0026-895X/90/040526-09$02.00/0

MOLECULAR PHARMACOLOGY, 37:526-534

Identification and Structural Characterization of α_1-Adrenergic Receptor Subtypes

B. I. TERMAN,[1] R. P. RIEK, A. GRODSKI, H.-J. HESS, and R. M. GRAHAM

Cardiac Unit, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114 (B.I.T., R.P.R., R.M.G.), and Medicinal Chemistry, Pfizer Central Research, Groton, Connecticut 06340 (A.G., H.-J.H.)

Received June 1, 1989; Accepted January 8, 1990

SUMMARY

Rat liver and brain membrane α_1-adrenergic receptors were purified >500-fold by successive chromatographic steps using heparin-agarose, an affinity matrix constructed by coupling a novel derivative of the α_1-selective antagonist prazosin to Affigel-102 and wheat germ agglutinin-agarose. Several lines of evidence were obtained for the existence in brain of an α_1-adrenergic receptor subtype that is structurally distinct from that previously characterized in liver and other tissues using photoaffinity labeling, protein purification, and DNA cloning techniques. The α_1-selective ligand chlorethylclonidine (CEC) (an alkylating agent) irreversibly inactivates 100% of [^{3}H]prazosin binding sites in partially purified preparations of rat liver. Under identical conditions, only 50% of brain receptors are irreversibly inactivated. Computer modeling of data obtained from the competition by the α antagonists WB4101 and phentolamine for [^{3}H]prazosin binding to partially purified preparations of rat liver is best fit by assuming a single class of low affinity sites for both ligands. However, analysis of partially purified brain preparations indicates the presence of two binding sites with different affinities for these antagonists. Additionally, prior alkylation of brain receptors with CEC results in the loss of low affinity phentolamine and WB4101 binding sites. The CEC-insensitive site in brain, which displays high affinity for phentolamine and WB4101, is resistant to photoaffinity labeling by [^{125}I]azidoprazosin. This is not due to a markedly lower affinity of the CEC-insensitive sites for the photoaffinity label, because competition studies with [^{127}I]azidoprazosin revealed a single class of high affinity sites in partially purified brain samples. Photoaffinity labeling of partially purified liver and brain samples not treated with CEC results in the specific labeling of a single protein of M_r 80,000. No specifically labeled protein is observed for partially purified brain samples that had previously been incubated with CEC. Treatment of photoaffinity-labeled liver and brain receptors with *N*-glycanase to cleave *N*-linked oligosaccharides results in a single M_r 55,000 protein. Taken together, these data provide evidence for the existence of a single receptor subtype (α_{1b}) in rat liver and for two subtypes (α_{1a} and α_{1b}) in rat brain. Furthermore, the insensitivity of the α_{1a} subtype to CEC and the resistance of the α_{1a} subtype to covalent labeling by an α_{1b}-selective photoaffinity probe suggest that the primary structures of the two receptor subtypes differ, such that an amino acid(s) in the α_{1b} subtype that incorporates CEC and the photoaffinity label is lacking in the α_{1a} subtype.

The following model (reviewed in Refs. 1 and 2) has evolved to describe how catecholamines activate cells at α_1-adrenergic receptors. Binding of agonist to the receptor leads to signal transduction across the membrane, resulting in activation of a phosphatidylinositol 4,5-bisphosphate-specific phospholipase C (1, 2). This signalling process involves an as yet unidentified guanine nucleotide-binding protein (3–6). Activation of phospholipase C leads to the generation of IP_3 and diacylglycerol, which act as second messengers (7–10). IP_3 mobilizes intracellular calcium (2, 7, 8) and diacylglycerol activates protein kinase C (9). Recent studies, however, suggest that α_1-adrenergic receptor signalling may be more complex than that suggested by this model. For example, stimulation of IP_3 formation by the α_1 agonist epinephrine is difficult to observe in some cells, and the time course of IP_3 production is not consistent with that for cellular activation (10). Other studies have identified early cellular events (other than production of IP_3), such as activation of phosphatidylcholine-specific phospholipase D (11) and activation of phospholipase A_2 (12, 13). In addition, it has been

This work was supported in part by National Institutes of Health Grants NS-19583 and HL-33107 and a Grant-In-Aid from the American Heart Association (86-1197) with funds from the Massachusetts Affiliate.

[1] Present address: Division of Protein Chemistry, Lederle Laboratories, Pearl River, NY 10965.

ABBREVIATIONS: IP_3, inositol-1,4,5-trisphosphate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EGTA, ethylene glycol-bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; WB4101, *N*-[2-(2,6-dimethoxyphenoxy)ethyl]-2,3-dihydro-1,4-benzodioxin-2-methanamine hydrochloride; WGA, wheat germ agglutinin. CP86,224, 1-(4-amino-6,7-dimethoxyquinazolin-2-yl)-4-(9-carboxynonanoyl) piperazine; [^{125}I]azidoprazosin, 2-[4-(4-azido-3-[^{125}I]iodobenzoyl)piperazin-1-yl]-4-amino-6,7-dimethoxyquinazoline; NH_4-prazosin, 2-[4-(4-aminobenzoyl)piperazin-1-yl]-4-amino-6,7-dimethoxyquinazoline; [^{127}I]azidoprazosin, 2-[4-(4-azido-3-[^{127}I]iodobenzoyl)piperazin-1-yl]-4-amino-6,7-dimethoxyquinazolin; CEC, chloroethylclonidine; CP86,225, 1-(4-amino-6,7-dimethoxyquinazolin-2-yl)-4-(9-carbomethoxynonanoyl)piperazine.

suggested that in some cells hormones can activate plasma membrane calcium transport systems by mechanisms that appear to be independent of IP_3 (14).

These findings indicate that different effector enzymes are involved in α_1-adrenergic activation, depending upon either the cell type or appropriate physiological circumstances. There is also reason to believe that different receptor subtypes are involved. Morrow *et al.* (15) have shown that, based upon radioligand binding studies, there appear to be two classes of binding sites for the α antagonists phentolamine and WB4101 in brain membranes. Work from Minneman's laboratory (16, 17) has indicated that the alkylating agent CEC irreversibly inactivates over 90% of receptors in liver, but less than 60% in different regions of the brain. However, because each of the above-mentioned studies used membrane preparations, it is difficult to draw conclusions as to whether the heterogeneity in antagonist interactions with brain α_1 receptors is due to structurally distinct receptor subtypes or to differences in some other membrane component that mediates this heterogeneity in ligand recognition.

There has been recent progress in characterizing structural aspects of α_1-adrenergic receptors. Using receptor-selective photoaffinity labels, it has been observed that in liver (18, 19), heart (20), and smooth muscle cell lines (21) the receptor is a M_r 80,000 glycoprotein. The receptor has been purified from both liver (22) and hamster DDT_1 smooth muscle cells (23), and the receptor from DDT_1 cells has been cloned and its primary structure deduced from nucleotide sequence data (24). Liver and DDT_1 cells contain exclusively the low affinity site for WB4101 and phentolamine (Fig. 3 for liver; Ref. 24 for DDT_1 cells), and CEC irreversibly inactivates greater than 90% of [^{3}H]prazosin binding sites in these cells.[2] Experimental approaches, such as photoaffinity labeling and protein purification, for the study of α_1 receptor structure have not been applied to tissues, such as brain, that contain the high affinity site for phentolamine and WB4101 and are insensitive to CEC treatment.

In view of the recent evidence suggesting heterogeneity in α_1 receptor function, we set out to examine structural aspects of these receptors in brain, a tissue that demonstrates the highest degree of heterogeneity to receptor inactivation by CEC. In the study described in this manuscript, we used protein purification and photoaffinity labeling techniques towards the goal of identifying and structurally characterizing α_1-adrenergic receptor subtypes.

Experimental Procedures

Materials. [^{125}I]Azidoprazosin and [^{127}I]azidoprazosin were synthesized as described previously (18). [^{3}H]Prazosin was from New England Nuclear (Boston, MA). Digitonin was from Gallard-Schlessinger (Carle Place, NY). Prazosin was a gift from Pfizer Central Research (Groton, CT). Phentolamine was from CIBA-GEIGY (Basle, Switzerland). CEC and WB4101 were from Research Biochemicals, Inc. (Natick, MA). Heparin- and WGA-agarose were from Sigma (St. Louis, MO). Affigel-102 and chemicals for SDS-PAGE were from Bio-Rad (Richmond, CA). *N*-Glycanase was given Genzyme (Cambridge, MA). All other reagents were from standard sources.

Synthesis of the CP86,224-agarose affinity resin. To develop an affinity resin for the purification of the α_1-adrenergic receptor, an analog of the α_1-selective antagonist prazosin that could be readily coupled to an affinity matrix was synthesized. The synthesis of this compound (CP86,224) and the parent compound CP86,225) were performed as described below. The rationale for the synthesis of the CP86,224-agarose affinity resin is detailed in Results.

Construction of CP86,225. To a stirred solution of 1-(4-amino-6,7-dimethoxyquinazolin-2-yl)-4-piperazine (11.8 g, 0.0408 mol) in 300 ml of anhydrous dimethyl formamide, under a N_2 atmosphere, was added sebacic acid monomethyl ester (8.8 g, 0.0407 mol), followed by 1-hydroxybenzotriazole hydrate (8.3 g, 0.0614 mol). The resulting solution was stirred for 15 min and then 1,3-dicyclohexylcarbodiimide (9.9 g, 0.048 mol) was added. The reaction was stirred for 18 hr and poured onto ice water (2 1) to give, after filtration and drying, 206 g of crude product. Two chromatography steps on Brinkmann silica gel (230–400 mesh), eluting with a gradient of $CHCl_3$/ethanol, gave the desired material (12.1 g, 60.8%).

For analysis, a sample was further purified by chromatography on Brinkmann silica gel (230–400 mesh), eluting with a gradient of $CHCl_3$/ethanol. The resulting material was recrystallized from ethyl acetate, m.p. 165–168°. Analysis for $C_{25}H_{37}N_5O_5$: calculated, 61.58% C, 7.65% H, 14.36% N; found, 61.69% C, 7.72% H, 14.17% N. Mass spectrum, (m/z): Found, 487.2791 (M^+); calculated, 487.2795. UV (methanol): λ_{212} (ϵ = 21,484), λ_{250} (ϵ = 58,834), λ_{275} (ϵ = 15,069), λ_{342} (ϵ = 6,497). IR (KBr): 1740 cm^{-1}, 1630 cm^{-1} (C = O). Thin layer chromatography (E. Merck silica gel 60) (CH_3CN/H_2O, 9:1): R_F = 0.44.

Construction of CP86,224. To a stirred solution of CP86,225 (7.0 g, 0.014 mol) in 50 ml of anhydrous methanol, under a N_2 atmosphere, was added 1 M KOH/methanol (100 ml, 0.1 mol). The resulting solution was stirred for 0.5 hr and then refluxed for 1 hr. The reaction was diluted with 50 ml of water and extracted with CH_2Cl_2 (2 × 50 ml). The aqueous layer was acidified to pH 6 with dilute hydrochloric acid, and the resulting precipitated solid was filtered and washed several times with water to give 5.3 g (77.7%) of crude product.

For analysis, a sample was further purified by chromatography on Brinkmann silica gel (230–400 mesh), eluting with a gradient of $CHCl_3$/ethanol. The resulting material was recrystallized from isopropanol, m.p. 154–157°. Analysis of $C_{24}H_{35}N_5O_5$: calculated, 60.87% C, 7.45% H, 14.79% N; found, 60.48% C, 7.60% H, 14.44% N. Mass spectrum (m/z): found, 473.2646 (M^+); calculated, 473.2638. UV (methanol): λ_{212} (ϵ = 21,705), λ_{249} (ϵ = 50,353), λ_{343} (ϵ = 6.727). IR (KBr): 1635 cm^{-1}, 1695 cm^{-1} (C = O). Thin layer chromatography (E. Merck silica gel 60) (CH_3CN/H_2O, 5:1): R_F = 0.34. Coupling of CP86,224 to Affigel-102 and quantitation of the degree of substitution were performed essentially as described previously (22, 25).

Membrane preparation and solubilization. Unless otherwise stated, all steps were done at 4°. Livers and brains were obtained from lactating Sprague-Dawley rats and placed into buffer A, which consisted of 300 mM sucrose, 10 mM Tris·HCl, pH 7.4, 5 mM $MgCl_2$, and 2.5 mM EGTA. Protease inhibitors (0.02% phenylmethylsulfonyl fluoride, 2 μg/ml bacitracin, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, and 2 μg/ml soybean trypsin inhibitor) were included in all steps of the purification. The tissues were washed several times with buffer A to remove blood cells, minced, and homogenized. The homogenates (25 ml/g of tissue) were centrifuged at 300 × g for 10 min and the supernatants were harvested and centrifuged at 45,000 × g for 30 min. The resulting pellets were suspended in buffer B, which consisted of 10 mM Tris·HCl, pH 7.4, 2.5 mM EGTA, 250 mM NaCl, 50 μM epinephrine, 500 μM ascorbic acid, and protease inhibitors. The pellets were washed three times in buffer B and finally suspended in solubilization buffer, which consisted of 1% (w/v) digitonin (the ratio of digitonin to protein was 2:1 by weight), 10 mM Tris·HCl, pH 7.4, 250 mM NaCl, 2.5 mM EGTA, 50 μM epinephrine, 500 μM ascorbic acid, 25% glycerol, and 3 times the concentration of protease inhibitors listed above. After a 1-hr incubation at 4°, the sample was diluted 1:2.5 with 10 mM Tris·HCl, pH 7.4, 2.5 mM EGTA, and centrifuged at 45,000 × g for 30 min. The supernatant was collected for subsequent chromatography.

Partial purification of α_1-adrenergic receptors. The solubilized

[2] B. I. Terman, P. R. Riek, and R. M. Graham; unpublished results.

liver extract was applied directly to heparin-agarose (20 ml of sample/ml of heparin-agarose). The brain extract was diluted to 60 mM NaCl with 10 mM Tris·HCl, 2.5 mM EGTA, 10% glycerol, plus protease inhibitors, and then applied to the column. The samples were loaded at a flow rate of 30 ml/hr in a 4° cold room. The column was washed with 5 column volumes of 0.1% digitonin, 10 mM Tris·HCl, 2.5 mM EGTA, 100 mM NaCl (for liver) or 60 mM NaCl (for brain), plus protease inhibitors. The column was eluted with 0.1% digitonin buffer containing 700 mM NaCl. The α_1 receptor eluted as a sharp peak after 1 column volume.

The heparin eluate was applied to an affinity column consisting of an analog of the α_1-selective antagonist prazosin (see Fig. 1) coupled to Affigel-102. Typically, 9 ml of the heparin eluate were applied to 3 ml of affinity resin. This chromatographic step was done at room temperature in the following manner. One milliliter of ice-cold heparin eluate was applied by gravity to the affinity column, which was at room temperature. Ten minutes later, another 1 ml of heparin eluate was applied; this was repeated until the entire sample was loaded onto the column. Typically, 70% of the receptor was adsorbed to the column. The resin was placed into a 4° cold room; 30 min later, it was washed with 30 ml of 0.1% digitonin buffer containing 700 mM NaCl. The column was put at room temperature; 30 min later, it was washed with 3 column volumes of 0.1% digitonin plus 700 mM NaCl. The column was then eluted with this buffer, containing 0.1 mM phentolamine, at a flow rate of 1 ml/5 min. The receptor typically eluted between 3 and 6 ml.

The eluate from the affinity column was mixed batchwise with 0.5 ml of WGA-agarose overnight at 4°. The resin was poured into a column and then washed with 30 ml of 0.1% digitonin buffer plus 700 mM NaCl. The column was eluted with this buffer containing 300 mM *N*-acetylglucosamine; the receptor eluted between 0.4 and 0.8 ml. In all experiments, 100% of the receptor was adsorbed to the resin at this step.

For the experiments described in Table 3, brain receptors (Table 3, row D) were partially purified as follows. Heparin-agarose-purified receptors (5 ml), prepared as described above, were mixed batchwise overnight at 4° with 1.0 ml of WGA-agarose that had been previously equilibrated with 0.1% digitonin, 10 mM Tris·HCl, pH 7.4, 10% glycerol, 2.5 mM EGTA, 700 mM NaCl, plus protease inhibitors (0.1% digitonin buffer). The resin was poured into a column and washed with 30 ml of 0.1% digitonin buffer. The receptor was eluted with 0.1% digitonin buffer plus 300 mM *N*-acetylglucosamine; 1.0-ml fractions were collected. Fractions containing receptor were pooled. Following each purification protocol (Table 3, rows A–E), samples (80 fmol) were incubated for 19 hr at 4°, with or without 50 μM CEC, in a total volume of 200 μl. The samples were passed over a G-50 desalting column that had been previously equilibrated with 0.1% digitonin buffer. [^{3}H]Prazosin (3 nM) binding was determined in duplicate on the resulting samples, as described below.

[^{3}H]Prazosin binding. Binding assays were performed by incubating partially purified receptor (20 fmol) with 3 nM [^{3}H]prazosin and other drugs, in a total volume of 250 μl. Assays were initiated by addition of receptor and were carried out in buffer containing 0.1% digitonin, 10 mM Tris·HCl, 10% glycerol, 2.5 mM EGTA, 100 mM NaCl, and protease inhibitors. After a 1-hr incubation at room temperature, the samples were placed at 4° for 15 min. The samples were then applied to G-50 Sepharose desalting columns that had been previously equilibrated with sample buffer. The void volumes from the desalting columns were collected in liquid scintillation minivials. Ecoscint A liquid scintillation cocktail (6 ml; National Diagnostic) was added, and the samples were counted at an efficiency of 45%. Specific binding was defined as the difference in counts seen in the absence and in the presence of 10 μM prazosin. Nonspecific binding accounted for 10–15% of the total counts. When epinephrine was added, 0.5 mM ascorbic acid was included to prevent oxidation. The results shown are the results of duplicate determinations for each sample. Replicate samples did not vary by more than 10%. Protein content was measured by the method of Bradford (26).

Photoaffinity labeling and SDS-PAGE autoradiography. All steps were done in the dark. Solubilized receptor (40 fmol) was incubated with 1 nM [^{125}I]azidoprazosin for 1 hr at room temperature. Phentolamine (10 μM) was used to define nonspecific labeling. The samples were placed in an ice bath and 1.0 mM glutathione was added. The receptor was then photolyzed for 20 min with a hand-held long-wavelength UV mineral lamp. The incorporated probe was separated from free using a G-50 Sepharose column that had been previously equilibrated with H_2O. The samples were lyophilized before addition of 100 μl of gel sample buffer, which contained 50 mM Tris·HCl, pH 6.9, 4% (w/v) SDS, 7 M urea, 10% glycerol, and 0.02% (w/v) bromophenol blue. The samples were incubated at 37° for 1 hr and then loaded onto a 10% (w/v) acrylamide gel, which was prepared and run according to the method of Laemmli (27). Electrophoresis was performed at 200 mV constant voltage until the tracking dye reached the bottom of the gels, which were then dried with a gel dryer. Autoradiography was carried out by exposing Kodak XAR-5 film at −70°, using DuPont image-intensifying screens, typically for 3 days. Molecular weight standards (catalogue no. 6041LA; Bethesda Research Laboratory) were also loaded on the gels; the molecular weights of the photoaffinity-labeled proteins were calculated from the center of the bands on the autoradiographs.

***N*-Glycanase treatment.** Photolabeled receptors were treated with *N*-glycanase in the following manner. Receptors were photolabeled and lyophilized as described above. The samples were suspended in 50 μl of the 1.0% Nonidet P40, 0.1% SDS, 50 mM NaP_i, pH 8.6, 10 mM 2-mercaptoethanol, plus protease inhibitors, and heated to 90° for 5 min. The sample was aliquoted into two 20-μl samples; *N*-glycanase (0.15 unit) was added to one of these and both samples were incubated for 18 hr at 37°. At this time, 100 μl of gel sample buffer were added and SDS-PAGE and autoradiography were done as described above.

Data analysis. The dissociation constant (K_D) and maximum number of binding sites (B_{max}) were determined from Scatchard analysis (28) of saturation binding isotherms. The line of the Scatchard plot was fit by linear regression analysis. Competition binding curves were analyzed by a computer program (LIGAND) that performs iterative nonlinear regression (29). The statistical analysis of the data is given in the text and figure legends. Unless otherwise stated, all results shown are typical of those obtained for at least three independent experiments.

Results

Partial purification of α_1 receptor from liver and brain. An analog of the α_1-selective antagonist prazosin, CP86,224 (Fig. 1), was synthesized specifically to construct an affinity matrix for the purification of the receptor. The rationale for its design was that it contains a long (10-carbon) spacer arm extending from the ligand. Because the binding site on the β-adrenergic receptor appears to be within the membrane-spanning regions (30, 31), we reasoned that, if this is also the case for the α_1-adrenergic receptor, then a long hydrocarbon side chain would prevent steric hindrance between the receptor and the ligand on the Affigel-102 matrix. Moreover, Affigel-102 itself contains a 6-carbon spacer and allows coupling of the ligand via a stable ether linkage. An affinity column, constructed by coupling CP86,224 to Affigel-102, allowed the digitonin-solubilized α_1 receptor to be purified ≈60-fold when chromatography was done under conditions described in Experimental Procedures (Table 1).

CP86,224 competed for [^{3}H]prazosin binding to α_1 receptors on DDT_1 cell membranes with a dissociation constant of 25 nM. For comparison, the dissociation constant of prazosin determined with these membranes was 100 pM (data not

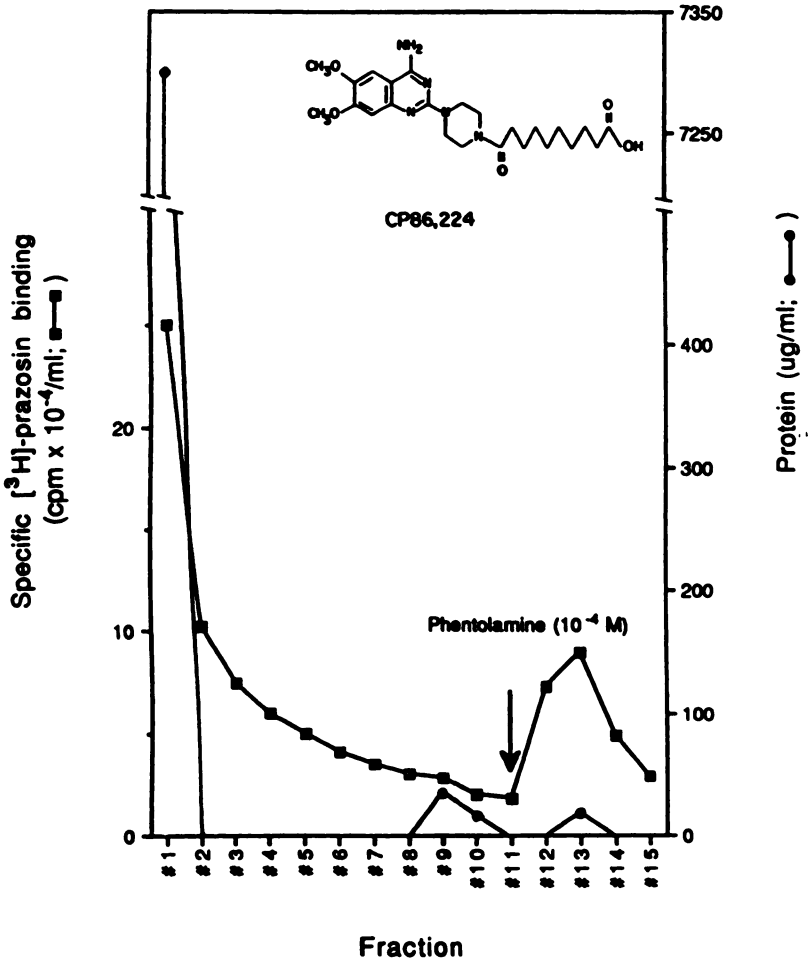

Fig. 1. Elution profile for CP86,224-Affigel 102 affinity chromatographic purification of rat brain α_1-adrenergic receptors. Rat brain membranes were solubilized with digitonin and purified using heparin-agarose chromatography and then the fractions containing the peak specific [^{3}H]prazosin-binding activity were applied to a CP86,224-Affigel 102 column and biospecifically eluted with phentolamine (10^{-4} M). After desalting, the eluted fractions were assayed for specific [^{3}H]prazosin-binding activity and protein concentration. These procedures were all performed as detailed in Experimental Procedures. *Inset*, structure of CP86,224 used for the synthesis of the affinity resin. Details of the synthesis of CP86,224 and of the affinity resin are given in Experimental Procedures.

TABLE 1

Summary of the purification of rat liver and brain α_1-adrenergic receptors

Fifteen rat brains and 10 livers were used. Details of the experimental protocols are given in Experimental Procedures. Values are representative of five independent purification procedures for both liver and brain.

Purification step	Recovered receptor	Recovery	Protein	Specific activity	Purification
	pmol	%	*mg*	*pmol/mg*	*fold*
Brain					
Digitonin extract	15.7		350	0.045	
Heparin-agarose	6.7	43	100	0.067	1.5
CP86,224-agarose	1.54	10	0.5	3.1	68
WGA-agarose	0.7	4.5	0.028	25	555
Liver					
Digitonin extract	170		1,377	0.12	
Heparin-agarose	95	56	180	0.525	4.3
CP86,224-agarose	18.3	11	0.6	30.5	250
WGA-agarose	8.8	5.2	0.11	80	650

shown). Because the long hydrocarbon spacer arms on CP86,224 and Affigel-102 may promote nonspecific hydrophobic interactions between the digitonin-solubilized proteins and the affinity column, a low salt (100 mM) wash of the column before elution was included. This step improved the purification of the receptor to 100-fold.

Table 1 summarizes the results for the partial purification of the α_1-adrenergic receptor from liver and brain. The greatest degree of purification was achieved when an affinity step using the CP86,224-Affigel-102 resin was employed. The elution profile for purification of brain α-adrenergic receptors using this affinity step is shown in Fig. 1. The recovery from this chromatographic step was somewhat low (20–35%); we suspect that the reason for this is denaturation of the receptor, because the step was done at room temperature. (Only 30% of the α_1 receptor in the heparin eluate was adsorbed to the resin when the sample was mixed batchwise with the resin overnight in the cold room.) The recovery was improved if the sample was applied quickly to the resin; for this reason, it proved useful to concentrate the solubilized receptor preparation using heparin-agarose, to reduce the loading time, before the affinity step.

The interaction of the soluble receptor with the different resins was very similar for the liver and brain receptors. The only chromatographic difference was that, whereas 100% of the liver receptor bound to heparin-agarose in 100 mM NaCl, only 50% of the brain receptor did so. All of the brain receptor bound to a heparin-agarose column in 60 mM NaCl (data not shown). No differences were observed in the degree of CEC sensitivity for digitonin-solubilized brain receptors that adsorbed to heparin-agarose in 100 mM NaCl, compared with receptors adsorbed in 60 mM NaCl (data not shown).

Interaction of antagonists with partially purified α_1-adrenergic receptors. In order to test the hypothesis that there exist subtypes of the α_1 receptor, we compared the interaction of antagonists with partially purified liver and brain receptors. In studies using membrane and tissue preparations, Minneman *et al.* (17) have reported differential sensitivity of

rat liver and brain α_1 receptors to the alkylating agent CEC. Fig. 2 shows that we have defined conditions for similar results using partially purified receptors. Incubating heparin-agarose-purified rat liver receptors at 4° for 16 hr with increasing concentrations of CEC resulted in the irreversible inactivation of increasing amounts of receptor; at 10 μM CEC, >90% of liver receptors were inactivated (Fig. 2). The loss in radioligand binding was due to irreversible inactivation of receptor binding sites and not to retained and unreacted CEC, because the alkylating agent was removed from the receptor sample (by desalting on G-50 columns) before radioligand binding. (If, instead of a 16-hr incubation, 50 μM CEC was added immediately before desalting, no loss in receptor number was observed.) Fig. 2 also shows the result of a similar experiment done with heparin-agarose-purified brain receptors. Under conditions that inactivate >90% of liver receptors, only 50% of brain receptors are irreversibly inactivated.

Although CEC irreversibly inactivates 50% of brain receptors, the addition of 50 μM CEC to binding assays completely blocks specific [^{3}H]prazosin binding to the receptors (data not shown). Also, 50 μM CEC completely blocks specific [^{3}H]prazosin binding to samples of brain receptors that have previously been irreversibly inactivated by the alkylating agent (data not shown). One explanation of these results is that CEC competitively blocks prazosin binding to both liver and brain receptors, but the site of alkylation is missing on 50% of the brain receptors.

Fig. 3 shows the results of competition for [^{3}H]prazosin binding by the α_1-adrenergic receptor antagonists WB4101 and phentolamine. For liver, computer analysis of radioligand binding to heparin-agarose-purified receptor can be fit by assuming a single class of low affinity binding sites for both antagonists, with dissociation constants of 4.3 nM for WB4101 and 48 nM for phentolamine (Fig. 3 and Table 2). In contrast, computer analysis for antagonist binding to partially purified brain receptors is best fit by assuming two sites, with a K_H = 0.059 nM (35% of receptors) and K_L = 5.4 nM (65% of receptors) for

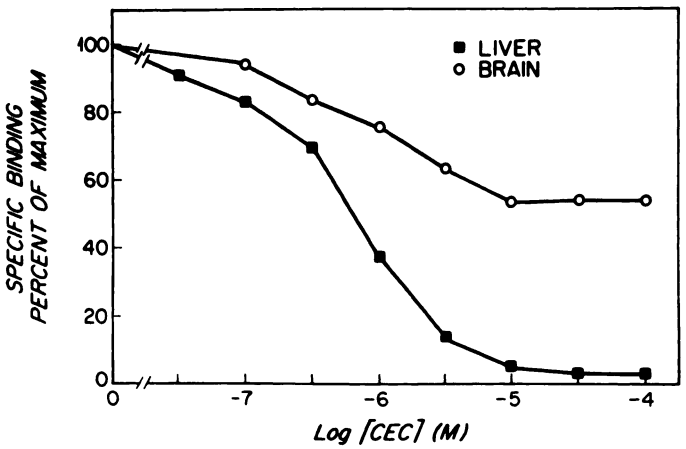

Fig. 2. Irreversible inactivation of partially purified rat liver and brain α_1 receptors by CEC. α_1-Adrenergic receptors from digitonin-solubilized rat liver (■) and brain (○) membranes were partially purified on heparin-agarose columns, as described in Experimental Procedures. Samples (80 fmol) were incubated for 19 hr at 4° with increasing concentrations of CEC, in a total volume of 200 μl. The samples were passed over a G-50 desalting column that had been previously equilibrated with 10 mM Tris·HCl, pH 7.4, 2.5 mM EGTA, 0.1% (w/v) digitonin, 10% (v/v) glycerol, plus protease inhibitors. [^{3}H]Prazosin (3 nM) binding was done on the resulting samples in duplicate, as described in Experimental Procedures.

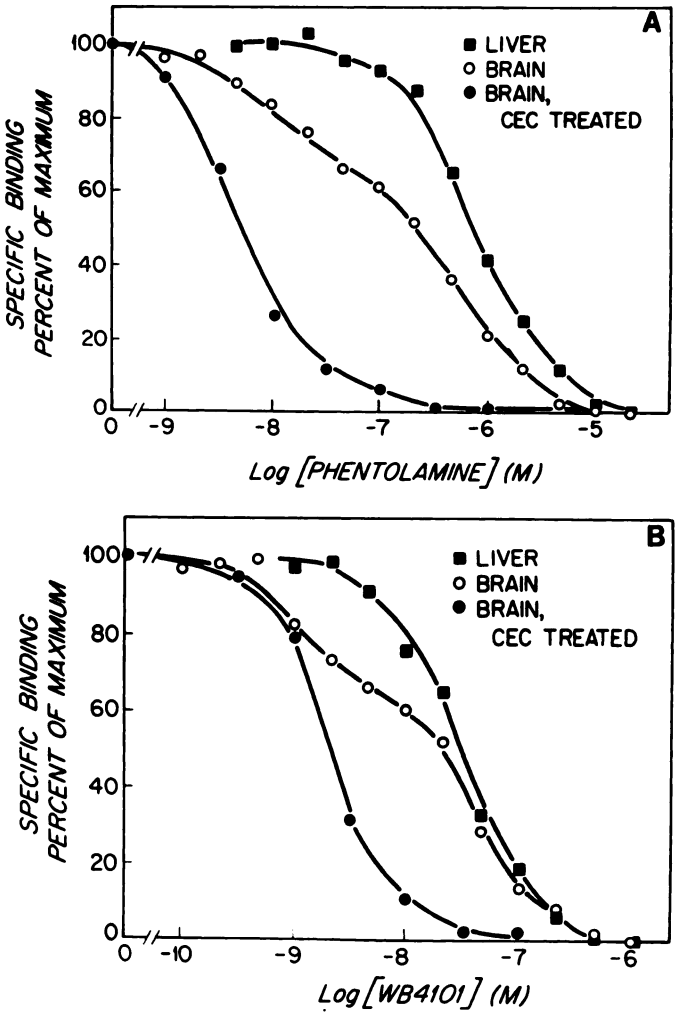

Fig. 3. Phentolamine (A) and WB4101 (B) competition of [^{3}H]prazosin binding to heparin-purified rat liver and brain α_1-adrenergic receptors. Digitonin-solubilized α_1 receptors from rat liver and brain were purified on heparin-agarose columns, as described in Experimental Procedures. Aliquots (400 fmol) of brain receptor preparations were incubated with 50 μM CEC for 19 hr and then desalted on G-50 columns to remove unincorporated CEC. Samples (20 fmol) of liver (■), brain (○), and CEC-treated brain (●) receptors were incubated in duplicate with 3 nM [^{3}H]prazosin in the presence of increasing concentrations of phentolamine or WB4101. Receptor-bound [^{3}H]prazosin was determined as described in Experimental Procedures. For brain samples not treated with CEC, the data were best fit by LIGAND to two binding sites, as described in the text and Table 2, whereas a one-site model best described the data from liver and CEC-treated brain samples.

WB4101 and K_H = 1.2 nM (34% of receptors) and K_L = 71 nM (66% of receptors) for phentolamine (Fig. 3 and Table 2). Both liver and brain receptors have a single class of binding sites for the agonist (−)-epinephrine and the antagonist prazosin (Table 2). Analysis of the results of competition binding studies using partially purified brain samples and [^{127}I]azidoprazosin revealed a single class of binding sites (K_D = 2.8 nM) for this ligand. Additionally, equilibrium binding studies using partially purified rat brain preparations and [^{3}H]prazosin revealed saturable binding to a single class of sites with a K_D = 0.4 nM and a binding site concentration of 64 fmol/ml. Binding to α_2 receptors of the α_{2b} subclass is unlikely, because the α_2 antagonists yohimbine and rauwolscine competed for [^{3}H]prazosin binding only with low affinity (K_D = 0.4 and 2.9 μM, respectively).

TABLE 2

WB4101, phentolamine, (−)-epinephrine, and NH_2-prazosin competition for [^{3}H]prazosin binding to α_1-adrenergic receptors

Partial purification of rat liver and brain α_1 receptors on heparin-agarose columns and competition by WB4101, phentolamine, (−)-epinephrine, and NH_2-prazosin for [^{3}H]prazosin binding to receptors were performed as described in Experimental Procedures and in the legend to Fig. 3.

Competing ligand/tissue	Dissociation constant[a]		
	K_H	K_L	K_D
		nM	
Phentolamine			
Brain, untreated	1.2 ± 1.1 (34%)	71 ± 9 (66%)	
Brain, CEC-treated			0.69 ± 0.17
Liver			48 ± 18
WB4101			
Brain, untreated	0.059 ± 0.049 (35%)	5.4 ± 1.3 (65%)	
Brain, CEC-treated			0.21 ± 0.04
Liver			4.3 ± 0.3
(−)-Epinephrine			
Brain, untreated			580 ± 70
Liver			830 ± 50
NH_2-Prazosin			
Brain, untreated			5.8 ± 0.9
Brain, CEC-treated			5.5 ± 0.8
Liver			3.4 ± 0.3

[a] Dissociation constants were determined from competition binding data using LIGAND (27). When the data was best fit to a two-site model, the dissociation constants for the high (K_H) and low (K_L) affinity sites are given. Values in parentheses are the fractions of high and low affinity sites, expressed as a percentage of the total sites. Data shown are the mean values ± standard errors of at least three separate studies for each drug.

TABLE 3

Irreversible inactivation of α_1-adrenergic receptors by CEC

α_1 Receptors for rows A, B, C, and E were partially purified as described in Experimental Procedures. The error for each of the values shown ranges from 11 to 15%.

Tissue	Purification protocol	Receptors Sensitive to CEC (%)
Liver	A. Heparin-agarose	>95
	B. As described in Table 1	>95
Brain	C. Heparin-agarose	50
	D. Heparin-agarose/WGA-agarose	60
	E. As described in Table 1	69

Fig. 3 shows that treatment of partially purified brain samples with CEC selectively inactivated the low affinity (for WB4101 and phentolamine) form of the receptor. Thus, computer analysis of WB4101 and phentolamine competition for [^{3}H]prazosin binding to samples of heparin-purified brain receptors (Fig. 3) that had been irreversibly inactivated with CEC could be best fit by assuming a single class of high affinity sites (Table 2). Previous studies in the literature have designated α_1 receptors that have low affinity sites for phentolamine and WB4101 as α_{1b} and receptors with high affinity sites for these antagonists as α_{1a} (14, 24). We will adopt this nomenclature for the remainder of the manuscript.

The results in Figs. 2 and 3 are from experiments using receptors that were partially purified on heparin-agarose columns. Liver receptors purified by the additional chromatographic steps listed in Table 1 display a sensitivity to CEC similar to that of heparin-purified receptors (Table 3, compare A and B). CEC-insensitive receptors were detected in brain preparations purified >500-fold, although at a lower percentage than receptors purified by heparin-agarose alone (Table 3), compare rows C and E.

Photoaffinity labeling of partially purified receptors with [^{125}I]azidoprazosin. We next sought to define the structural properties of both α_{1a}- and α_{1b}-adrenergic receptors, using the photoaffinity ligand [^{125}I]azidoprazosin. Digitonin-solubilized liver, brain, and heart α_1 receptors [heart being a tissue that contains a significant proportion of CEC-insensitive receptors (16)] were partially purified by successive chromatography on heparin- and WGA-agarose. Samples were photoaffinity labeled with [^{125}I]azidoprazosin, as described in the legend to Fig. 4A. It can be seen that a number of proteins are labeled under these conditions, but addition of prazosin blocks only a single M_r 80,000 protein in all three tissues. The legend to Fig. 4B describes experimental conditions that reduced the amount of nonspecifically labeled proteins in brain samples; under these conditions only the specifically labeled M_r 80,000 protein is visualized. Results similar to those shown in Fig. 4 are observed for receptors purified as described in Table 1 (Fig. 5 for brain; data not shown for liver). The fact that only a single specifically labeled protein is observed in brain might indicate that the molecular weight of α_{1a} is identical to that of α_{1b}, or, alternatively, that the α_{1a} receptor is not labeled.

In order to distinguish between these possibilities, we treated partially purified brain receptors (prepared as described in Table 1) with 50 μM CEC for 14 hr and then photolabeled the samples. Under these conditions, only the high affinity form of the receptor for phentolamine and WB4101 could be observed. No specifically labeled proteins were observed for these samples (data not shown). This finding does not conclusively prove that the α_{1a} receptors cannot be photoaffinity labeled, because it is possible that CEC alkylates a site on the α_{1a} subtype required for photoaffinity labeling but not for [^{3}H]prazosin binding. Other approaches were sought then to determine whether the α_{1a} form of the receptor could be photoaffinity labeled.

The α_{1b} receptor is a glycoprotein, and treatment of the photoaffinity-labeled receptor with *N*-glycanase results in a specifically labeled protein of M_r 52,000–55,000 (19–21). We reasoned then that, although the molecular weights of the α_{1a} and α_{1b} subtypes may be identical, the molecular weights of their protein backbones may differ and, thus, allow these subtypes to be distinguished. However, as shown in Fig. 5, only a single protein, of molecular weight 55,000 was evident after treatment of partially purified brain receptors with *N*-glycanase. Thus, either the α_{1a} subtype is not photolabeled or its peptide backbone is of a similar molecular weight to the α_{1b} receptor.

Taken together, our results obtained using photoaffinity labeling (i.e., only a single M_r 80,000 protein is specifically labeled in brain samples, prior incubation of brain receptor with CEC prevents specific labeling of the M_r 80,000 protein, and *N*-glycanase treatment of photolabeled brain samples results in the identification of only a single M_r 55,000 protein) suggest that the α_{1a} subtype is likely not photolabeled. It is possible that the amino acid(s) on the α_{1b} receptor where the photolabel incorporates differs in the α_{1a} receptor or that its affinity for [^{125}I]azidoprazosin is much lower in α_{1a} than α_{1b} receptors. Attempts to compare the affinity constant of [^{125}I] azidoprazosin for the two receptor subtypes proved difficult, because of the marked hydrophobicity and, thus, high nonspecific binding of the aryl azide radioligand. Nevertheless, CEC-

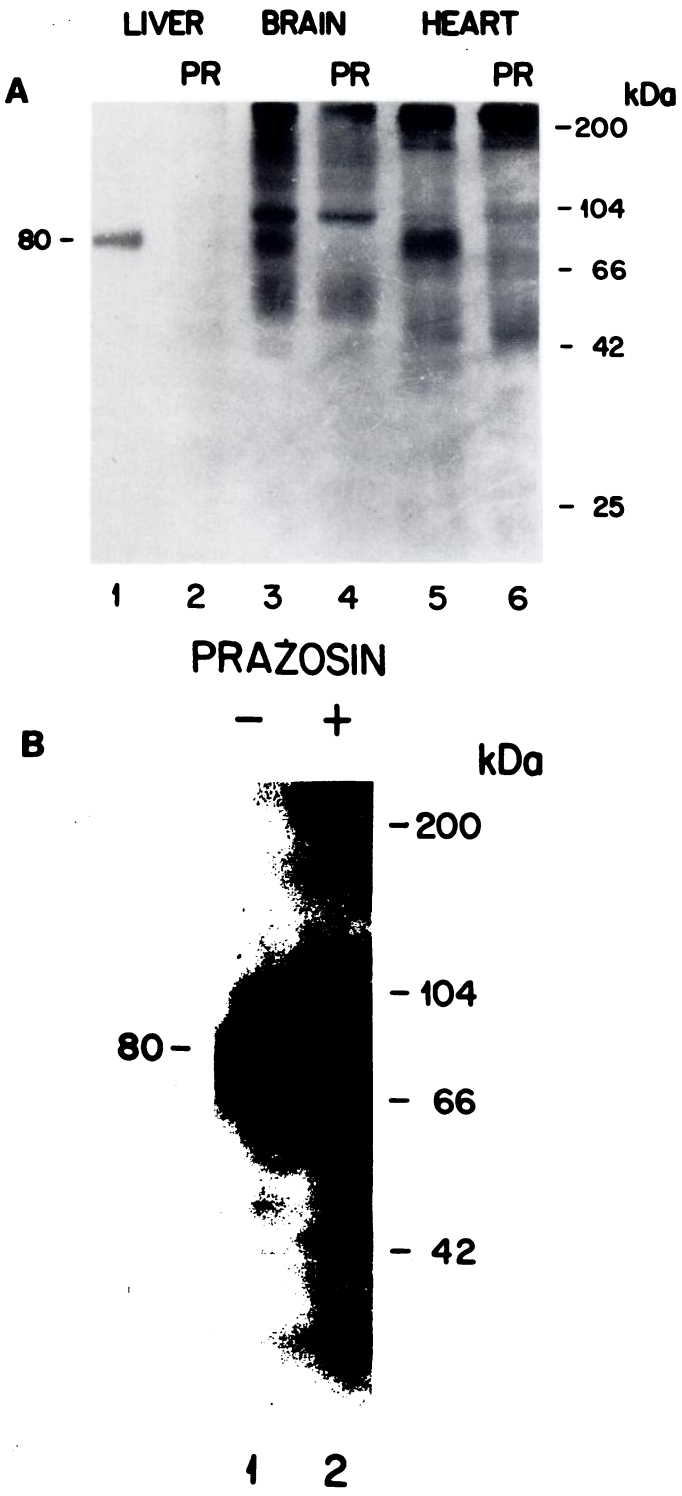

Fig. 4. A, Photoaffinity labeling of rat liver, brain, and heart α_1-adrenergic receptors. α_1-Adrenergic receptors from digitonin-solubilized rat liver (*lanes 1* and *2*), brain (*lanes 3* and *4*), and heart (*lanes 5* and *6*) membranes were purified by successive chromatography on heparin-agarose and WGA-Sepharose. Samples (40 fmol) of partially purified receptors were incubated with 0.5 nM [^{125}I]azidoprazosin in the presence (*lanes 2*, *4*, and *6*) or absence (*lanes 1*, *3*, and *5*) of 10 μM phentolamine, for 1 hr at room temperature in the dark. Photolysis and SDS-PAGE autoradiography were done as described in Experimental Procedures. The migration positions of molecular weight markers are indicated on the *right*. A specifically labeled M_r 80,000 protein is seen in all three tissues. *PR*, prazosin. B, Photoaffinity labeling of heparin-agarose-purified rat brain α_1-adrenergic receptors. α_1-Adrenergic receptors from digitonin-solubilized rat brain membranes were purified with heparin-agarose, as described in Experimental Procedures. Samples (40 fmol) were incubated with 1 nM [^{125}I]azidoprazosin in the presence (*lane 2*) or absence (*lane 1*) of 10 μM prazosin, for 1 hr at room temperature. The samples were put on ice for 15 min and 1 mM glutathione was added. The samples were then desalted by centrifugation through a dry G-25 column, that had previously been equilibrated with 0.1% (w/v) digitonin, 10 mM Tris·HCl, 2.5 mM EGTA, 10% (v/v) glycerol, plus protease inhibitors. Photolysis and SDS-PAGE autoradiography were done as described in Experimental Procedures. A specifically labeled M_r 80,000 protein is shown.

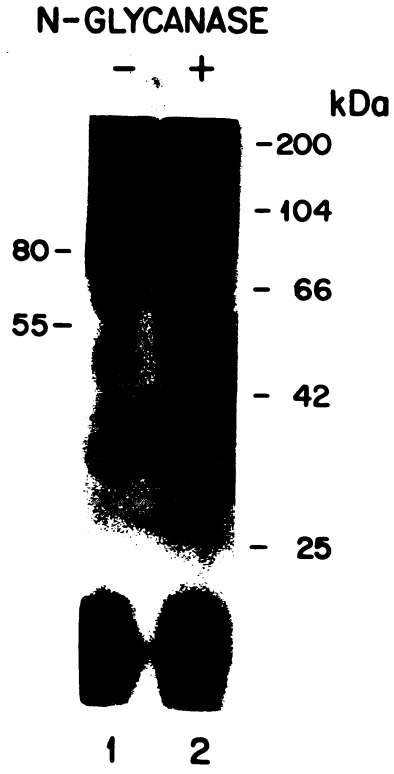

Fig. 5. Effect of *N*-glycanase on photoaffinity-labeled α_1-adrenergic receptors. α_1-Adrenergic receptors from digitonin-solubilized brain membranes were purified more than 500-fold, as described in Experimental Procedures and Table 2. Purified receptor (80 fmol) was photoaffinity labeled with [^{125}I]azidoprazosin, as described in Experimental Procedures. After photolysis, the sample was desalted against H_2O and lyophilized. The sample was resuspended in 50 μl of 1% Nonidet P-40, 0.1% SDS, 20 mM NaP_i, pH 8.5, 10 mM β-mercaptoethanol, plus protease inhibitors. The sample was then heated to 90° for 5 min and aliquoted into two 20-μl samples. *N*-Glycanase (0.12 units) was added to one sample (*lane 2*), and both samples were incubated for 18 hr at 37°. At this time, 100 μl of gel sample buffer were added. SDS-PAGE autoradiography was done as described in Experimental Procedures. A single M_r 80,000 labeled species was visualized in the sample not treated with *N*-glycanase, and a single M_r 55,000 labeled species is seen for the sample treated with *N*-glycanase.

treated brain (α_{1a} subtype) and liver (α_{1b} subtype) receptors recognized NH_2-prazosin (the parent compound used to synthesize the aryl azide photoaffinity label) with similar affinities (Table 2). Additionally, partially purified brain samples recognize [^{127}I]azidoprazosin with only a single class of binding sites (K_D = 2.8 nM).

Discussion

Differences in the sensitivity of liver and brain receptors to CEC provided evidence for structurally distinct receptor sub-

types. In samples that were partially purified to a similar extent, liver receptors were completely inactivated (loss of [^{3}H]prazosin binding) by prior treatment with the alkylating agent CEC, whereas 50% of brain receptors were insensitive to this treatment. The conclusion that the insensitive receptors represent a structurally distinct population of α_1 receptors from those in liver is supported by the finding that only a single high affinity site for WB4101 and phentolamine is found after CEC treatment. The finding that CEC-insensitive sites persist in the brain samples and that CEC-sensitive sites persist in the liver samples after >500-fold purification of both preparations provides additional evidence that structurally distinct forms of the receptor (receptor subtypes) exist and that the heterogeneity of ligand binding observed with membrane preparations is not due to differences in some other membrane component that interacts with the receptor.

The decreased proportion of CEC-insensitive receptors, relative to CEC-sensitive receptors, in the more purified brain samples is most likely due to a lower stability and thus loss of the α_{1a} subtype during the course of the purification protocols. It is not clear why the percentage of α_{1b} subtype receptors in brain determined by CEC sensitivity (50%) is greater than that determined by computer analysis of WB4101 and phentolamine competition (35%). It is possible that not all α_{1b} receptors are inactivated during CEC treatment, but no evidence for two binding sites for antagonists was obtained using LIGAND for brain samples treated with CEC (Table 2). Minneman *et al.* (17) also observed that the percentage of high affinity WB4101 and phentolamine binding sites in brain membranes is less than that predicted by their CEC sensitivity.

Our results represent the first report on the partial purification of the α_{1a} subtype, and the chromatographic properties observed provide insights on structural aspects of this receptor. The α_{1a} subtype must possess a number of structural similarities to the previously purified α_{1b} subtype, because they copurify through three chromatographic steps (Table 3). The interaction of the α_{1a} subtype with WGA indicates that this receptor is glycosylated. The oligosaccharide component of α receptors has been studied extensively, in both liver (19) and DDT$_1$ smooth muscle cells (19, 21). However, each of these cells contains receptors of the α_{1b} subtype (17, 24).

The photoaffinity ligand [^{125}I]azidoprazosin has in the past proven to be a valuable tool for the structural characterization of α_1 receptors. It is difficult to draw conclusions from previously published reports employing the probe as to the existence of receptor subtypes. Subtle molecular weight differences have been observed for receptors derived from different cell types, but heterogeneity in the glycan moiety could account for these molecular weight differences (21). The results from our current studies would indicate that conditions that readily label the α_{1b} subtype are not sufficient to label the α_{1a} receptor. Only a single M_r 80,000 protein was photoaffinity labeled in partially purified preparations of rat brain receptors, and no specifically labeled protein could be observed after treatment of brain samples with CEC. Treatment of photoaffinity-labeled brain receptors with *N*-glycanase resulted in a single M_r 55,000 protein; this is identical to results previously published for the effect of *N*-glycanase on photoaffinity-labeled α_{1b} receptors. Because the affinity of the α_{1b} receptor for both prazosin and NH_2-prazosin is identical to that of the α_{1a} subtype and because only a single class of sites are observed for [^{127}I]azidoprazosin with partially purified brain preparations, it is likely that the amino acid(s) at which the photoaffinity probe incorporates on the α_{1b} receptor is missing or different in the α_{1a} receptor.

TABLE 4

α_1-Adrenergic receptor subtype characteristics

Characteristic	Subtype	
	α_{1a}	α_{1b}
Tissue distribution	Brain/aorta/ vas deferens	Liver/spleen
GTP-binding protein	?	Gp[a]
Ligand binding		
WB4101	High affinity	Low affinity
Phentolamine	High affinity	Low affinity
Methoxamine activation	+++	+
CEC sensitivity	–	+++
Spare receptors	+	–
PI turnover[b]	–	+++
Ca^{2+} influx	+++	–
Glycosylation	++	++
M_r	?	80,000
[^{125}I]Azidoprazosin photolabeling	–	+++

[a] See also ref. 6.
[b] Phosphatidylinositol turnover.

The resistance of the α_{1a} subtype to photoaffinity labeling by [^{125}I]azidoprazosin is of interest, because work with aryl azides has generally assumed that, once generated, the reactive nitrene interacts rapidly and nonspecifically with its immediate environment, including aliphatic residues. However, as discussed by Richards and co-workers (32), aryl nitrenes may, in fact, not react rapidly on a molecular time scale, many having half-lifes in the millisecond time range, and insertion of an aryl nitrene into a aliphatic C—H bond is likely to be one of the slowest processes. Nitrenes are also less indiscriminate than carbenes in their reactions with primary, secondary, and tertiary C—H bonds and are somewhat electrophilic, preferring an O—H over a C—H bond (33). In fact, aryl azides frequently seek out and bond to tryptophan residues (30, 34), because the indole ring of this amino acid is an excellent donor for the formation of charge-transfer complexes with electrophilic nitrenes (35). In this regard, Wong *et al.* (30) recently demonstrated that the β-adrenergic photoaffinity reagent [^{125}I]iodoazidobenzylpindolol labels tryptophan^{330} in the β-adrenergic receptor. This residue is located in the extracellular half of the putative seventh transmembrane-spanning region and is in a position that is homologous to the site of attachment of retinal via a Schiff's base to lysine^{216} and lysine^{296} in bacteriorhodopsin (36) and rhodopsin (37), respectively. By analogy, one might speculate, therefore, that the α_{1a} subtype lacks an essential nucleophile, such as a tryptophan, that is present in the α_{1b} receptor.

The existence of α_{1a}-adrenergic subtypes raises many questions as to how this receptor subtype mediates cellular responses to catecholamines. The physiological responses, second messengers, and coupling mechanisms activated by binding of agonists to both the α_{1a} and α_{1b} receptors need to be clarified. Nevertheless, based on the findings of this study as well as the observations of others (38, 39), a number of distinct characteristics (Table 4) have now been identified that can aid in distinguishing between α_{1a} and α_{1b}-adrenergic receptor subtypes.

Acknowledgments

We thank Dr. C. J. Homcy for constructive comments and D. Duhamel and J. Holl for typing the manuscript.

References

1. Berridge, M. J. Inositol trisphosphate and diacylglycerol as second messengers. *Biochem. J.* **220**:345–360 (1984).
2. Graham, R. M., and S. M. Lanier. Identification and characterization of *alpha*-adrenergic receptors, in *The Heart and Cardiovascular System* (H. M. Fozzard, E. Haber, R. Jennings, A. Katz, and H. Morgan, eds.), Vol. 2. Raven Press, New York, 1059–1095 (1986).
3. Goodhardt, M., N. Ferry, P. Geynet, and J. Hanoune. Hepatic α_1-adrenergic receptors show agonist-specific regulation by guanine nucleotides. *J. Biol. Chem.* **257**:11577–11583 (1982).
4. Boyer, J. L., A. Garcia, C. Posadas, and J. A. Garcia-Sainz. Differential effect of pertussis toxin on the affinity state for agonists of renal α_1- and α_2-adrenoceptors. *J. Biol. Chem.* **259**:8076–8079 (1984).
5. Terman, B. I., S. R. Slivka, R. J. Hughes, and P. A. Insel. α_1-Adrenergic receptor-linked guanine nucleotide-binding protein in muscle and kidney epithelial cells. *Mol. Pharmacol.* **31**:12–20 (1987).
6. Im, M.-J., and R. M. Graham. Identification of a novel guanine nucleotide-binding regulatory protein (G-protein) coupled to the *alpha*$_1$-adrenergic receptor. *Clin. Res.* **37**:267A (1989).
7. Michell, R. H. Inositol phospholipids and cell surface receptor function. *Biochim. Biophys. Acta* **415**:81–147 (1975).
8. Putney, J. W. Formation and actions of calcium-mobilizing messenger, inositol 1,4,5-trisphosphate. *Am. J. Physiol.* **252**:G149–G157 (1987).
9. Nishizuka, Y. The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature (Lond.)* **308**:693–698 (1984).
10. Ambler, S. K., R. D. Brown, and P. Taylor. The relationship between phosphatidylinositol metabolism and mobilization of intracellular calcium elicited by *alpha*$_1$-adrenergic receptor stimulation in BC3H-1 muscle cells. *Mol. Pharmacol.* **26**:405–413 (1984).
11. Irving, H. R., and J. H. Exton. Phosphatidylcholine breakdown in rat liver plasma membranes: roles of guanine nucleotides and P_2-purinergic agonists. *J. Biol. Chem.* **262**:3440–3443 (1987).
12. Slivka, S. R., and P. A. Insel. α_1-Adrenergic receptor-mediated phosphoinositide hydrolysis and prostaglandin E_2 formation in Madin-Darby canine kidney cells: possible parallel activation of phospholipase C and phospholipase A_2. *J. Biol. Chem.* **262**:4200–4207 (1987).
13. Burch, R. M., A. Luini, and J. Axelrod. Phospholipase A_2 and phospholipase C are activated by distinct GTP-binding proteins in response to α_1-adrenergic stimulation in FRTL5 thyroid cells. *Proc. Natl. Acad. Sci. USA* **83**:7201–7205 (1986).
14. Han, C., P. W. Abel, and K. P. Minneman. α_1-Adrenoceptor subtypes linked to different mechanisms for increasing intracellular Ca^{2+} in smooth muscle. *Nature (Lond.)* **329**:333–335 (1987).
15. Morrow, A. L., G. Battaglia, A. B. Norman, and I. Creese. Identification of subtypes of [^{3}H]prazosin-labelled α_1 receptor binding sites in rat brain. *Eur. J. Pharmacol.* **109**:285–287 (1985).
16. Han, C., P. W. Abel, and K. P. Minneman. Heterogeneity of α_1-adrenergic receptors revealed by chlorethylclonidine. *Mol. Pharmacol.* **32**:505–510 (1987).
17. Minneman, K. P., C. Han, and P. W. Abel. Comparison of α_1-adrenergic receptor subtypes distinguished by chlorethylclonidine and WB4101. *Mol. Pharmacol.* **33**:509–514 (1988).
18. Seidman, C. E., H.-J. Hess, C. J. Homcy, and R. M. Graham. Photoaffinity labeling of the *alpha*$_1$-adrenergic receptor using an ^{125}I-labeled aryl azide analogue of prazosin. *Biochemistry* **23**:3756–3770 (1984).
19. Sawutz, D. G., S. M. Lanier, C. D. Warren, and R. M. Graham. Glycosylation of the mammalian *alpha*$_1$-adrenergic receptor by complex type *N*-linked oligosaccharides. *Mol. Pharmacol.* **32**:565–571 (1987).
20. Terman, B. I., and P. A. Insel. Photoaffinity labeling of α_1-adrenergic receptors of rat heart. *J. Biol. Chem.* **261**:5603–5609 (1986).
21. Terman, B. I., J. F. Reece, R. D. Brown, and P. A. Insel. The oligosaccharide component of α_1-adrenergic receptors from BC_3H_1 and DDT_1 muscle cells. *Biochem. J.* **253**:363–370 (1988).
22. Graham, R. M., H.-J. Hess, and C. J. Homcy. Biophysical characterization of the purified *alpha*$_1$-adrenergic receptor and identification of the hormone binding subunit. *J. Biol. Chem.* **257**:15174–15181 (1982).
23. Lomasney, J. W., L. M. F. Leeb-Lundberg, S. Cotecchia, J. W. Regan, J. F. DeBernardis, M. G. Caron, and R. J. Lefkowitz. Mammalian α_1-adrenergic receptor: purification and characterization of the native receptor ligand binding subunit. *J. Biol. Chem.* **261**:7710–7716 (1986).
24. Cotecchia, S., D. A. Schwinn, R. R. Randall, R. J. Lefkowitz, M. G. Caron, and B. K. Kobilka. Molecular cloning and expression of the cDNA for the hamster α_1-adrenergic receptor. *Proc. Natl. Acad. Sci. USA* **85**:7159–7163 (1988).
25. Parini, A., and R. M. Graham. Glycerol, sodium phosphate, and sodium chloride permit the solubilization and partial purification of rat hepatic α_1-receptors by 3-(3-cholamidylpropyl)-dimethylammonio-1-propanesulfonate. *Anal. Biochem.* **176**:375–381 (1989).
26. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
27. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature (Lond.)* **227**:680–685 (1970).
28. Scatchard, G. The attractions of proteins for small molecules and ions. *Ann. N. Y. Acad. Sci.* **51**:660–672 (1949).
29. Munson, P. J., and D. Rodbard. LIGAND: a versatile computerized approach for characterization of ligand-binding systems. *Anal. Biochem.* **107**:220–239 (1980).
30. Wong, S. K.-F., C. Slaughter, A. E. Ruoho, and E. M. Ross. The catecholamine binding site of the β-adrenergic receptor is formed by juxtaposed membrane-spanning domains. *J. Biol. Chem.* **263**:7925–7928 (1988).
31. Dixon, R. A. F., I. S. Sigal, E. Rands, R. B. Register, M. R. Candelore, A. D. Blake, and C. D. Strader. Ligand binding to the β-adrenergic receptor involves its rhodopsin-like core. *Nature (Lond.)* **326**:73–77 (1987).
32. Brunner, J., H. Senn, and F. M. Richards. 3-Trifluoromethyl-3-phenyldiazirine: a new carbene generating group for photolabeling reagents. *J. Biol. Chem.* **255**:3313–3318 (1980).
33. Bayley, H., and J. R. Knowles. Photoaffinity labeling. *Methods Enzymol.* **46**:69–114 (1977).
34. Brunner, J., and F. M. Richards. Analysis of membranes photolabeled with lipid analogues: reaction of phospholipids containing a disulfide group and a nitrene or carbene precursor with lipids and with gramicidin A. *J. Biol. Chem.* **255**:3319–3329 (1980).
35. Creighton, T. E. *Proteins: Structure and Molecular Properties.* W. H. Freeman and Company, New York (1984).
36. Bayley, H., K.-S. Huang, R. Radhakrishnan, A. H. Ross, Y. Takagaki, and H. G. Khorana. Site of attachment of retinal in bacteriorhodopsin. *Proc. Natl. Acad. Sci. USA* **78**:2225–2229 (1981).
37. Wang, J. K., J. H. McDowell, and P. A. Hargrave. Site of attachment of 11-*cis*-retinal in bovine rhodopsin. *Biochemistry* **19**:5111–5117 (1980).
38. Minnerman, K. P. α_1-Adrenergic receptor subtypes, inositol phosphates, and sources of cell Ca^{2+}. *Pharmacol. Rev.* **40**:87–119 (1988).
39. Tsujimoto, G., A. Tsujimoto, E. Suzuki, and K. Hashimoto. Glycogen phosphorylase activation by two different α_1-adrenergic receptor subtypes: methesamine selectively stimulates a putative α_1-adrenergic receptor subtype (α_{1a}) that couples with Ca^{2+} influx. *Mol. Pharmacol.* **36**:166–176 (1989).

Send reprint requests to: Robert M. Graham, M.D., Department of Heart and Hypertension Research, Cleveland Clinic Foundation/FF3, 9500 Euclid Avenue, Cleveland, OH 44195.