 daltons with monoclonal and autoantibody immunoaffinity chromatography and sodium dodecyl sulfate polyacrylamide gel electrophoresis. Target size analysis of mammalian lung membrane β_2-adrenergic receptors indicates a functional molecular weight for the β_2-receptor *in situ* of 109,000 ± 5,300 daltons. These data suggest that the mammalian lung β_2-receptor may be a dimer of two identical subunits of 59,000 daltons.

Model systems commonly used in the study of β-adrenergic receptors are the plasma membranes of avian and amphibian erythrocytes which contain β_1 and β_2-receptors, respectively (1). Reports on the subunit molecular size of β_1 and β_2-receptors from these tissues have strongly suggested that these receptor sub-types represent distinct protein species (2,3). Our laboratory has recently purified turkey erythrocyte β_1-receptors using a β-receptor specific monoclonal antibody immunoaffinity column. The purified β_1-receptor exhibits an SDS[1]-polyacrylamide gel molecular weight of approximately 70,000 with a possible 31,000 dalton subunit (2). These data contrast with reports on the subunit size for frog erythrocyte β_2-receptors of 58,000 daltons (3).

The validity of using avian and amphibian erythrocyte β-receptors as models for mammalian receptor sub-types has not been unequivocally estab-

[1] Abbreviations: SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; IHYP, [^{125}I]-iodohydroxybenzylpindolol; NHNP-NBE, N-[2-hydroxy-3-1-(naphthoxy)-propyl]-N-bromoacetylethylenediamine; PBS, phosphate buffered saline.

0006-291X/82/210021-09$1.00/0

lished. However, in comparison to avian and amphibian erythrocyte β-receptors, mammalian β-receptors are significantly less stable following detergent solubilization from membranes (4). The instability of mammalian β-receptors coupled with the extremely low quantities of β-receptors in mammalian tissues have made the purification and molecular characterization of these receptors a formidable task.

The β_2-adrenergic receptor is a physiologically significant macromolecule exerting control over such functions as airway diameter, blood pressure and uterine smooth muscle tone. The physiology and pharmacology of this receptor have been discussed in a multitude of manuscripts since 1906 (5). In this present study, we have utilized autoantibodies to β_2-receptors (6) and monoclonal antibodies which recognize β_2-receptors (2) for the immunoaffinity isolation and subunit characterization of mammalian lung β_2-adrenergic receptors. Furthermore, we have used target size analysis to determine the functional size of β_2-adrenergic receptors *in situ* in lung membrane preparations.

MATERIALS AND METHODS

Materials - Chromatography materials including Sephacryl S-300, Sepharose 4B and Protein A-Sepharose CL-4B were obtained from Pharmacia. Ammonium persulfate, TEMED, acrylamide, N,N'-methylene-bis-acrylamide were obtained from BioRad. [^{125}I]-iodohydroxybenzylpindolol (IHYP), Protosol, Econofluor and Formula 963 were from New England Nuclear. All other reagents were obtained from Sigma. N-[2-hydroxy-3-1-(naphthoxy)-propyl]-N-bromoacetyl-ethylenediamine (NHNP-NBE) was kindly synthesized for us by Dr. David J. Triggle using a procedure described previously (7) with modifications.

Membrane Preparations - Lung membranes were prepared as previously described (4) except that further fractionation of the 48,000 x g pellet was omitted.

Binding of [^{3}H]-NHNP-NBE to Lung Membranes - Membranes (∿3 mg protein/ml) were equilibrated with 0.1 μM (+)- or (-)-propranolol in 20 mM KPO_4, 2 mM $MgCl_2$, 1 mM EDTA buffer, pH 7.0 for 20 minutes at 30° C. [^{3}H]-NHNP-NBE was added at a final concentration of 0.1 μM, the reaction was incubated for 10 minutes at 30° C and then diluted with cold buffer. Membranes were centrifuged at 48,000 x g for 30 minutes and β-receptors were solubilized from membranes as described (4).

Immunoaffinity Chromatography - Immunoaffinity columns were prepared as follows in ISCO polypropylene columns: 1) A 10% suspension of Protein A - Sepharose CL-4B in phosphate buffered saline (PBS) was pipetted into the column. The column was washed once with PBS and 100 μl of Protein A-purified IgG from patient #10 (6), containing autoantibodies to β_2-receptors, were applied to each column and allowed to equilibrate at room temperature for 15 minutes. Columns were washed with PBS and then 250 μl of partially

purified [^{3}H]-NHNP-NBE-β-receptor were applied to each column. The columns were washed extensively with PBS, dried by centrifugation and the bound protein was eluted with 250 µl of buffer containing 2% SDS, 5% β-mercaptoethanol, 10% glycerol, or 2) Monoclonal antibody FV-104, purified by isoelectric focusing (2,8), was coupled to CN-Br activated Sepharose 4B (2 mg protein/ml gel). Lung β-receptors (500 µl) partially purified by gel exclusion chromatography, were applied to monoclonal antibody affinity columns. The columns were washed extensively with PBS and adsorbed protein was eluted with 10 µM (-)-propranolol. The eluate was radiolabeled with Na^{125}I using lactoperoxidase (9), concentrated in a Millipore stirred cell, and SDS, β-mercaptoethanol and glycerol added to final concentrations of 2, 10 and 5%, respectively. Samples were incubated at 100° C for 5 minutes in SDS and applied to SDS-polyacrylamide gels for analysis.

SDS-polyacrylamide Gel Electrophoresis - Eluates from immunoaffinity columns were characterized by electrophoresis on 10% polyacrylamide gels in SDS using the discontinuous buffer system described by Laemmli (10). Following electrophoresis, gels were sliced horizontally into 2 mm thick sections and assayed for radioactivity in a Beckman 4000 gamma counter or incubated overnight in a mixture of 3% Protosol in Econofluor prior to liquid scintillography.

Target Size Analysis - The method utilized is essentially that of Jung and coworkers (11). Purified cell membranes were layered at a depth of 0.5 mm in open aluminum trays at protein concentrations of 1-2 mg/ml. Membranes were frozen by immersion of the trays in liquid N_2 and irradiated with a Van de Graaff generator producing a 0.5 mA beam of 1.5 MeV electrons. The irradiation chamber was cooled with flowing liquid N_2. Chamber temperature was measured as described (12). Sample temperature was constantly in the range -45° C to -52° C during irradiation procedures. Calibration of the radiation dosage was performed with each run by dosimetry. Film (DuPont MSC-300) bleaching was calibrated against a chemical dosimeter (13). Further standardization was performed by irradiation of a standard enzyme, eel acetylcholinesterase as described (11).

Data were analyzed according to radiation target theory (14) where it is assumed that the biological activity of the protein in question (a single target) is destroyed by a single high energy electron hit occurring within its molecular volume. An empirical equation,

$$M_r = \frac{6.4 \times 10^{11}}{D_{37}}$$

where D_{37} is the radiation dose in rads required to reduce the measured activity to 37% of the original control was derived by Kempner and Macey (15) from the radiation inactivation of known molecules. Under appropriate conditions, the molecular weight obtained from target size analysis will be the oligomeric molecular weight of a multisubunit structure.

RESULTS

Immunoaffinity Chromatography of Lung β_2-Adrenergic Receptors using Autoantibodies - As illustrated in Figure 1, when isoelectric focusing purified bovine lung β_2-adrenergic receptors stereospecifically labeled with [^{3}H]-NHNP-NBE were further purified by immunoaffinity chromatography on Protein A Sepharose 4B-CL containing adsorbed autoantibodies to β_2-receptors from a patient with allergic rhinitis, and subsequently analyzed on SDS-poly-

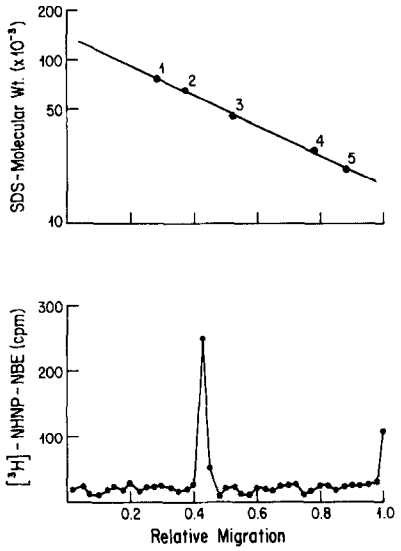

FIGURE 1 - SDS-PAGE of Autoantibody Affinity Purified Bovine Lung β_2- Adrenergic Receptors. Triton X-100 solubilized β_2-receptors labeled with the irreversible affinity ligand, [^{3}H]-NHNP-NBE, were partially purified by gel permeation chromatography and isoelectric focusing followed by immunoaffinity chromatography on IgG-Protein-A Sepharose as described in Methods. The bottom panel represents an SDS-polyacrylamide gel (10%) of immunoaffinity purified β_2-receptors. The top panel is a linear plot of the molecular weight standards used for 10% SDS-PAGE; 1) lactoperoxidase (M_r = 77,500), 2) bovine serum albumin (M_r = 66,000), 3) ovalbumin (M_r = 46,000), 4) chymotrypsinogen A (M_r = 27,500) and 5) soybean trypsin inhibitor (M_r = 21,500).

acrylamide gels, a single peak of radiolabeled protein at 59,000 daltons is obtained. Immunoaffinity chromatography significantly enhanced the resolution of the 59,000 dalton component on SDS-polyacrylamide gels when compared to analysis of isoelectric focusing purified β-receptors (not shown), indicating that additional purification of the β_2-receptor had been achieved with this technique. The stereospecificity of [^{3}H]-NHNP-NBE labeling of this protein at 59,000 daltons along with the additional specificity offered by autoantibody immunoaffinity chromatography strongly suggested that the protein isolated with these techniques represented a β_2-receptor subunit which contained the adrenergic ligand binding site.

Immunoaffinity Chromatography of Lung β_2-Adrenergic Receptors using Monoclonal Antibodies - When bovine lung β_2-adrenergic receptors, partially purified by gel permeation chromatography, are further purified by immunoaffinity chromatography on monoclonal antibody FV-104-Sepharose 4B,

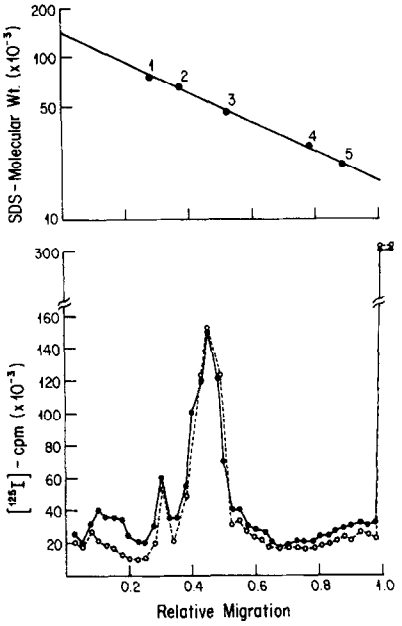

FIGURE 2 - SDS-PAGE of Monoclonal Antibody Affinity Purified Bovine Lung β_2-Adrenergic Receptors. Triton X-100 solubilized β_2-receptors, specifically labeled with IHYP, were partially purified by gel permeation chromatography followed by immunoaffinity chromatography on FV-104-Sepharose as described in Methods. The bottom panel represents an SDS-polyacrylamide gel (10%) of immunoaffinity purified β_2-receptors under reducing (○) and non-reducing conditions (●). The molecular weight standards in the top panel are identical to those described in the legend for Figure 1.

SDS-PAGE of the iodinated eluate again reveals a major protein of molecular weight 59,000 daltons (Figure 2). In addition, proteins of molecular weights 115,000 and 68,000 daltons are also obtained. In an attempt to elucidate the relationship between these three proteins, SDS-polyacrylamide gels were run under reducing and non-reducing conditions (Figure 2). Under non-reducing conditions, all three proteins are evident on SDS-polyacrylamide gels. When SDS-PAGE of eluates pre-incubated with β-mercaptoethanol is performed, the protein at 115,000 daltons is eliminated and the amount of radiolabel in the 59,000 dalton peak is increased. These data suggest that the 59,000 dalton protein may be formed from the 115,000 dalton material by reduction of disulfide bond(s).

To further confirm that the 59,000 dalton protein was indeed generated from the 115,000 dalton protein, gel slices containing the 115,000 dalton protein were re-electrophoresed under reducing conditions. Only one component

was identified on SDS-polyacrylamide gels which had a molecular weight of 59,000 (not shown), and therefore, this strongly suggested that the 115,000 dalton protein is composed of subunits of 59,000 daltons. These data on the SDS-subunit composition of bovine lung β_2-receptors purified by monoclonal antibody immunoaffinity chromatography are in excellent agreement with the data obtained using autoantibodies.

We assume that the 68,000 dalton protein represents a small complement of β_1-receptors found in bovine lung tissue, as its molecular weight is identical to that determined by us for turkey erythrocyte β_1-receptors (2). Previous data on ligand binding to lung β-receptors suggests that lung tissue contains both β_1 and β_2-receptor sub-types (16). As monoclonal antibody FV-104 recognizes an antigenic determinant within the ligand binding site of β-receptors common to both β-receptor sub-types, it is not surprising that FV-104 immunoaffinity chromatograpy would co-purify both β_1 and β_2-receptor subclasses.

Target Size Analysis of Lung β_2-Adrenergic Receptors - The data from the monoclonal antibody immunoaffinity chromatography of mammalian lung β_2-receptors suggested that the β_2-receptor may be a dimer of two identical components of molecular weight 115,000. To further investigate this hypothesis, we turned to target size analysis or radiation inactivation of lung β_2-receptors. Target size analysis is presently the only technique readily available for determination of the functional size of a protein within the cell membrane.

As shown in Figure 3, when canine or bovine (not shown) lung membranes were subjected to high energy electron bombardment, the β_2-adrenergic receptor, as measured by IHYP specific binding, was inactivated as a simple exponential of the radiation dosage. IHYP binding was assessed over a wide range of ligand concentrations both above and below the K_d for IHYP binding to the β-receptor (150 pM). The target size molecular weight of the β_2-receptor was independent of the ligand concentration or membrane protein concentration. Saturation isotherms demonstrated a loss of IHYP binding sites without evidence for affinity changes, consistent with the one target-one hit theory of radiation

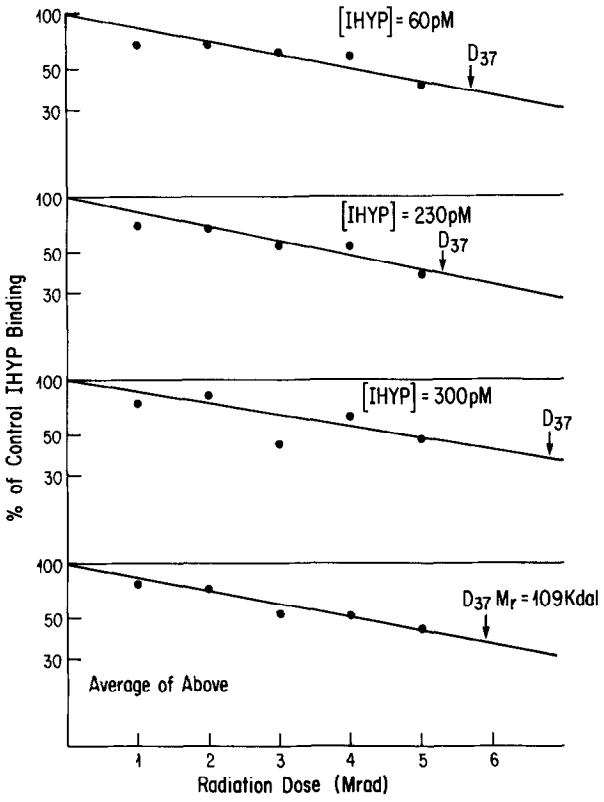

FIGURE 3 - Target Size Analysis of Lung β_2-Adrenergic Receptors. Canine lung membranes were frozen in thin layers in aluminum trays and subjected to high energy electron bombardment as described in Methods. Radiation induced inactivation of the β_2-receptor was assessed by measuring IHYP specific binding at the indicated ligand concentrations. The figure represents the loss of specific IHYP binding as a function of increasing doses of radiation. These experiments were performed three times. Each point represents the mean of triplicate determinations from each experiment. Lines were drawn by least squares linear regression and molecular weight values calculated as described in Methods.

inactivation (14). The average mammalian lung β_2-adrenergic receptor molecular weight calculated from all radiation inactivation studies was 109,000 ± 5,300 daltons.

Radiation inactivation standardization was performed with eel acetylcholinesterase at the same temperature. Target size analysis of this protein yielded a target size of 248,000 daltons, similar to that previously reported from other radiation inactivation studies (11,17).

DISCUSSION

The purification and resolution of β-adrenergic receptor structure is an essential step toward fully understanding the function and mechanism of action

of this important regulatory protein. Mammalian β-receptor purification has been complicated by the low density of receptor molecules present in virtually all tissues and by instability of receptors upon solubilization from membranes by detergents (4).

To help overcome these problems in β-receptor purification and characterization, we have combined immunoaffinity chromatography using autoantibodies and monoclonal antibodies to β-adrenergic receptors with target size analysis. The specificity of both autoantibody and monoclonal antibody preparations for β_2-adrenergic receptors has allowed for the isolation of β-receptor subunits from small quantities of relatively impure receptor preparations. We have identified the major subunit of mammalian lung β_2-receptors as a protein of 59,000 daltons on SDS-polyacrylamide gels. These findings are in good agreement with the SDS-subunit composition reported for frog erythrocyte β_2-receptors isolated by affinity chromatography (3).

In order to determine whether the 59,000 dalton subunit represented all or only a portion of the lung β_2-receptor in the membrane, we turned to target size analysis. Radiation inactivation provides a molecular weight of 109,000 daltons for the lung β_2-receptor. These data, along with the appearance of higher molecular weight species of β_2-receptor on SDS-PAGE, suggest that the 59,000 dalton subunit arises from a receptor complex in the membrane with an average molecular weight of 110-120,000 daltons. This number would suggest that the β_2-receptor is at least a dimer of two identical 59,000 dalton subunits, both of which contain the adrenergic ligand binding site.

ACKNOWLEDGEMENTS

This investigation was supported by a grant to JCV from NIH #AI-19346 and a grant to CMF from the American Heart Association #82-881 with funds contributed in part from the Heart Association of Western New York.

REFERENCES

1. Maguire, M.E., Ross, E.M. and Gilman, A.G. (1977) in Advances in Cyclic Nucleotide Research (eds., Greengard, P. and Robison, G.A.), Vol. 8, pp. 1-74, Raven Press, New York.
2. Fraser, C.M. and Venter, J.C. (1980) Proc. Nat. Acad. Sci. USA, 77, 7034-7038.

3. Schorr, R.G.L., Lefkowitz, R.J. and Caron, M.G. (1981) J. Biol. Chem., 256, 5820-5826.
4. Strauss, W.L., Ghai, G., Fraser, C.M. and Venter, J.C. (1979) Arch. Biochem. Biophys., 196, 566-573.
5. Dale, H.H. (1906) J. Physiol. London, 34, 163.
6. Venter, J.C., Fraser, C.M. and Harrison, L.C. (1980) Science, 207, 1361-1363.
7. Atlas, D., Steer, M.L. and Levitski, A. (1976) Proc. Nat. Acad. Sci. USA, 73, 1921-1925.
8. Venter, J.C. and Fraser, C.M. (1981) in Monoclonal Antibodies in Endocrine Research (eds., Fellows, R. and Eisenbarth, G.), pp. 119-134, Raven Press, New York.
9. Marchalonis, J.J., Cone, R.E. and Santer, Y. (1971) Biochem. J., 124, 921-927.
10. Laemmli, U.K. (1970) Nature, 277, 680-685.
11. Saccomani, G., Sachs, G., Cuppoletti, J. and Jung, C.Y. (1981) J. Biol. Chem. 256, 7727-7729.
12. Fluke, D.J. (1966) Radiat. Res., 28, 677-693.
13. Fricke, H. and Hart, E.S. (1966) in Radiation Dosimetry (eds., Attix, F.H. and Roesch, W.Z.), Vol. II, pp. 167-239, Academic Press, New York.
14. Pollard, E.Z. (1953) Adv. Biol. Med. Phys. 3, 153-189.
15. Kempner, G.R. and Macey, R.I. (1968) Biochim. Biophys. Acta, 163, 188-203.
16. Rugg, E.L., Barnett, D.B. and Nahorski, S.R. (1978) Mol. Pharmacol., 14, 996-1005.
17. Levinson, S.R. and Ellory, J.Z. (1974) Biochem. J., 137, 123-125.