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Alpha$_1$-Adrenergic Receptor Structure

J. CRAIG VENTER, PETER HORNE, BARBARA EDDY, ROGER GREGUSKI, AND CLAIRE M. FRASER

Department of Molecular Immunology, Roswell Park Memorial Institute, Buffalo, New York 14263

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SUMMARY

The structure of the *alpha*$_1$-adrenergic receptor was investigated by comparing polypeptides identified by sodium dodecyl sulfate ($NaDodSO_4$)-polyacrylamide gel electrophoresis with the size of the intact receptor in cell membranes as determined by target size analysis. The *alpha*$_1$-adrenergic receptor from rat liver membranes affinity-labeled with [^{3}H]phenoxybenzamine, a covalent affinity reagent, appeared as a single polypeptide with a molecular mass of 85,000 daltons (Da) on $NaDodSO_4$-polyacrylamide gels. In the absence of protease inhibitors, smaller peptides of 58–62 kDa and 40–45 kDa, specifically labeled with [^{3}H]phenoxybenzamine, were also apparent on $NaDodSO_4$ gels. In order to determine whether the 85-kDa protein represented all or only a portion of the *alpha*$_1$-receptor, radiation inactivation (target size analysis) was undertaken. Radiation-induced receptor inactivation was measured by the loss of specific [^{3}H]phenoxybenzamine and [^{3}H]prazosin binding and by the loss of affinity-labeled *alpha*$_1$-adrenergic receptors on $NaDodSO_4$ gels. Target size analysis of rat liver *alpha*$_1$-receptors indicated that the intact membrane-bound receptor has an average molecular mass of 160,000 Da. These data suggest that the intact *alpha*-receptor may exist in the membrane as a dimer of two 85,000-Da subunits. The structure of the *alpha*$_1$-receptor was further studied by limited proteolysis of the 85-kDa protein isolated from $NaDodSO_4$ gels. Trypsin, chymotrypsin, and papain produce smaller peptides similar to those produced during membrane isolation in the absence of protease inhibition. Limited proteolysis of the membrane-bound receptor produces water-soluble peptides, the largest of which is 45,000 Da. This peptide contains the ligand-binding domain and protrudes from the membrane into the extracellular space.

INTRODUCTION

The *alpha*$_1$-adrenergic receptor is one of the principal neurotransmitter receptors of the autonomic and central nervous systems. *Alpha*$_1$-adrenergic receptors are found in most tissues, including brain, blood vessels, airway smooth muscle, cardiac muscle, and liver. Although the rat liver *alpha*$_1$-receptor has undergone extensive physiological and pharmacological characterization, structural studies on the *alpha*-adrenergic receptor have been very limited. In 1979, Guellaen *et al.* (1) reported the hydrodynamic size for the *alpha*$_1$-receptor to be 96,000 Da.[1] More recently, this same group has reported that the rat liver *alpha*$_1$-adrenergic receptor has a molecular mass of 45,000 Da from $NaDodSO_4$-polyacrylamide gel electrophoresis (2).

In structural studies on *alpha*$_1$-adrenergic receptors, we utilized high specific activity [^{3}H]POB as an affinity reagent (3). The [^{3}H]POB was found to be highly selective for the *alpha*$_1$-adrenergic receptor, and can readily be utilized in the 0.5– 1.0 nM range to label specifically and covalently *alpha*$_1$-receptors in the membrane (3). In initial experiments on the affinity labeling of rat liver *alpha*$_1$-receptors (see Results), we observed a number of lower molecular weight species specifically labeled by [^{3}H]POB, including peptides of 58,000 and 45,000 Da. In the presence of high concentrations of protease inhibitors, however, a specifically labeled protein with a molecular mass of 80,000–85,000 Da was found on $NaDodSO_4$-polyacrylamide gels (3). Nevertheless, it was difficult to eliminate completely the 58,000-Da peptide with protease inhibitors. It was assumed, but not proven, that the 58,000-Da species arose from the 85,000-Da protein (3). These data are not unlike those observed in the analysis of the structure of the muscarinic cholinergic receptor from tissues with high intrinsic protease activity, inasmuch as lower molecular weight species were generated by proteolytic cleavage of one receptor protein (4).

This study was undertaken to resolve further the structure of the *alpha*$_1$-adrenergic receptor, to ascertain whether the smaller peptides of 58,000 and 45,000 Da

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[1] The abbreviations used are: Da, dalton; $NaDodSO_4$, sodium dodecyl sulfate; POB, phenoxybenzamine.

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derive from the 85,000-Da protein, to determine the overall size of the *alpha*$_1$-receptor in the membrane, and to provide specific information concerning the membrane orientation and structure of the *alpha*$_1$-receptor binding subunit.

EXPERIMENTAL PROCEDURES

Materials

[^{3}H]POB-HCl (45 Ci/mmol) and [^{3}H]prazosin (20 Ci/mmol) were obtained from New England Nuclear Corporation (Boston, Mass.). Trypsin, chymotrypsin, and papain were from Worthington Biochemicals (Freehold, N. J.). Protease inhibitors were from Sigma Chemical Company (St. Louis, MO.). Prazosin was a gift from Pfizer (New York, N. Y.). All other materials were as previously described (3, 4).

Methods

Tissue and membrane preparation. Rat liver plasma membranes were prepared as described previously (5) with extreme care to maintain the temperature between 1 and 3°. Buffers contained the protease inhibitors aprotinin (20 kallikrein inhibitor units per milliliter), bacitracin (100 μg/ml), benzamidine (1 mM), benzethonium chloride (0.1 mM), pepstatin (0.1 μg/ml), phenylmethylsulfonyl fluoride (0.1 mM), leupeptin (10 μg/ml), soybean trypsin inhibitor (10 μg/ml), and iodoacetamide (10 mM). Protease inhibitors were made up individually and added to the homogenization buffer immediately prior to use. Membranes were snap-frozen in liquid N_2 and stored at −78° until use.

Receptor assays. [^{3}H]POB specific binding was assessed essentially as described previously (3) at 23° in 50 mM K^+ phosphate buffer (pH 7.5) containing 4 mM $MgSO_4$. Specific binding is defined as the total binding minus the binding in the presence of 0.1 μM prazosin or 100 μM *R*(−)-epinephrine. Specific binding represented between 65 and 85% of the total binding. As $NaDodSO_4$ gel analysis shows that the nonspecific [^{3}H]POB binding is present at the dye front, it is concluded that the majority of this nonspecific binding is to small peptides and/or membrane lipids (3) (see Figs. 1 and 2). For $NaDodSO_4$-gel analysis or proteolysis studies (unless otherwise indicated), membranes were labeled at a protein concentration of 0.15 mg/ml in the presence of 1 nM [^{3}H]POB at 23° for 4 min. Membranes were then cooled to 0° and centrifuged at 42,000 × *g* for 15 min followed by two additional centrifugations in assay buffer. The resultant pellet was resuspended in 2% $NaDodSO_4$ at a protein concentration of 2–4 mg/ml and incubated at 100° for 5 min. Labeled receptors were characterized by electrophoresis on polyacrylamide gels in $NaDodSO_4$ according to the method of Laemmli (6).

Limited proteolysis.

Soluble receptor. Alpha$_1$-receptors were affinity-labeled with [^{3}H]POB and solubilized with 2% $NaDodSO_4$. The soluble [^{3}H]POB receptor complexes were either directly treated with proteolytic enzymes, as below, or first subjected to preparative $NaDodSO_4$-polyacrylamide gel electrophoresis as described previously (4, 7). Solubilized receptor was diluted 10-fold with 50 mM $NaPO_4$ buffer (pH 7.4) and subjected to trypsin, chymotrypsin, or papain (25 μg/ml) digestion for 30 min at 37°. Reactions were terminated by treatment at 100° for 2 min. Samples were centrifuged (42,000 × *g*) for 20 min and then concentrated approximately 10-fold over a YM-10 membrane (Amicon). The proteolytic fragments were subjected to $NaDodSO_4$-polyacrylamide gel electrophoresis as described previously (4).

Alpha$_1$-adrenergic receptors, purified by $NaDodSO_4$-polyacrylamide gel electrophoresis, were eluted from the gel in 0.1% $NaDodSO_4$ (7) and treated with trypsin, chymotrypsin, or papain as above. Samples were concentrated and subjected to analytical $NaDodSO_4$-polyacrylamide gel electrophoresis as described (Fig. 1).

Membrane-bound receptor. Alpha$_1$-adrenergic receptors were covalently labeled with [^{3}H]POB, and membranes were washed extensively by centrifugation and redilution with buffer. Labeled membranes were treated with the indicated enzymes (25 μg/ml) for 30 min at 37°. The incubations were terminated by centrifugation at 42,000 × *g* for 20 min at 4°, $NaDodSO_4$ and β-mercaptoethanol were added to the supernatant fluid to final concentrations of 2 and 5%, respectively, and the supernatant fluid was incubated at 100° for 5 min. The receptor fragments released by protease treatment were analyzed by $NaDodSO_4$-polyacrylamide gel electrophoresis.

Radiation inactivation. The radiation inactivation was performed as described by Venter *et al.* (8) for the slow inward calcium channel and the muscarinic receptor (4).

By inclusion of enzyme standards of known molecular weight, the key criteria for radiation inactivation of frozen samples can be satisfied: (a) that the radiation inactivation occur at a temperature within the specimen well below that at which any thermal inactivation commences (b) that empirical correction factors for temperature be eliminated, and (c) that the determination should not depend upon knowledge of the absolute dose of radiation received by the specimen.

Criterion a was satisfied by maintenance of the samples between −45° and −52° throughout irradiation. In all experiments, *alpha*$_1$-adrenergic receptor binding activity from control frozen samples was within 95–100% of the receptor binding of control, nonfrozen membranes. Criteria b and c were satisfied as described (4, 8–10) by the use of enzymes of known molecular weight as internal standards. Enzyme molecular weight and subunit composition were analyzed by $NaDodSO_4$-polyacrylamide gel electrophoresis.

In addition to the globular proteins used as standards for radiation inactivation of the *alpha*$_1$-adrenergic receptor, we also used the muscarinic receptor, an integral membrane protein for which the target size has been shown to be identical with the molecular size determined by other means (4). Other workers have also demonstrated close agreement between the target size of membrane proteins and their known molecular size (9, 10). When the standard enzymes (see below) were subjected to high-energy electron bombardment, enzymatic activity declined as a simple exponential of the radiation dosage (Figs. 4 and 5). Enzymes simultaneously irradiated had their inactivation ratio, S_X/S_G, determined by the ratio of the slopes of the semilogarithmic plots of each enzyme (S_X) to the slope of either β-galactosidase (S_G) or horse liver alcohol dehydrogenase (ADH) (S_{ADH}). The ratio S_X/S_G for each standard (X) was related to its molecular weight, M_X, normalized to the molecular weight (M_G) of β-galactosidase (Fig. 6). This relationship was based on a transformation of radiation target theory (11):

$$2.3 \log A/A_0 = \frac{\bar{V}}{N_0} M \cdot D$$

where A is the enzyme activity upon irradiation at dose D, A_o is the initial control activity, N_o is Avogadro's number, V is the partial specific volume of the protein, and M is the protein molecular weight.

The slope of $\log A/A_o$ versus D is proportional to M. Therefore,

$$S_X/S_G = M_X/M_G$$

The requirement for the absolute value of D is eliminated and, along with it, any temperature correction factors.

Radiation procedure. Purified cell membranes prepared as above, and/or the calibrating enzymes were layered at a depth of 0.5 mm in open aluminum trays. Membranes and/or enzymes were quickly frozen by immersion of the trays in liquid nitrogen. The aluminum trays containing the thin film of frozen sample were placed in an aluminum chamber which was then flushed for 3 min with liquid N_2 to replace the air with a nitrogen atmosphere. The irradiation chamber was continuously cooled with flowing liquid N_2 throughout the irradiation procedure to maintain sample temperature constantly in the range of −45° to −52°. The irradiation room is maintained at low humidity and has a high-velocity ventilation system to minimize frost and ozone accumulation. Samples were irradiated with a 0.5-mamp beam of 1.5 MeV electrons produced with a Van de Graaf generator. Calibration of the radiation dose was performed with dosimetry (12).

Enzymes. Horse liver alcohol dehydrogenase, yeast alcohol dehydrogenase, pyruvate kinase, and β-galactosidase over a range of 0.4–4.0 mg/ml were frozen in trays as above either in combination with

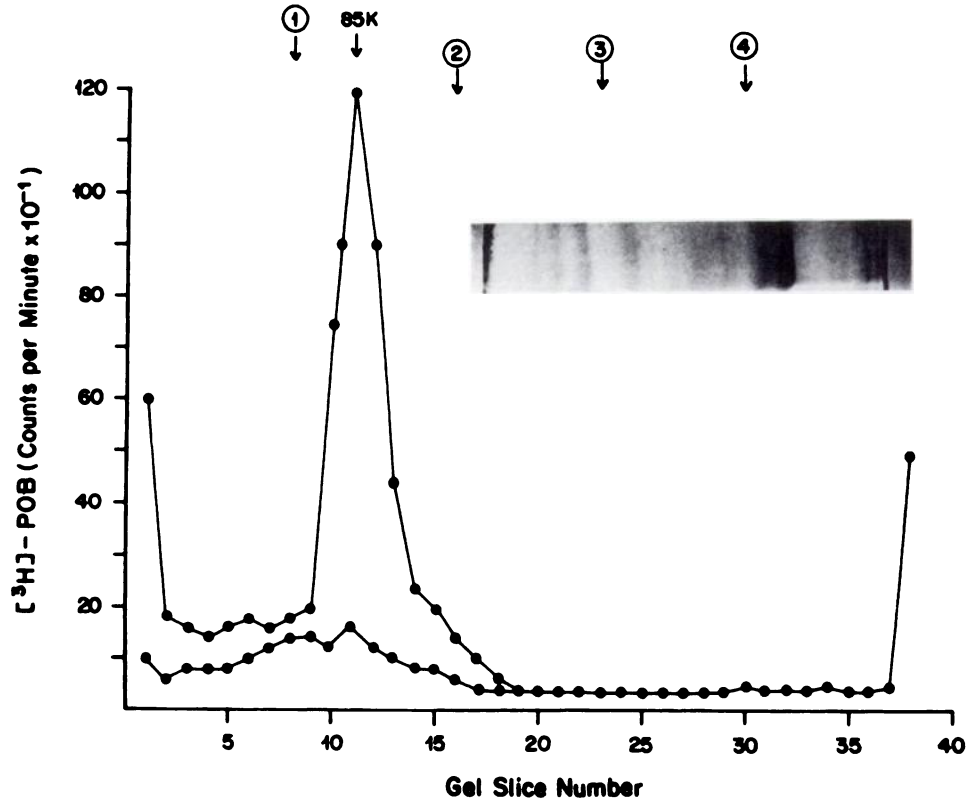

FIG. 1. *NaDodSO$_4$-polyacrylamide gel electrophoretic analysis of the rat liver alpha-adrenergic receptor*

Rat liver membranes were purified and affinity-labeled with [^{3}H]POB as described under Experimental Procedures. [^{3}H]POB/α_1-receptor complexes were solubilized with 2% NaDodSO$_4$, treated at 100° for 5 min in the presence of 5% β-mercaptoethanol, and analyzed on 10% NaDodSO$_4$-polyacrylamide gels. Gels were sliced into 2.0-mm slices and counted in a scintillation counter subsequent to overnight incubation in Econofluor (New England Nuclear Corporation) containing 3% Protosol (New England Nuclear Corporation). Data shown are representative of data from 18 experiments. The background nonspecific binding (*lower line*) is representative of [^{3}H]POB binding in the presence of 0.1 μM prazosin.

Standard proteins were analyzed on each gel. Protein standards are as follows: *1*, phosphorylase *b*, 94,000 Da; *2*, albumin, 67,000 Da; *3*, ovalbumin, 43,000 Da; *4*, carbonic anhydrase, 30,000 Da. The *inset* shows the gel prior to slicing.

membranes or individually, and irradiated. In the case of pyruvate kinase, bovine albumin (10 mg/ml) was added to prevent enzyme aggregation upon freezing and thawing (4, 8). Enzymes included with membranes or other enzymes for irradiation had radiation inactivation profiles identical with those of the pure enzymes irradiated alone. Enzyme activity was measured on a recording Gilford spectrophotometer as described (4, 8). Samples were stored at −80° both prior to and subsequent to irradiation procedures.

RESULTS

Alpha$_1$-adrenergic receptor subunit molecular weight. When rat liver membranes isolated at 2° in the presence of protease inhibitors are affinity-labeled with [^{3}H]POB, solubilized with 2% NaDodSO$_4$, and analyzed on NaDodSO$_4$-polyacrylamide gels, we obtain the results shown in Fig. 1. The largest single polypeptide appears as a protein with a molecular mass of 85,000 ± 5,500 Da (n = 18). The 85-kDa protein specifically labeled as 0.1 μM prazosin completely blocks [^{3}H]POB binding to this protein (Fig. 1). The molecular weight of the *alpha$_1$*-receptor subunit is not altered by the inclusion or exclusion (data not illustrated) of disulfide bond-reducing agents, supporting the concept that the 85-kDa protein is a single polypeptide.

When protease inhibitors are eliminated from the membrane preparation, and/or isolation times are prolonged, and/or strict temperature control (2°) is not exercised throughout the isolation procedure, a variety of specifically labeled peptides appears on NaDodSO$_4$-gels (Fig. 2). As our tissue isolation techniques were altered (3) and improved, the peptide patterns observed on NaDodSO$_4$ gels showed an increased proportion of higher molecular weight proteins (Fig. 2A–D). *Alpha$_1$*-adrenergic receptor-associated peptides include those with molecular masses in the range of 37–42 kDa, 45–50 kDa, 58–62 kDa, and 78–85 kDa (Fig. 2). The bands of [^{3}H]POB labeling are rather broad and may reflect the fact that the *alpha$_1$*-receptor is a glycoprotein. Indeed, it has been demonstrated that *alpha$_1$*-receptors are capable of binding to lectin affinity columns, consistent with this hypothesis (13). We interpret our data to suggest that the lower molecular weight peptides are generated by tissue proteases from the *alpha$_1$*-receptor monomer (85,000-Da protein).

A number of ligands were tested for their ability to inhibit [^{3}H]POB labeling of the *alpha$_1$*-receptor (Table 1). These data demonstrate a specific order of potency and stereoselection indicative of the *alpha$_1$*-adrenergic

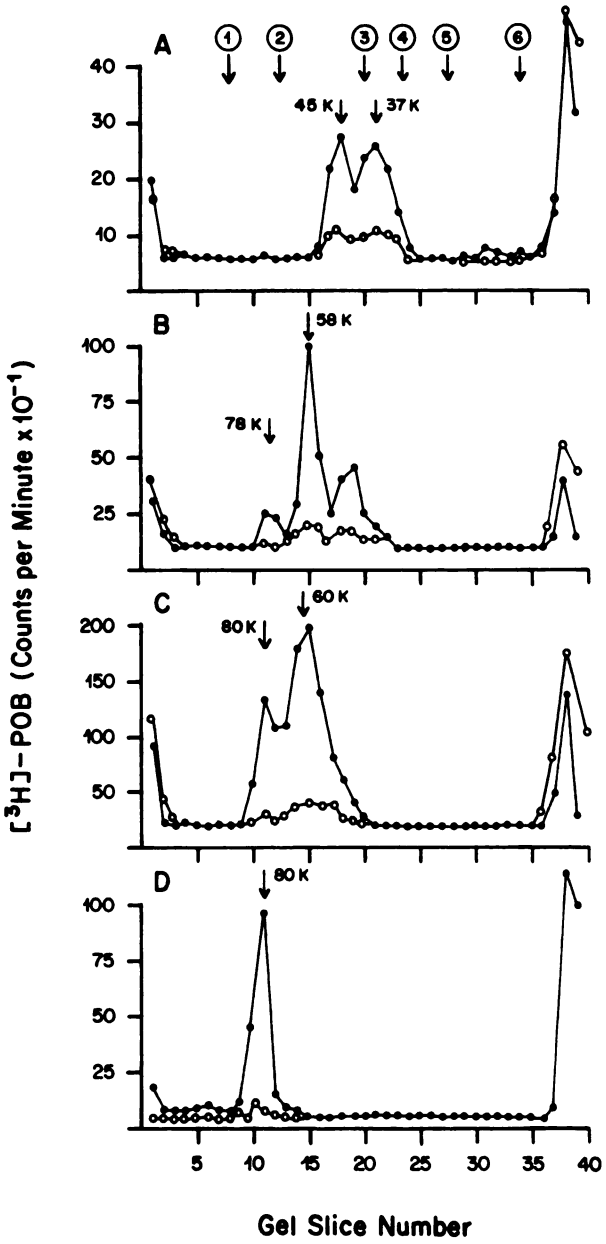

FIG. 2. *Effect of endogenous proteases on NaDodSO$_4$-polyacrylamide gel electrophoretic analysis of rat liver alpha$_1$-adrenergic receptors labeled with [^{3}H]POB*

Rat liver membranes were labeled with [^{3}H]POB and solubilized as described in legend to Fig. 1 and under Experimental Procedures. The four gel patterns represent experiments performed as described previously (3) in the absence of protease inhibitors, which gave variable results (A and B) or in the presence of protease inhibitors (3) (C). D, *Alpha*$_1$-receptor-[^{3}H]POB complexes from membranes prepared with protease inhibitors as described under Experimental Procedures; diligent attention was paid to the temperature of the samples throughout the isolation procedures (1–3°). ●, Total binding; ○, binding in the presence of 0.1 μM prazosin.

Gels were sliced and counted for radioactivity as in Fig. 1. Protein standards are as follows: *1*, phosphorylase *b*, 94,000 Da; *2*, albumin, 67,000 Da; *3*, ovalbumin, 43,000 Da; *4*, carbonic anhydrase, 30,000 Da; *5*, trypsin inhibitor, 20,000 Da; *6*, α-lactalbumin, 14,000 Da.

receptor. We have previously demonstrated that the IC_{50} values for inhibition of [^{3}H]POB binding by a variety of ligands correlate well with their ability to antagonize [^{3}H]prazosin binding (3).

TABLE 1

Effect of adrenergic ligands on [^{3}H]POB labeling of alpha$_1$-adrenergic receptors

Drug	EC_{50}[a]
	nM
POB	0.35
Prazosin	0.006
Phentolamine	30
Yohimbine	1,000
R(−)-Epinephrine	2,800
S(+)-Epinephrine	50,000
L-Propranolol	1,100
Phenylephrine	20,000

[a] EC_{50} values are the concentration of the drugs which produced a 50% inhibition of [^{3}H]POB (1.0 nM) binding to rat liver *alpha*$_1$-adrenergic receptors. The values represent averages from four experiments.

Further studies were performed to demonstrate the binding specificity to the various proteolytic peptides generated by the action of liver proteases. Figure 3 represents the NaDodSO$_4$-polyacrylamide gel (7.5%) profile of [^{3}H]POB proteins labeled in the absence of ligands and in the presence of 0.1 μM prazosin (17,000 xK_d), 10 μM *R*(−)-epinephrine (3.6 xK_d), 10 μM *S*(+)-epinephrine (0.2 xK_d), 10 μM phentolamine (300 xK_d), and 10 μM L-propranolol (10 xK_d).

These data demonstrate a similar specificity in inhibiting [^{3}H]POB labeling of the various receptor fragments that supports the concept that they are derived from the same protein.

Limited proteolysis of the alpha$_1$-receptor subunit. In order to investigate further the origin of the lower molecular weight receptor fragments, limited proteolysis of the isolated 85,000-Da *alpha*$_1$-receptor subunit was undertaken.

The *alpha*$_1$-receptor (85,000-Da protein) was isolated by preparative NaDodSO$_4$-polyacrylamide gel electrophoresis. The isolated receptor was subjected to papain digestion and reanalyzed on NaDodSO$_4$ gels (Fig. 4). Papain treatment of the 85-kDa receptor subunit produced proteolytic fragments with molecular masses in the range of 58,000–62,000, 40,000–45,000, 27,000–30,000, 23,000, and 18,000 Da (Fig. 4). The fragments generated by protease treatment of the intact 85-kDa subunit correspond to similar-sized peptides isolated from various tissue preparations (Fig. 2–4). These data support the hypothesis that the lower molecular weight fragments, e.g., 58,000 Da (3, 15) and 45,000 Da (2), derive from the 85,000-Da protein by the action of tissue proteases (3).

Radiation inactivation—target size analysis. Although the affinity-labeling experiments suggested that the largest *alpha*$_1$-receptor subunit containing the ligand binding site has a molecular mass of 80,000–85,000 Da, the question of whether this subunit represented the intact *alpha*$_1$-receptor or only a portion of it was answered in part by target size analysis. Radiation inactivation is the only technique available which can determine the functional size of a protein or protein complex in the intact membrane. This laboratory has recently applied this technique to the muscarinic cholinergic receptor (4), the

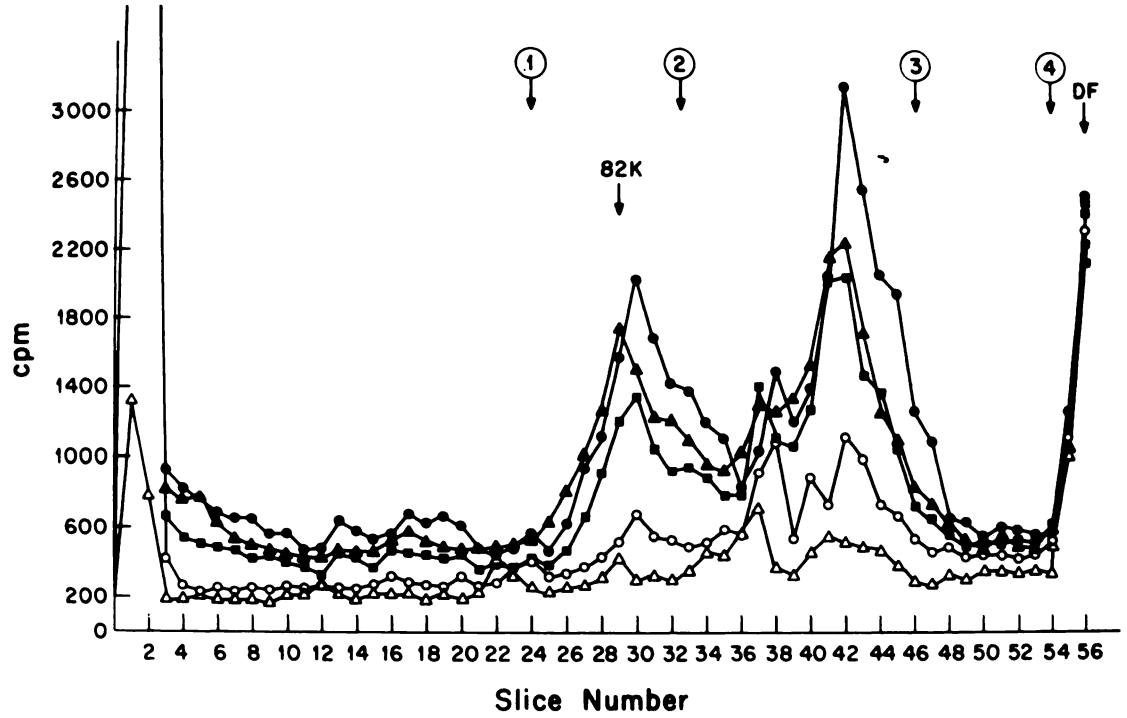

FIG. 3. *NaDodSO$_4$-polyacrylamide gel electrophoretic analysis of the effect of various ligands on [^{3}H]POB labeling of rat liver alpha$_1$-receptor*

Rat liver membranes were prepared and affinity-labeled with [^{3}H]POB in the absence (▲) and presence of 10 μM L-propranolol (●), 10 μM *S*(+)-epinephrine (■), 10 μM *R*(−)-epinephrine (○), and 10 μM L-phentolamine (△) as described under Experimental Procedures. Membranes were then solubilized and analyzed by NaDodSO$_4$-polyacrylamide gel electrophoresis on a 7.5% acrylamide gel; the radioactivity in 2-mm slices was determined as described in the legend to Fig. 1.

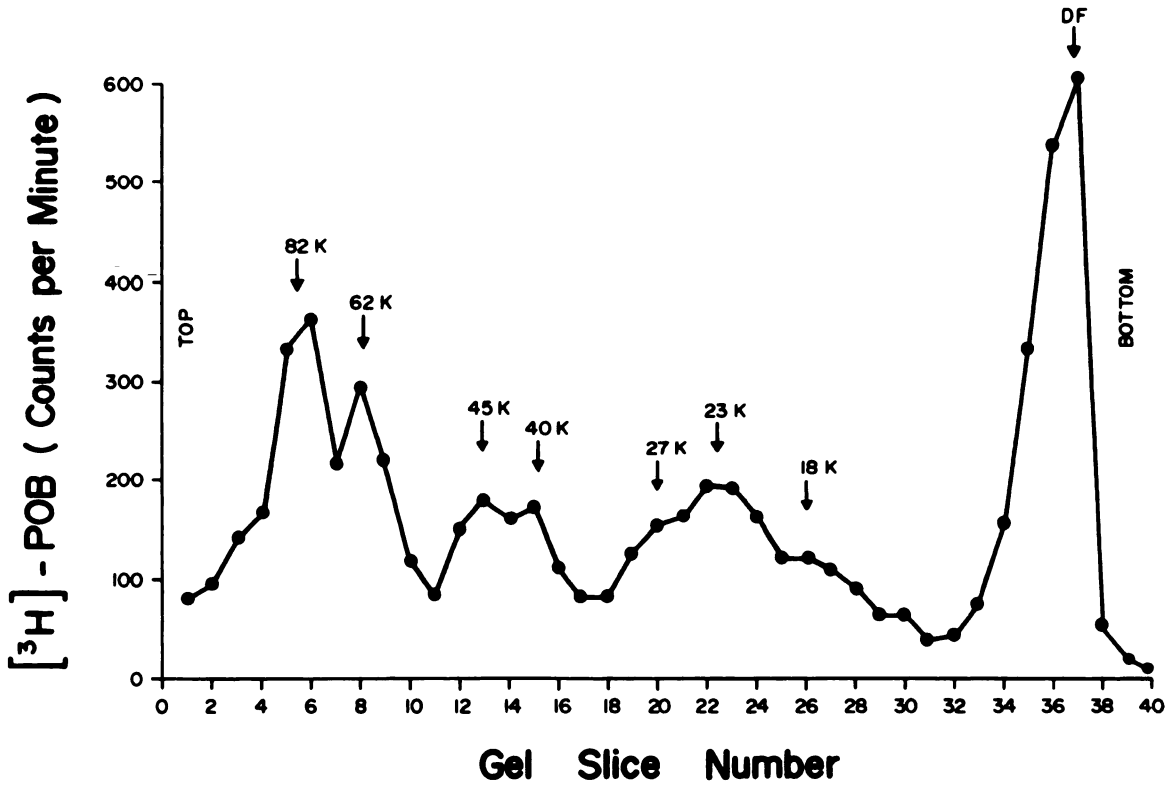

FIG. 4. *Limited proteolysis of rat liver alpha$_1$-adrenergic receptors*

Alpha$_1$-adrenergic receptors were affinity-labeled with [^{3}H]POB, solubilized with 2% NaDodSO$_4$, and purified by preparative NaDodSO$_4$-polyacrylamide gel electrophoresis. The soluble [^{3}H]POB-*alpha$_1$*-receptor complexes were subjected to trypsin, chymotrypsin, or papain (25 μg/ml) digestion for 30 min at 30° as described under Experimental Procedures. The proteolytic fragments were subjected to NaDodSO$_4$ polyacrylamide gel electrophoresis as described previously (4). The different proteolytic enzymes provided essentially identical results. Limited proteolysis of *alpha$_1$*-receptors with papain is illustrated.

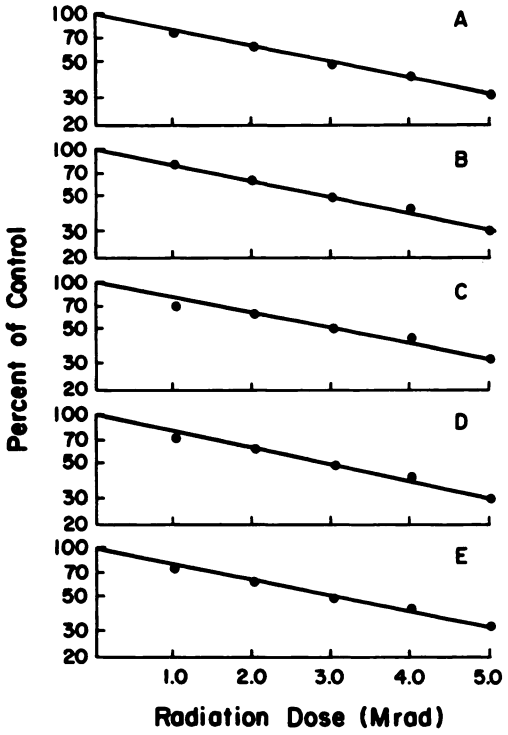

FIG. 5. *Radiation inactivation-target size analysis of $alpha_1$-adrenergic receptors as determined by [^{3}H]prazosin binding*

Rat liver membranes were purified and frozen in thin layers in aluminum trays along with enzyme standards and subjected to high-energy electron bombardment as described under Experimental Procedures. Radiation-induced loss of *$alpha_1$*-receptors was assessed by measuring specific [^{3}H]prazosin binding to membranes (50 μg of protein) at concentrations of 0.025, 0.05, 0.1, 0.5, and 2 nM, respectively, as shown in A–E. Specific binding is defined as total [^{3}H]prazosin binding minus the binding in the presence of 10 μM phentolamine.

$beta_2$-adrenergic receptor (16), the slow inward calcium channel (8), and the D_2-dopamine receptor (17).

When rat liver membranes are frozen in thin layers and subjected to high-energy electron bombardment (see Experimental Procedures), there is an exponential loss of the functional *$alpha_1$*-adrenergic receptor as a function of the radiation dosage (Figs. 5–8). The loss of *$alpha_1$*-receptors was assessed by the loss of [^{3}H]POB- and [^{3}H]prazosin-specific binding over a wide range of ligand concentrations both below and above the K_d (or apparent K_d) for ligand binding (Fig. 5). The loss of the receptor was linear over a broad dose of radiation, indicating a single homogeneous class of ligand binding sites (Figs. 5 and 8). Scatchard analysis of prazosin saturation isotherms (Fig. 6) indicated that the radiation produced only a loss of the number of sites with no apparent change in the receptor affinity for ligand at the remaining receptors, consistent with the one target-one hit theory of radiation inactivation of proteins (11). The target size of the liver *$alpha_1$*-receptor was also assessed by the loss of the 85,000-Da protein on $NaDodSO_4$ gels (Fig. 7).

The functional membrane-bound molecular size of *$alpha_1$*-receptor was determined as described under Experimental Procedures by the inclusion of enzyme standards of known molecular weight and subunit composition with the membrane preparations. As can be seen in Figs.

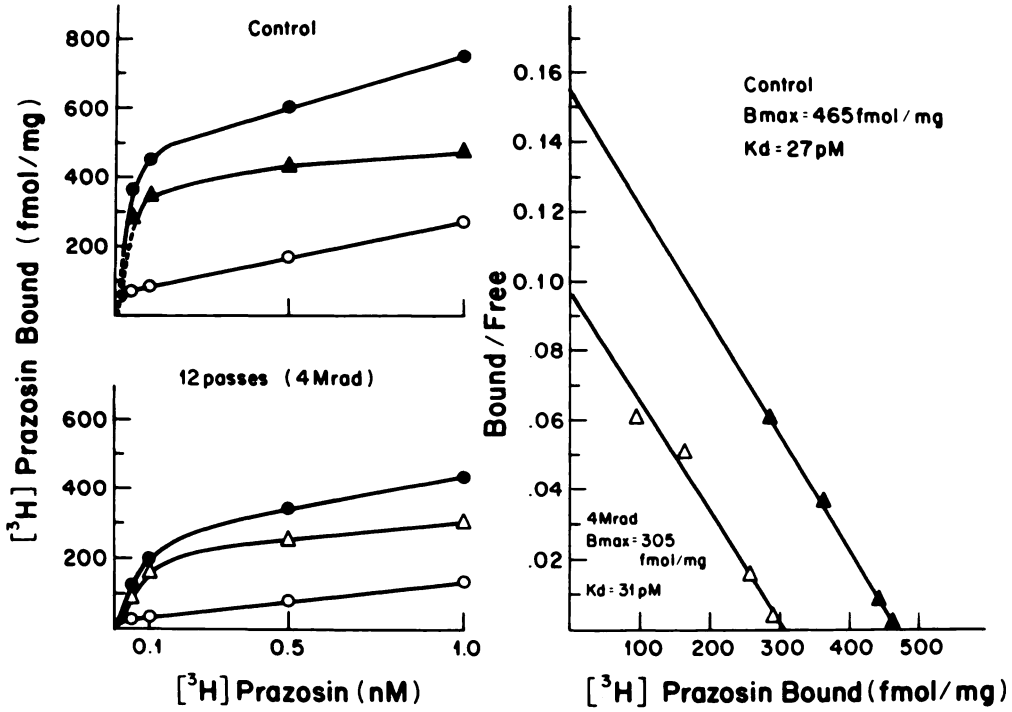

FIG. 6. *Scatchard analysis of [^{3}H]prazosin binding before and after radiation inactivation*

Rat liver membranes were subjected to electron bombardment as described in the legend to Fig. 5 and under Experimental Procedures. Membranes (50 μg of protein) were assayed for [^{3}H]prazosin binding, before and after 12 passes (approximately 4 Mrad total radiation). Nonspecific binding was determined in the presence of 10 μM phentolamine.

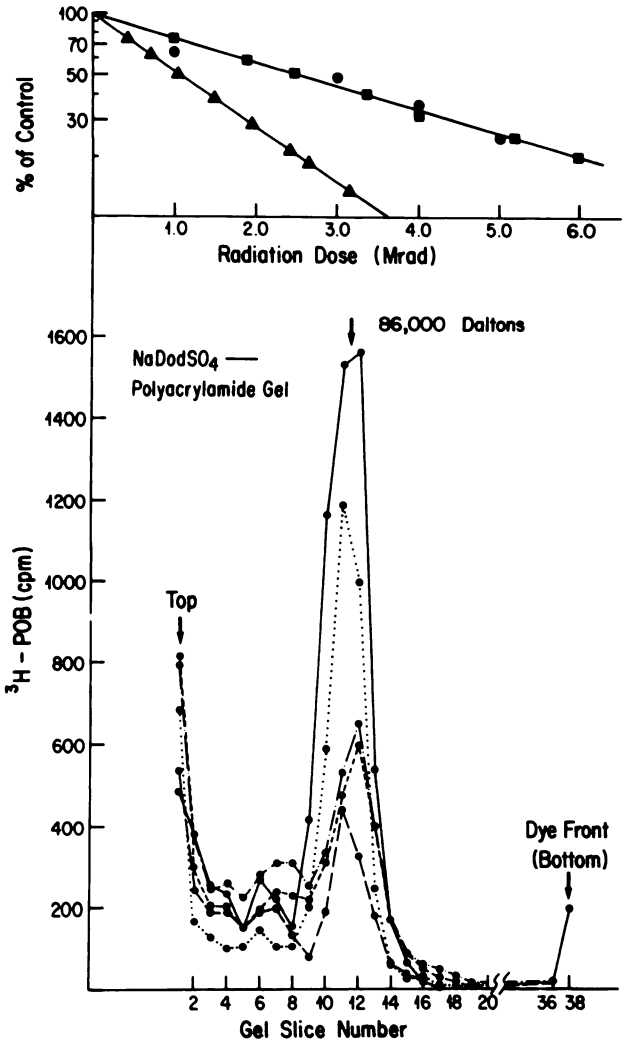

FIG. 7. *Target size analysis of alpha$_1$-adrenergic receptors as measured by the loss of the 85,000-Da alpha-receptor monomer on NaDodSO$_4$ gels*

Radiation inactivation of rat liver membranes was performed as described in the legend to Fig. 5. *Alpha$_1$*-receptors were affinity-labeled with [^{3}H]POB, solubilized with 2% NaDodSO$_4$, and subjected to NaDodSO$_4$-polyacrylamide gel electrophoresis.

Bottom, the superimposed NaDodSO$_4$ gel patterns from control membranes (●——●) and membranes subjected to inactivation with 1.0 Mrad (●·····●), 3.0 Mrad (●-·-●), 4.0 Mrad (●– – –●), and 5.0 Mrad (●— —●). *Top*, a semilogarithmic plot of the loss of the 85-kDa *alpha$_1$*-receptor monomer (●) from NaDodSO$_4$ gels. Compared on the same plot are the standard enzymes, yeast alcohol dehydrogenase (160 kDa, ■), and β-galactosidase (464 kDa, ▲). These data demonstrate that the 85-kDa protein derives from a 160-kDa complex in the membrane.

7 and 8, the radiation-induced loss of the *alpha$_1$*-receptor coincides with the loss of yeast alcohol dehydrogenase (molecular weight 160,000). Other enzyme standards, horse liver alcohol dehydrogenase (80,000 Da) and β-galactosidase (464,000 Da), are also shown (Fig. 7). The *alpha$_1$*-receptor molecular size was determined by the slope ratio method (Fig. 9).

The combined average of all experiments on the size of the *alpha$_1$*-receptor, determined by ligand binding and NaDodSO$_4$-gel electrophoresis (Figs. 5–8), yields a molecular size of 160,000 Da for the *alpha$_1$*-receptor in the native membrane (Fig. 9; Table 2). These data indicate that the 85,000-Da protein identified on NaDodSO$_4$-gels derives from a 160,000 molecular weight complex in the membrane.

Membrane orientation of the alpha$_1$-receptor monomer. Previous studies (18) have demonstrated that the ligand-binding site of the liver *alpha$_1$*-adrenergic receptor is exposed to the extracellular space. Since the affinity ligand, [^{3}H]POB, specifically and covalently labels this site, it is possible to obtain information relative to the portion of the *alpha$_1$*-receptor that contains the ligand binding site and protrudes into the extracellular space (4).

The approach of using limited proteolysis of affinity-labeled, membrane-bound receptors was undertaken as previously described by this laboratory for the muscarinic receptor (4). Trypsin, chymotrypsin, or papain treatment of [^{3}H]POB affinity-labeled *alpha$_1$*-receptors in intact liver membranes generates water-soluble fragments (Table 3). These water-soluble receptor fragments contain the ligand-binding site ([^{3}H]POB) and therefore represent in whole or in part that portion of the receptor not enveloped in the lipid phase of the membrane (4).

NaDodSO$_4$-polyacrylamide gel electrophoretic analysis of the water-soluble receptor fragments generated by protease treatment (Fig. 10) demonstrates that the largest tryptic fragment has a molecular mass of 45,000 Da. Lower molecular weight peptides are also generated by this treatment (Fig. 10). If we assume that there is only one ligand binding site per 85,000-Da subunit, then the 45,000-Da fragment produced by proteolysis would appear to have arisen from this higher molecular weight component. Similarly, the lower molecular weight water-soluble peptides must be derived from the 45,000-Da fragment, since they also retain the [^{3}H]POB binding site (Fig. 10).

DISCUSSION

These data suggest that the *alpha$_1$*-adrenergic receptor exists in the rat liver membrane as an oligomeric structure with an apparent molecular size of 160,000 Da. Since the NaDodSO$_4$-polyacrylamide gel electrophoresis data (Fig. 1) indicate a molecular mass of 80,000–85,000 Da for the *alpha$_1$*-receptor monomer, the simplest explanation for the 160,000-Da complex found in the membrane would be a dimer of two 85,000-Da *alpha$_1$*-receptor monomers. However, from the results presented here it is not possible to determine whether or not the components are identical or whether both contain ligand binding sites. It is equally possible that a yet-to-be identified receptor subunit(s) exists in the 160,000-Da complex which includes one 85,000-Da *alpha$_1$*-receptor ligand binding subunit.

Target size analysis is the only technique for determining the functional molecular size of a membrane protein while it is still associated with the lipid bilayer. This technique has been employed by our laboratory for the analysis of the structure of the muscarinic cholinergic receptor (4), the *beta$_2$*-adrenergic receptor (15), the D_2-

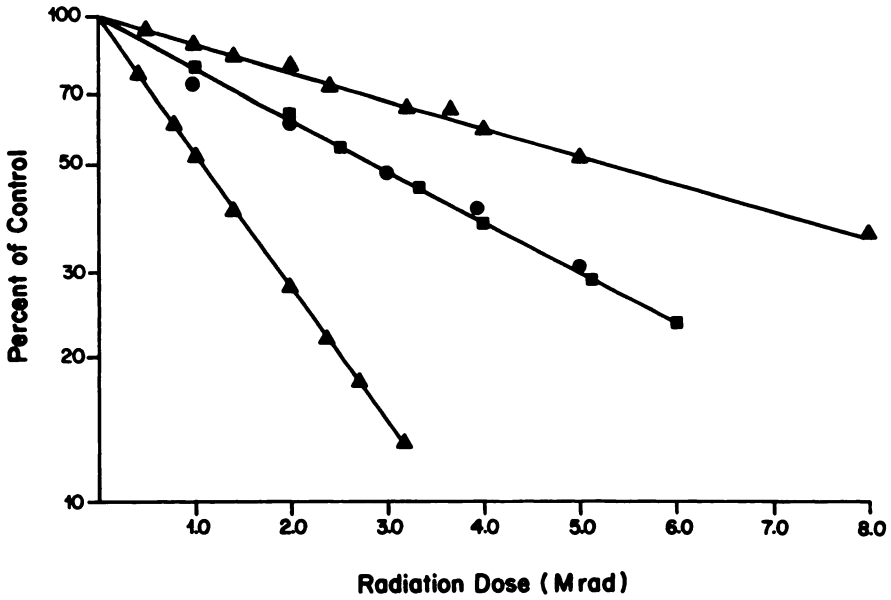

FIG. 8. *Radiation inactivation-target size analysis of $alpha_1$-adrenergic receptors and standard enzymes*

Rat liver membranes were purified and frozen in thin layers in aluminum trays along with enzyme standards and subjected to high-energy electron bombardment as described under Experimental Procedures. Radiation-induced loss of $alpha_1$-receptors was assessed by measuring [^{3}H] POB and [^{3}H]prazosin specific binding over a wide variety of ligand concentrations both above and below the K_d or apparent K_d of each ligand (Fig. 4). ●, The survival of $alpha_1$-receptor specific binding. The data points represent the averages of triplicate determinations with the two ligands and five ligand concentrations from three radiation inactivation experiments. Inactivation of the *alpha*-receptor is compared on the same plot with inactivation of horse liver alcohol dehydrogenase (80 kDa) (*top curve*, ▲), yeast alcohol dehydrogenase (160 kDa) (■), and β-galactosidase (464 kDa) (*lower curve*, ▲).

Lines were drawn by least-squares linear regression; molecular weight values were calculated as shown in Fig. 9.

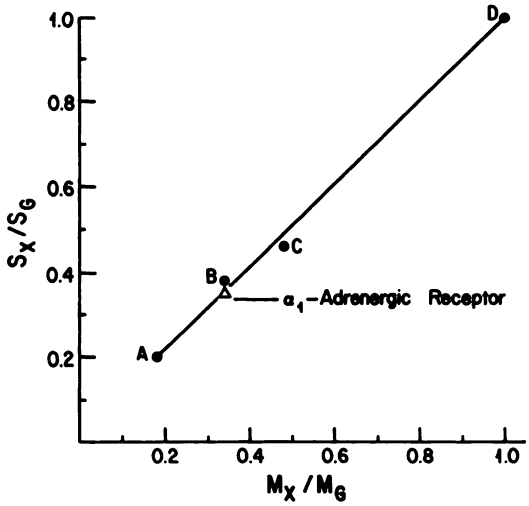

FIG. 9. *Radiation inactivation molecular weight calibration curve*

Standard enzymes [A, horse liver alcohol dehydrogenase, 84,000 Da, B, yeast alcohol dehydrogenase 160,000 Da, C, pyruvate kinase, 224,000 Da;. and D, *Escherichia coli* β-galactosidase, 464,000 Da (9)] were inactivated individually, in groups, and with membranes with identical results, and their inactivation ratios were determined. The least-squares slope of the line is 0.97. The $alpha_1$-adrenergic receptor (Δ) was related to the standard plot by determining the inactivation ratio $S_{\alpha\text{-receptor}}/S_{\text{yeast ADH}}$ or $S_{\alpha\text{-receptor}}/S_G$, which permits a direct comparison to the enzyme standard plot of the relationship between the inactivation ratio S_X/S_G and molecular weight ratio M_X/M_G. The inactivation ratio of the *alpha*-receptor was related to the standard plot by $S_X/S_G = (S_X/S_{ADH}) \cdot (S_{ADH}/S_G)$. These data indicate a molecular mass of 160,000 Da for the rat liver $alpha_1$-adrenergic receptor.

TABLE 2

Molecular properties of $alpha_1$-adrenergic receptors

Subunit molecular mass (Da) ($NaDodSO_4$-polyacrylamide gel electrophoresis)	85,000 ± 5,500
Membrane molecular mass (Da) (target size)	160,000
Stokes radius	6.0 nm (3)[a]
Isoelectric point (p_I)	3.9 (14)[a]
"Putative" receptor structure	Dimer

[a] Numbers in parentheses are references.

dopamine receptor (17), and the slow inward calcium channel (8) and by others for the nicotinic cholinergic receptor (9) and hormone-activated adenylate cyclase (19).

In this present study, target size analysis of $alpha_1$-adrenergic receptors demonstrated that the 85,000-Da protein (Fig. 1) is derived from a 160,000-Da complex in the intact membrane (Figs. 7–9). These data are in dramatic contrast to results from a similar study on the muscarinic cholinergic receptor (4), where the molecular mass of the receptor determined from $NaDodSO_4$-gels (80,000 Da) agrees exactly with the size of the membrane-bound receptor as determined by radiation inactivation. Previous studies have demonstrated that target size analysis can provide information concerning the oligomeric structure of a protein (8, 9, 16); however, this may depend upon having either a covalent linkage (e.g., di-

sulfide bond) or an extremely close physical proximity between interacting subunits for them to inactivate as the oligomeric structure (10).

Although earlier studies have suggested a lower molecular weight for the *alpha*$_1$-receptor (2, 15), our data indicate that the 45,000- and 58,000-Da peptides are probably proteolytic fragments of the intact 85,000-Da *alpha*$_1$-receptor monomer (Figs. 2–4). The predominant proteolytic activities found in rat liver are associated with trypsin, chymotrypsin, elastase, and cathepsin (20), enzymes inhibited by the protease inhibitors we add during the membrane preparations. Limited proteolysis of the isolated 85-kDa protein by papain, trypsin, or chymotrypsin produced receptor fragments (Fig. 4) that correspond to those found in various preparations of liver membranes from the action of endogenous proteases (Fig. 2). This situation with the *alpha*$_1$-adrenergic receptor is very similar to that of other receptor proteins, where the first peptides identified were subsequently shown to be proteolytic fragments of the intact receptor (4). Indeed, it is conceivable that the 85,000-Da subunit reported here could be a rapidly liberated proteolytic fragment of the 160-kDa receptor identified by radiation inactivation analysis, although no evidence exists at present to support this idea.

Limited proteolysis of affinity-labeled membrane-bound *alpha*$_1$-adrenergic receptors (Fig. 10; Table 3) indicates that a considerable portion of the *alpha*$_1$-receptor monomer protrudes from the plasma membrane into the aqueous environment. These results are similar to those found for other integral membrane proteins, such as the nicotinic acetylcholine receptor, where 50% of the receptor protrudes from the membrane into the extracellular

TABLE 3

Protease-produced water-soluble fragments from the membrane-bound alpha$_1$*-adrenergic receptor*

	Specific cpm			
	Control	Trypsin	Chymotrypsin	Papain
Membrane-bound	26,427	18,200	12,126	11,800
Water-soluble fraction	3,340	7,600	13,640	14,820

Rat liver *alpha*$_1$-adrenergic receptors were covalent affinity-labeled with 1 nM [^{3}H]POB as described under Experimental Procedures in the presence and absence of 0.1 μM prazosin, washed three times by centrifugation (42,000 × *g*) for 20 min, and resuspended at 37° in fresh buffer prior to recentrifugation. Affinity-labeled membranes (4 mg/ml) were incubated with the indicated enzyme (25 μg/ml) in a final volume of 1 ml for 30 min at 37°. Reactions were terminated by centrifugation (42,000 × *g*) at 4° for 20 min. Aliquots (100 μl) were counted from the supernatant fluid and from the resuspended pellets. Values shown are the average of triplicate determinations. This experiment was repeated three times with the same results. The water-soluble material was analyzed by NaDodSO$_4$-polyacrylamide gel electrophoresis (Fig. 10).

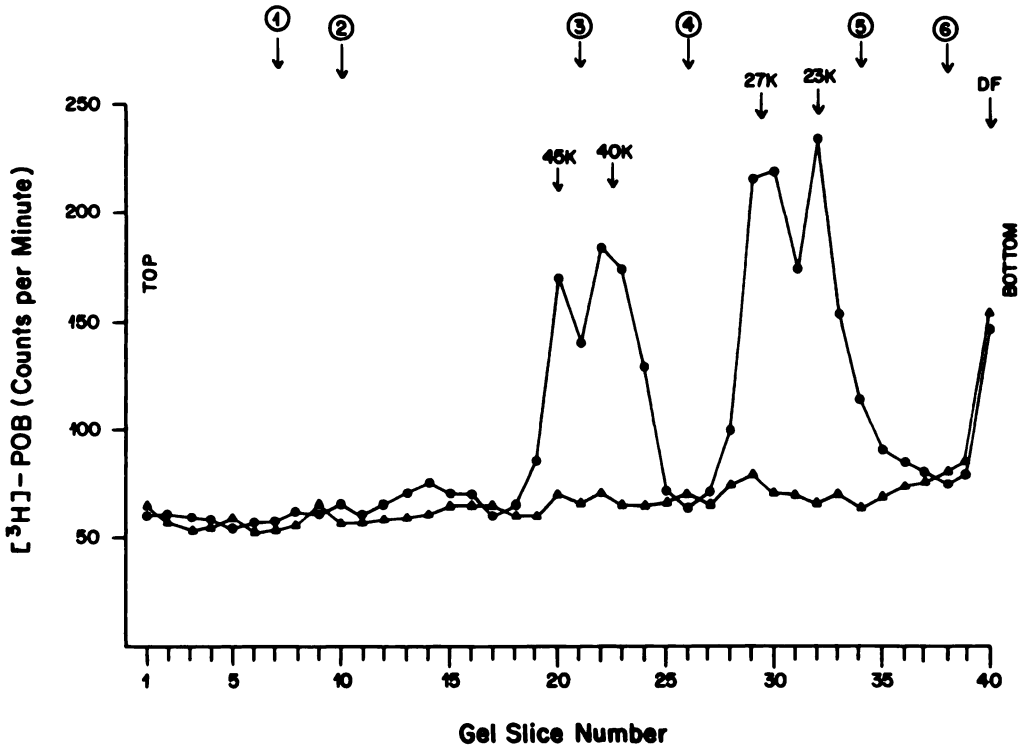

FIG. 10. *Determination of the extracellular domain of the alpha*$_1$*-adrenergic receptor*

Rat liver membranes were labeled with [^{3}H]POB in the presence (▲) or absence (●) of 0.1 μM prazosin and extensively washed. The affinity-labeled membranes were subjected to chymotrypsin digestion (25 μg/ml) for 30 min at 37°. Incubations were terminated by centrifugation at 42,000 × *g* for 20 min at 4°, and the supernatant fluid was treated at 100° for 5 min in 2% NaDodSO$_4$ and 5% β-mercaptoethanol. Chymotrypsin-solubilized (water-soluble) receptor fragments were subjected to NaDodSO$_4$-polyacrylamide gel electrophoresis. Gels were sliced and counted as described in legend to Fig. 1. Protein standards are as in Fig. 2. The water-soluble proteolytic fragments contain the ligand-binding site of the *alpha*$_1$-receptor and therefore represent that portion of the receptor exposed to and contained in the extracellular aqueous environment.

space and approximately 14% protrudes from the cytoplasmic side of the membrane (21). A previous study from this laboratory demonstrated that approximately 50% of the muscarinic cholinergic receptor extends from the membrane into the extracellular environment (4).

Studies utilizing polymeric-immobilized epinephrine (15, 22) have shown that the ligand-binding site of the rat liver $alpha_1$-adrenergic receptor is on the outer surface of the plasma membrane. [^{3}H]POB, which is highly $alpha_1$-receptor-selective (3), interacts covalently with the ligand-binding site of the $alpha_1$-receptor. Because of this specific interaction, proteolytic fragments produced from the receptor can be analyzed with reference to the ligand binding site (4).

Limited proteolysis of $alpha_1$-receptors in membranes, either by endogenous or added proteases, most readily reduces the receptor size from 85 kDa to 58–62 kDa. More prolonged protease activity will produce lower molecular weight fragments (Figs. 2, 3, and 9). The 58,000- to 62,000-Da fragments require detergents for liberation from the membrane and therefore appear to contain the membrane domain sequence of the $alpha_1$-receptor in addition to the ligand binding site. This is again analogous to the muscarinic cholinergic receptor (4), where a 58,000- to 64,000-Da peptide containing the membrane domain and extracellular domain is readily produced from the intact 80,000- Da muscarinic receptor by limited proteolysis of membranes.

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Send reprint requests to: Dr. J. Craig Venter, Department of Molecular Immunology, Roswell Park Memorial Institute, 666 Elm Street, Buffalo, N.Y. 14263.