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Photoaffinity Cross-Linking of a Radioiodinated Probe, ^{125}I-A55453, into *Alpha*$_1$-Adrenergic Receptors

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SUMMARY

We have synthesized and characterized a high-affinity *alpha*$_1$-adrenergic receptor probe, 4-amino-6,7-dimethoxy-2[4′-[5″-(3‴-^{125}I-iodo-4‴-aminophenyl)pentanoyl]-1′-piperazinyl]quinazoline (^{125}I-A55453). This ligand binds reversibly to rat hepatic plasma membranes with high affinity (K_D = 77 ± 6 pM), and it labels the same number of "specific" prazosin-competable sites as the *alpha*$_1$-adrenergic receptor-selective radioligand [^{125}I]iodo-2-[β-(4-hydroxyphenyl)-ethylaminomethyl]tetralone. Specific binding is stereoselective and competed for by *alpha*-adrenergic agents with an *alpha*$_1$-adrenergic receptor specificity. ^{125}I-A55453 can be covalently photoincorporated into peptides of rat hepatic and splenic membranes using the bifunctional photoactive cross-linker, *N*-succinimidyl-6-(4′-azido-2′-nitrophenylamino)hexanoate. Following photolysis, sodium dodecyl sulfate-polyacrylamide gel electrophoresis of labeled hepatic membranes reveals a major "specifically" labeled peptide of M_r = 82,000 (±1,000) with minor peptides at M_r = 50,000 (±500), and 40,000 (±300). Covalent incorporation of ^{125}I-A55453 into the M_r = 82,000 peptide is inhibited by adrenergic drugs with an *alpha*$_1$-adrenergic receptor specificity. Labeled splenic membranes demonstrate a broad band of photoincorporated radioactivity centered at M_r = 82,000, and covalent incorporation into this peptide is also attenuated with an *alpha*$_1$-adrenergic receptor specificity. This new high-affinity radioiodinated probe has features which should make it useful for the molecular characterization of *alpha*$_1$-adrenergic receptors in tissues.

INTRODUCTION

Since the original designation of adrenergic receptors as *alpha* and *beta* (1), it has become generally accepted that there exist at least two subtypes of mammalian *alpha*-adrenergic receptors. The early attempts to subclassify *alpha*-adrenergic receptor systems were based on proposed differences of anatomical location (2). More recently, however, *alpha*-adrenergic receptors have been differentiated into *alpha*$_1$- and *alpha*$_2$-adrenergic receptors using pharmacological criteria (3, 4). The biochemical characterization of *alpha*-adrenergic receptors has been facilitated by the introduction of specific high-affinity radioligands. A number of tritiated ligands have been available for reversibly labeling *alpha*-adrenergic receptors. These have included the nonsubtype-selective antagonists [^{3}H]dihydroergocryptine and [^{3}H]phentolamine and the subtype-selective antagonists [^{3}H]prazosin (*alpha*$_1$) and [^{3}H]yohimbine (*alpha*$_2$). More recently, the radioiodinated *alpha*$_1$ ligand ^{125}I- HEAT[3] has been introduced (5) which has a theoretical specific radioactivity of 2200 Ci/mmol.

In order to elucidate the structural and molecular properties of *alpha*-adrenergic receptors, the availability of appropriate irreversible affinity or photoaffinity ligands would be advantageous. The alkylating agent [^{3}H] phenoxybenzamine has been used to label covalently rat hepatic *alpha*$_1$-adrenergic receptors and permit soluble *alpha*$_1$-adrenergic receptors to be detected (6). However, phenoxybenzamine is a poor affinity ligand because of its low specific radioactivity and selectivity for *alpha*$_1$-

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[3] The abbreviations used are: ^{125}I-HEAT, [^{125}I]iodo-2-[β-(4-hydroxyphenyl)-ethylaminomethyl]tetralone; ^{125}I-A55453, 4-amino-6,7-dimethoxy-2[4′-5″-(3‴-[^{125}I]-iodo-4‴-aminophenyl)pentanoyl]-1′-piperazine]quinazoline; ^{125}I-APDQ, 4-amino-6,7-dimethoxy-2[4′-[5″-(3‴-[^{125}I]-iodo-4‴-azidophenyl)-pentazoyl]-1′-piperazine]quinazoline; CP-59,430, 2-[4-(4-azido-benzoyl-piperazin-1-yl]-4-amino-6,7-dimethoxyquinazoline; SANAH, *N*-succinimidyl-6-[4′-azido-2′-nitrophenylamino)hexanoate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; BSA, bovine serum albumin; TLC, thin-layer chromatography.

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adrenergic receptors (7). A nonradioactive azide analogue of prazosin, CP59430, has also been described (8) which irreversibly inactivates membrane-bound and soluble rat hepatic *alpha*$_1$-adrenergic receptors. However, this compound exhibits low affinity for membrane-bound *alpha*$_1$-adrenergic receptors (K_D = 85 nM), which may limit its usefulness. We therefore set out to develop probes for *alpha*$_1$-adrenergic receptors that had the desirable features of high ^{125}I-specific radioactivity and affinity while possessing the potential for covalent photoincorporation into *alpha*$_1$-adrenergic receptors. To this end we synthesized compounds based on the structure of the highly selective *alpha*$_1$-adrenergic receptor antagonist prazosin and applied techniques similar to those recently used successfully for the development of photoaffinity probes for *beta*-adrenergic receptors (9–11).

We report here the development of a novel radioiodinated probe for *alpha*$_1$-adrenergic receptors, ^{125}I-A55453 (see Fig. 1). The purpose of this paper is to describe the characteristics of the reversible binding and covalent incorporation of ^{125}I-A55453 into *alpha*$_1$-adrenergic receptors using a heterobifunctional cross-linking agent. The findings of this study indicate that ^{125}I-A55453 possesses desirable features which should make it useful in the eventual molecular and biochemical characterization of this receptor.

EXPERIMENTAL PROCEDURES

Materials

^{125}I-HEAT (5) and carrier-free Na^{125}I were purchased from New England Nuclear Corporation (Boston, Mass.). Rats (Sprague-Dawley) were from Charles River Breeding Laboratories (Wilmington, Mass.). Premixed SDS-PAGE standards (phosphorylase *b*, M_r = 94,000; BSA, M_r = 67,000; ovalbumin, M_r = 43,000; carbonic anhydrase, M_r = 30,000; soybean trypsin inhibitor, M_r = 20,100; and α-lactalbumin, M_r = 14,000) were from Pharmacia Fine Chemicals (Piscataway, N. J.) and were iodinated by the chloramine T method of Greenwood *et al.* (12). Coomassie brilliant blue R-250 was from Bio-Rad Laboratories (Richmond, Calif.), as were all electrophoresis reagents. SDS was obtained from BDH Chemicals, Ltd. (Poole, England). X-ray film XAR-5 and developing solutions were from Kodak (Rochester, N. Y.) Intensifying screens (Cronex Lighting Plus) were from DuPont. Adrenergic compounds were from sources shown in ref. 13 as follows: HEAT (Beiersdorf AG, Hamburg, Federal Republic of Germany); Oxymetazoline (Draco AB, Lund, Sweden); clonidine (Boehringer Ingelheim). BSA, essentially fatty acid-free, was purchased from Sigma Chemical Company (St. Louis, Mo.). SANAH was from Pierce Chemical Company (Rockford, Ill.). Other biochemical reagents were from Sigma Chemical Company, and chemicals were usually from Aldrich Chemical Company (Milwaukee, Wisc.).

PRAZOSIN

A-55453

[^{125}I]-A55453

FIG. 1. *Structures of the novel compounds are compared with that of the selective alpha*$_1$ *antagonist prazosin*

Methods

Chemical synthesis and characterization of ^{125}I-A55453. Details concerning the preparation and complete chemical characterization of the nonradioactive model compound will be described elsewhere. The procedures for the radioiodination of A55453 are presented below.

Radioiodination of A55453. A55453 (6 μg/6 μl of CH_3OH, 11 nmol) was added to 1.0 M sodium acetate buffer (24 μl, pH 5.6) at room temperature. Na^{125}I (10 mCi, ~17 Ci/mg in 0.1 N NaOH, 4.5 nmol) was then added followed by chloramine T (6 μg/6 μl of water, 21 nmol). After 1 min the reaction was halted with sodium metabisulfite (8 μg/8 μl, 42 nmol) and basified with 1 N NaOH (4 μl). Approximately 1 μl of the reaction mixture was applied to a TLC plate (Scientific Products silica gel 60 F-254, 5 × 20 cm) and co-spotted with I-A55453. The remainder of the reaction mixture was applied across two TLC plates. Both were developed in 15% CH_3OH/CH_2Cl_2/1 mM phenol and were visualized by autoradiography. A single radioactive product which co-migrated with I-A55453 was observed from the analytical TLC (R_F = 0.47, where A55453 R_F = 0.38). From the preparative TLC, the band of silica containing ^{125}I-A55453 was removed with a spatula and eluted with $CHCl_3/CH_3CN$/triethylamine (85:35:5, 5 × 0.5 ml). The eluate was filtered, concentrated immediately, and stored in ethyl acetate/1 mM phenol (1 ml) under N_2 in the dark at −4°. The isolated ^{125}I-A55453 was shown to co-migrate on TLC with I-A55453 using several solvent systems. Owing to the total separation of the radiolabeled product from A55453 and the use of carrier-free Na^{125}I, a specific activity of 2175 Ci/mmol was assumed.

Membrane preparation. Rat hepatic plasma membranes were prepared essentially as described previously (14) except for the following modifications. The diced livers were homogenized using eight strokes of a loose-fitting Dounce homogenizer, and liver plasma membranes were washed in 50 mM Tris-HCl (pH 7.4). Buffers were supplemented with a protease inhibitor mix containing soybean trypsin inhibitor (10 μg/ml), bacitracin (200 μg/ml), leupeptin (10 μg/ml), 100 μM phenylmethylsulfonyl fluoride, and 5 mM EDTA. Membranes (10 mg/ml protein) were stored at −80° until use.

Rat and porcine spleens were placed in ice-cold 10 mM $NaHCO_3$/5 mM EDTA (pH 7.4). This and all subsequent buffers were supplemented with the above protease inhibitor mix. The spleens were diced with scissors and homogenized in 10 volumes of this buffer by three 10-sec bursts of a Brinkmann Polytron homogenizer. The homogenate was centrifuged at 12,000 × *g* for 10 min at 4° in a Sorvall SS-34 rotor. The supernatant was passed through two layers of cheesecloth and centrifuged at 40,000 × *g* for 30 min. The pellet was extracted with 0.6 M KCl 20 mM imidazole (pH 7.0) and recentrifuged at 40,000 × *g* for 30 min at 4°. The pellet was washed twice by centrifugation using 50 mM Tris-HCl (pH 7.4)/5 mM EDTA, and the final pellet was stored at −80° in this buffer at a protein concentration of 5 mg/ml. Rabbit aortic smooth muscle cell membranes were prepared as previously described (15). Protein concentrations were determined by the method of Lowry *et al.* (16), using BSA (Sigma Chemical Company) as standard.

Radioligand Binding Assays

^{125}I-A55453. Assays were performed in polypropylene tubes (75 × 12 mm) in a 0.1-ml total volume. The assay buffer was 150 mM NaCl/5 mM EDTA/50 mM Tris-HCl (pH 7.4). Membranes were thawed, and 50 μl of diluted membranes (≅10 μg of protein) were added to 25 μl of drug or buffer; the reaction was initiated by the addition of 25 μl of ^{125}I-A55453 (in 1% ethanol/3 mM HCl). The reaction proceeded for 90 min at 25° and was terminated by a 50-fold dilution with ice-cold 25 mM sodium acetate/acetic acid buffer (pH 5.5) containing 0.2% BSA

(essentially fatty acid-free), followed by rapid filtration on Whatman GF/C filters. The membranes were washed with 20 ml of the BSA-containing buffer, and the filters were counted in a Packard Auto gamma counter at 75% efficiency. In order to reduce nonspecific filter binding to low levels, the GF/C filters were siliconized with a 1:100 solution of Prosil-28 (PCR Research Chemicals, Gainesville, Fla.) and rinsed in distilled water before use.

^{125}I-A55453 is a hydrophobic compound which, like ^{125}I-HEAT (see ref. 17), adsorbs avidly to surfaces. Such adsorption is barely attenuated by NaCl or lowered pH. It is therefore essential to minimize the surface area of tubes in contact with ligand and to avoid mixing by vortexing or vigorous shaking. All measurements of total radioligand concentration were therefore made by taking aliquots of the reaction mixture at the time of filtration.

^{125}I-HEAT. Incubations were conducted as described in ref. 18. Specific binding to hepatic *alpha*$_1$-adrenergic receptors was defined as that binding competed for by 10 μM prazosin.

Binding data were analyzed by a nonlinear least-squares computer program as previously described (19). Association and dissociation rate constants were determined by graphical analysis of kinetic data using published methods (20).

Membrane labeling with ^{125}I-A55453. In a 50-ml polycarbonate tube were incubated membranes (1 mg of protein, 400 fmol of total receptor sites); "incubation buffer" (150 mM NaCl/50 mM Tris-HCl (pH 7.4)/5 mM EDTA); buffer or competing agent; 50–150 pM radioligand in a total volume of 10 ml. The reaction was initiated by the addition of radioligand (in 1% ethanol/3 mM HCl) and continued for 90 min at 25°. The incubation mixture was diluted to 40 ml with cold 150 mM NaCl/10 mM sodium phosphate buffer (pH 7.4) containing 0.5% BSA (essentially fatty acid-free; Sigma Chemical Company) and centrifuged for 7 min at 50,000 × *g*. The membranes were homogenized in cold 150 mM NaCl/10 mM sodium phosphate buffer (pH 7.4) using a motor-driven Teflon pestle, recentrifuged, and resuspended in 5 ml of this buffer. Under dark conditions, 30 μl of a solution of 5 mM SANAH (freshly dissolved in dimethyl sulfoxide) was added to the membrane suspension, and the reaction proceeded for 10 min at 25°. The reaction was quenched by the addition of 100 μl of 1 M glycine. The cooled reaction mixture was photolyzed for 90 sec in a Petri dish placed 12 cm from a 450 W medium pressure mercury arc lamp (Hanovia) filtered with 5 mm of Pyrex glass. Measurable deiodination did not occur as a result of this protocol. Following photolysis, the membranes were washed twice by centrifugation with cold "incubation buffer," resuspended in 1.5 ml of 150 mM NaCl/50 mM Tris-HCl (pH 6.8)/5 mM EDTA, and pelleted by centrifugation for 15 min in a Fisher Microfuge (13,000 × *g*). The final pellets were denatured in 150 μl of 10% SDS/10% glycerol/25 mM Tris-HCl (pH 6.8)/5% β-mercaptoethanol for 45–60 min at 25°.

SDS-PAGE. Gel electrophoresis was performed on the solubilized membrane samples according to the method of Laemmli (21), using 10% polyacrylamide slab gels run at a constant current of 3 mamp/lane. Identical amounts of total protein were applied to the lanes (generally 100–300 μg), and gels were dried using a slab gel drier (Hoefer Scientific Instruments, San Francisco, Calif.). Autoradiography was performed at −80° on the dried gels using Kodak XAR-5 film. In general, the exposure time for gels was 2–3 days. Autoradiograms were scanned on a Zeineh scanning densitometer Model SL-504-XL (Biomed Instruments, Inc.) using laser light.

RESULTS

Characteristics of ^{125}I-A55453 binding to rat hepatic membranes. Incubation of rat hepatic membranes with ^{125}I-A55453 revealed high-affinity binding of ligand to a uniform population of sites (Fig. 2). At a K_D concentration of radioligand, nonspecific binding of radioligand represented only 10–15% of the total binding in this system. The equilibrium dissociation constant (K_D) calculated for ^{125}I-A55453 was 77 ± 6 pM (n = 4). The

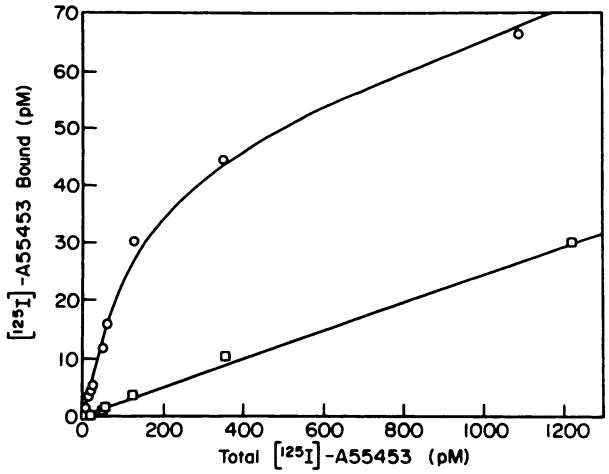

FIG. 2. *Saturation isotherm of ^{125}I-A55453 binding to rat liver membranes*

Increasing concentrations of radioligand were incubated with rat liver membranes (11 μg of protein), and, after the attainment of equilibrium, bound ligand was separated from free ligand by procedures described under Methods. Total receptor binding (○) and nonspecific binding (□), measured in the presence of 10 μM prazosin, are plotted. Each point represents the mean of triplicate determinations, and the lines represent the computer-modeled best fit to the experimental data (^{125}I-A55453, K_D = 88 pM, B_{max} = 410 fmol/mg of protein) The data shown are representative curves of experiments performed four times.

number of binding sites determined with ^{125}I-A55453 (405 ± 20 fmol/mg of protein) was in excellent agreement with values obtained for the same membrane preparation using ^{125}I-HEAT (data not shown) and [^{3}H]prazosin (22).

Figure 3 shows the association and dissociation kinetics of ^{125}I-A55453 binding to rat hepatic membranes. Steady state was attained after 45 min at 25°, and the addition of an excess of unlabeled prazosin led to total dissociation of receptor-bound ^{125}I-A55453. Similar results were obtained using phentolamine as the competitor (data not shown). The dissociation reaction was a first-order process at 25° with a mean $t_{½}$ = 21 (±2) min (n = 3) (see Fig. 3, *inset*). The association (k_{+1}) and dissociation (k_{-1}) rate constants were 5.94 × 10^{-4} (±1 × 10^{-4}) min^{-1} pM^{-1} (n = 3) and 3.3 × 10^{-2} (±2 × 10^{-3}) min^{-1} (n = 3), respectively. The dissociation constant, calculated as k_{-1}/k_{+1}, was 56 pM, in excellent agreement with values determined by equilibrium binding measurements (77 pM, see above).

The pharmacological specificity of ^{125}I-A55453 binding to rat hepatic membranes is shown in Fig. 4A and B and Table 1. Adrenergic agents competed for the specific binding sites with characteristics expected of an interaction with *alpha*-adrenergic receptors. Thus, the catecholamines (−)-epinephrine and (−)-norepinephrine were equipotent and approximately 250-fold more potent than the specific *beta*-adrenergic agonist isoproterenol. The neurotransmitters serotonin and dopamine were extremely poor competitors with specific ^{125}I-A55453 binding (see Table 1). The (−)-isomers of epinephrine and norepinephrine had affinities for the labeled sites which were 12- to 27-fold greater than the (+)-isomers, thereby demonstrating the stereoselective nature of the binding. The order of potencies of the agonists, oxyme-

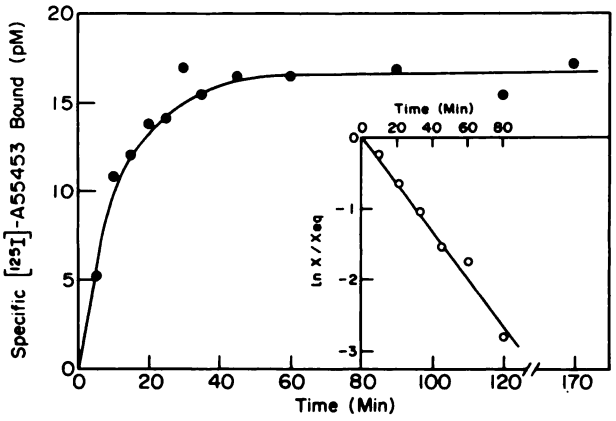

FIG. 3. *Association and dissociation of ^{125}I-A55453 to rat hepatic membranes*

Membranes were incubated with ^{125}I-A55453 at 25° for the specified times, and the amount of ^{125}I-A55453 bound to the membranes was determined following rapid filtration as described under Methods. "Specific binding" to *alpha*$_1$-adrenergic receptors was calculated by subtracting values of membrane-bound ^{125}I-A55453 measured at similar times in the presence of prazosin (10 μM). Specific binding is plotted versus time (●). Following the attainment of steady state (90 min at 25°), an excess of prazosin (10 μM) was added, and specific binding was measured at timed intervals. *Inset.* First-order plot of the dissociation reaction, where X is the amount of radioligand specifically bound at time t, and X_{eq} is the amount bound at equilibrium. Each point represents the mean of duplicate determinations of a representative experiment performed three times.

tazoline > clonidine > phenylephrine, was identical with that determined for rat cerebral cortex *alpha*$_1$-adrenergic receptors (17) and suggested that ^{125}I-A55453 labeled *alpha*$_1$-adrenergic receptors in these hepatic plasma membranes.

In support of this conclusion, the *alpha*$_1$-adrenergic receptor-selective antagonist prazosin was an extremely effective competitor for ^{125}I-A55453 binding, being 3- to 4-fold more potent than HEAT and 190-fold more potent than the nonselective agent phentolamine (Fig. 4B). Yohimbine and rauwolscine, two *alpha*$_2$-adrenergic receptor selective antagonists (4), were respectively 10- and 20-fold weaker than phentolamine. The competition curves that were obtained with all agonists and antagonists were uniphasic, with slope factors that were not significantly different from unity. Inhibition constants calculated for ^{125}I-A55453 binding were compared with values obtained using the *alpha*$_1$-selective radioligand ^{125}I-HEAT. Figure 5 shows, for a series of agonists and antagonists having affinities which differ by 5 log units, that there is an excellent correlation between the two sets of data (r = 0.991, slope = 0.93). These collective data indicate that ^{125}I-A55453 labels a homogeneous population of *alpha*$_1$-adrenergic receptors in rat hepatic membranes.

The degree of *alpha*$_1$-adrenergic receptor selectivity possessed by the compound A55453 was investigated by measuring its inhibition constant for an *alpha*$_2$-adrenergic receptor system. In competition studies of [^{3}H]yohimbine binding to human platelet *alpha*$_2$-adrenergic receptors, a value of 1 μM was determined (data not shown). Thus, A55453 exhibited approximately 1000-fold selectivity between hepatic *alpha*$_1$-adrenergic receptors and human platelet *alpha*$_2$-adrenergic receptors.

Covalent incorporation of ^{125}I-A55453 into the alpha$_1$*-adrenergic subunit of rat hepatic membranes.* A major aim in the development of this ligand was to investigate its potential utility for covalent incorporation into *alpha*$_1$-adrenergic receptors. Clearly it was of considerable interest to determine whether the arylamine present in A55453 could be covalently cross-linked to *alpha*$_1$-adrenergic receptors using methodology similar to that applied to studies of *beta*-adrenergic receptors (25, 26). The photoactive bifunctional cross-linker SANAH was examined in order to determine its suitability for this purpose. When rat hepatic membranes were incubated with ^{125}I-A55453, allowed to react with SANAH, photolyzed, and subjected to SDS-PAGE, a labeling pattern like that shown in Fig. 6 was detected. There was evi-

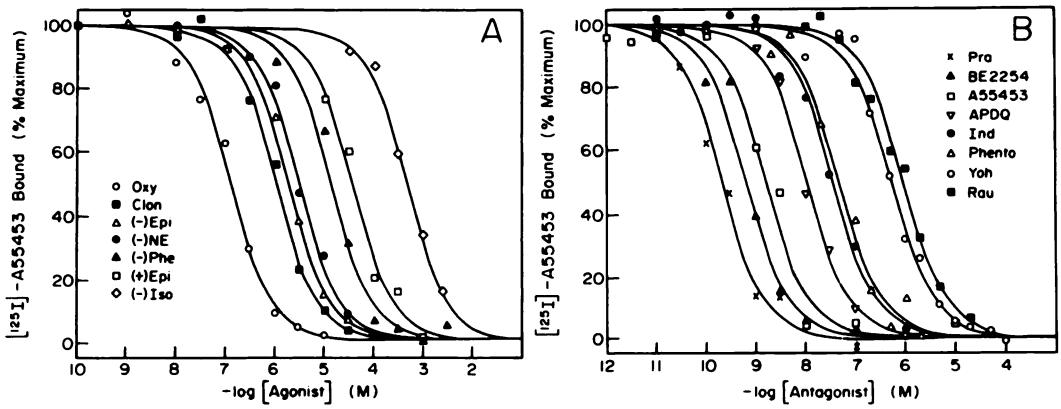

FIG. 4. *Competition curves of adrenergic agonists (A) and antagonists (B) for the specific ^{125}I-A55453 binding sites on rat hepatic membranes*

Membranes were incubated with ^{125}I-A55453 (60–100 pM) and competing drugs, and binding was determined as described under Methods. Each point represents the mean of duplicate experimental determinations, and the curve represents the computer-drawn best fit of the experimental data to a four-parameter logistic equation (23). One hundred per cent specific binding represents typically 18 pM. Results are typical of three to six experiments. *Oxy*, oxymetazoline; *Clon*, clonidine; *(−)Epi*, (−)-epinephrine; *(−)NE*, (−)-norepinephrine; *(−)Phe*, (−)-phenylephrine; *(+)Epi*, (+)-epinephrine; *(−)Iso*, (−)-isoproterenol; *Pra*, prazosin; *Ind*, indoramine; *Phento*, phentolamine; *Yoh*, yohimbine; *Rau*, rauwolscine.

TABLE 1

Inhibition constants of drugs for rat hepatic alpha$_1$-adrenergic receptors determined with ^{125}I-A55453

IC_{50} values were determined by fitting individual competition curves to a nonlinear, least-squares computer program (19), and K_i values (± standard error of the mean) were calculated from the Cheng and Prusoff equation (24). Values represent the mean of 3–6 experiments performed in duplicate.

Drug	K_i
	nM
Agonists	
Oxymetazoline	102 (±16)
Clonidine	490 (±30)
(−)-Epinephrine	1,140 (±120)
(+)-Epinephrine	14,050 (±800)
(−)-Norepinephrine	1,730 (±205)
(+)-Norepinephrine	47,800 (±3,600)
(−)-Phenylephrine	13,460 (±1,150)
(−)-Isoproterenol	296,000 (±15,600)
5-Hydroxytryptamine	63,800 (±8,000)
Dopamine	158,000 (±17,000)
Antagonists	
Prazosin	0.138 (±0.01)
HEAT	0.44 (±0.03)
A55453	1.02 (±0.18)
APDQ	5.8 (±1.1)
Phentolamine	26.5 (±1.8)
Indoramine	31 (±2)
Yohimbine	334 (±20)
Rauwolscine	535 (±50)

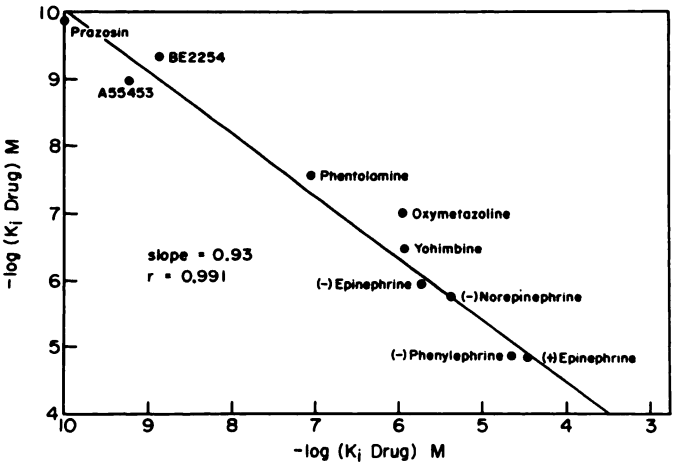

FIG. 5. *Correlation plot of log K_i (inhibition constants) determined for drugs in competition with ^{125}I-A55453 and ^{125}I-HEAT binding to hepatic membranes*

Incubations were performed at 25° using techniques reported under Methods. K_i values were calculated as described in the legend to Table 1. Ordinate data were obtained using ^{125}I-A55453; abscissa data were determined with ^{125}I-HEAT. Each point represents the mean of three to six experiments conducted in duplicate.

dence of "specific" labeling as shown by the ability of prazosin (0.1 μM) to inhibit covalent incorporation (Fig. 6, *curves a* and *b*). One major peak of "specific" radioactivity is apparent which corresponds to M_r = 82,000, and two minor peaks at M_r = 50,000 and 40,000 are partially protected by prazosin. By densitometric scanning, the M_r = 82,000 peptide represented 76% of the

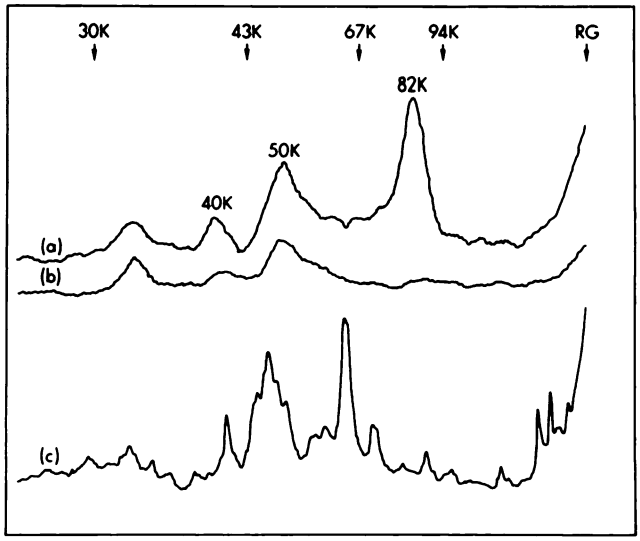

FIG. 6. *Photoaffinity cross-linking of ^{125}I-A55453 into rat hepatic membranes*

Rat hepatic membranes were incubated with 100–150 pM ^{125}I-A55453 in the absence (*curve a*) or presence (*curve b*) of 0.1 μM prazosin, washed, and allowed to react with SANAH. Following photolysis, the labeled membranes were processed for SDS-PAGE as described under Methods. The gel was stained with 0.1% Coomassie blue in 45% methanol/10% acetic acid and destained, and the bands of protein were scanned using a densitometer (*curve c*). The gel was dried, autoradiography was performed, and the autoradiogram was scanned as described above. Molecular weights of markers are shown × 1000 (*K*). *RG*, Running gel. Results are representative of seven experiments.

total protectable peptides, and the M_r = 50,000 and 40,000 peptides represented 20% and 4%, respectively (see Table 2). This pattern of radioactivity was clearly different from the protein profile (Fig. 6, *curve c*). Thus the scan of the Coomassie blue-stained gel yielded major peaks of protein at M_r = 62,000 and multiple bands centered at M_r = 48,000. Only modest amounts of membrane protein are coincident with the major peak of "specific" radioactivity. These data indicate that covalent incorporation of ^{125}I-A55453 is into discrete peptides that represent minor constituents of the total membrane protein.

In order to examine the pharmacological specificity of the peptide labeling, hepatic membranes were incubated with ^{125}I-A55453 in the presence of agonist and antagonist drugs and, after reaction with SANAH, photolabeled and subjected to SDS-PAGE. Figure 7 shows the autoradiograms obtained using agonists (A) and antagonists (B). In these preparations, three protectable bands of radioactivity are apparent which correspond to M_r values of 82,000, 50,000, and 40,000. A minor labeled peptide of high molecular weight ($M_r \cong$ 160,000) was not routinely observed and may represent cross-linked M_r = 80,000 subunits.

Labeling of the M_r = 82,000 band is clearly attenuated to the greatest extent by the agonists and antagonists used, and the pharmacological specificity is indicative of an *alpha*-adrenergic receptor. Thus, (−)-epinephrine and (−)-norepinephrine are equipotent, and both are more potent than (−)-isoproterenol in preventing covalent incorporation of ^{125}I-A55453. The (−)-isomers of both

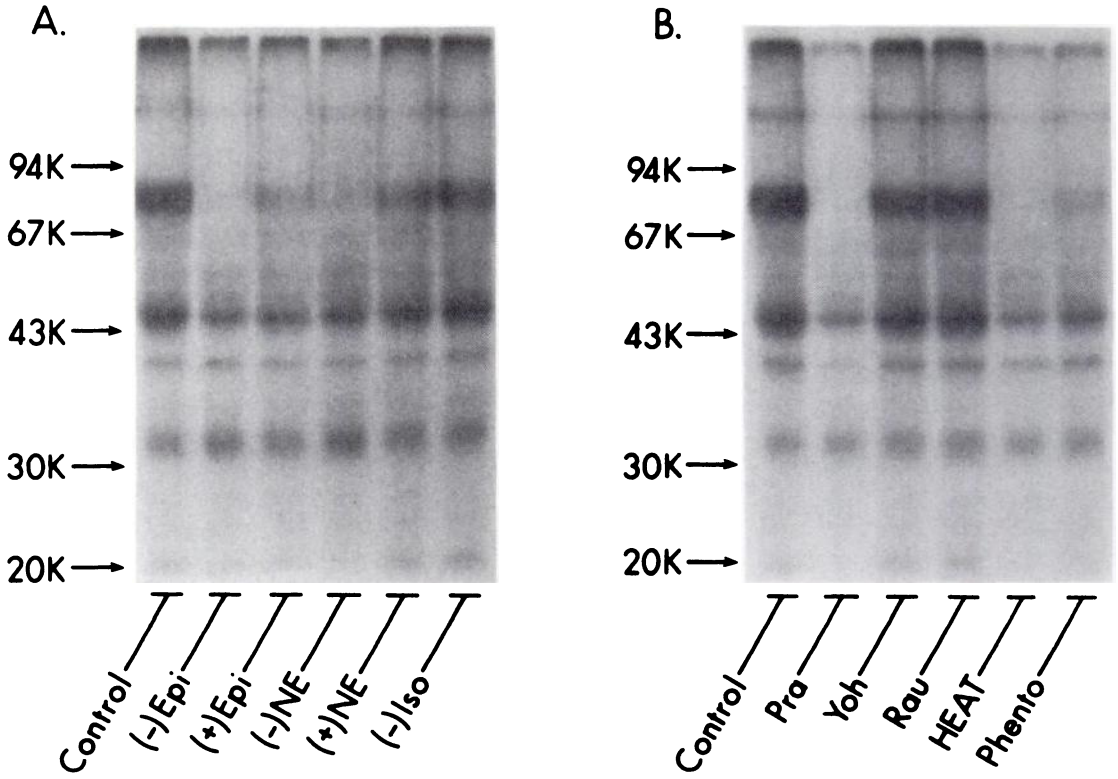

FIG. 7. *Photoaffinity cross-linking and pharmacological specificity of ^{125}I-A55453 incorporation into rat hepatic membranes*

A. Hepatic membranes were incubated with ^{125}I-A55453 (75–100 pM) in the absence or presence of (−)-epinephrine [*(−)Epi*], (+)-epinephrine [*(+)Epi*], (−)-norepinephrine [*(−)NE*], (+)-norepinephrine [*(+)NE*], and (−)-isoproterenol [*(−)Iso*], all at 30 μM; washed; allowed to react with 30 μM SANAH; photolyzed; and subjected to SDS-PAGE. Autoradiography was performed on the dried gels.

B. Membranes were incubated with ^{125}I-A55453 (60–90 pM) in the absence and presence of prazosin (*Pra*), yohimbine (*Yoh*), rauwolscine (*Rau*), HEAT, and phentolamine (*Phento*), all at 0.1 μM, and processed as described above. The results shown are identical with three other experiments.

epinephrine and norepinephrine are more effective protecting agents than are their respective (+)-isomers.

Results of using subtype-selective antagonists (Fig. 7B) clearly indicate that labeling of the M_r = 82,000 peptide (and to a lesser extent the M_r = 50,000 and 40,000 peptides) occurs with an *alpha*$_1$-adrenergic receptor specificity. Thus the relative ability of these antagonists to attenuate the labeling was prazosin ≡HEAT > phentolamine ≫ yohimbine ≡ rauwolscine. This rank order is identical with that determined for reversible radioligand binding to the hepatic *alpha*$_1$-adrenergic receptors (see Table 1).

Labeling of rat spleen alpha$_1$*-adrenergic receptors with ^{125}I-A55453.* The ability of ^{125}I-A55453 to label rat spleen *alpha*$_1$-adrenergic receptors was investigated. Membranes derived from this smooth muscle-containing tissue exhibited a B_{max} of 190 (±20) fmol/mg of protein (n = 5). Binding of ^{125}I-A55453 to rat spleen membranes is competed for by adrenergic agents, prazosin, yohimbine, and (−)-epinephrine, with an *alpha*$_1$-adrenergic receptor specificity as shown in Fig. 8A. The inhibition constants calculated for these agents (0.09 nM, 235 nM, and 760 nM, respectively) are similar to values obtained using rat hepatic membranes (see Table 1). These findings suggest that the pharmacological characteristics of rat hepatic and splenic *alpha*$_1$-adrenergic receptors are identical. Figure 8B shows results of experiments conducted to examine the structural properties of rat spleen *alpha*$_1$-adrenergic receptors. Rat spleen membranes were incubated with ^{125}I-A55453 in the absence (a) or presence of prazosin (b), yohimbine (c), and epinephrine (d), and, following a reaction with SANAH, photolyzed and subjected to SDS-PAGE. The densitometric scan of the autoradiogram shows four major peaks of radioactivity at M_r = 82,000, 52,000, 40,000, and 33,000. However, only the broad peak centered at M_r = 82,000 shows an appropriate *alpha*$_1$-adrenergic receptor specificity. Thus, covalent incorporation of ^{125}I-A55453 into this peak is attenuated by prazosin but is unaffected by yohimbine, and a saturating concentration of epinephrine eliminates the labeling of this peak completely. These data suggest that the same major *alpha*$_1$-adrenergic receptor peptide is labeled in rat spleen and liver by ^{125}I-A55453.

The photoaffinity labeling pattern obtained with this cross-linking technique was compared with that produced using the photoaffinity arylazide radioligand ^{125}I-APDQ (27). Membranes from a number of tissues were covalently labeled with both radioligands, and the autoradiogram was subjected to densitometric scanning. The relative mobilities and percentage of protectable peptides

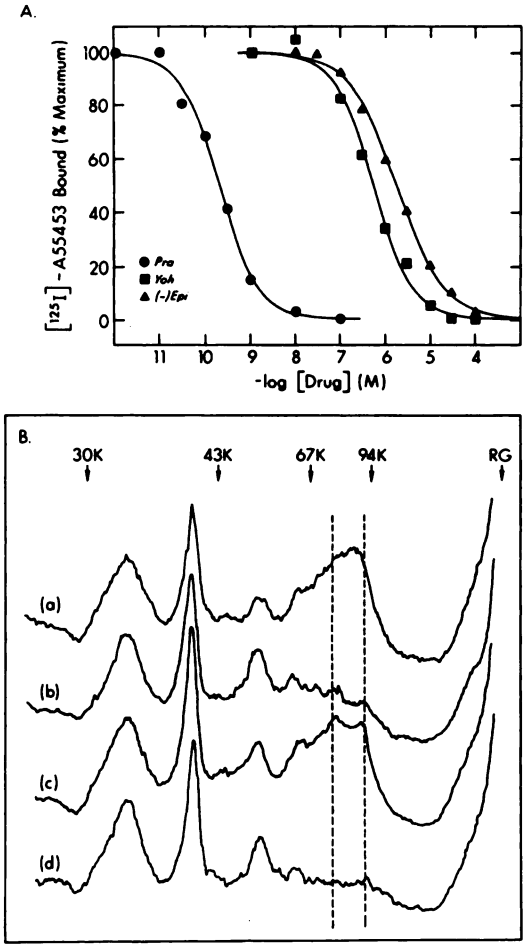

FIG. 8. *Reversible binding and photoaffinity cross-linking of ^{125}I-A55453 into rat spleen membranes*

A. ^{125}I-A55453 (60–100 pM) was incubated with rat spleen membranes and competing drugs: prazosin (*Pra*); yohimbine (*Yoh*), and (−)-epinephrine [*(−)Epi*], and binding was determined as described under Methods. The best-fit curves represent typical data replicated three times.

B. Rat spleen membranes were incubated with 100–150 pM ^{125}I-A55453 in the absence (*curve a*) or presence of 0.1 μM prazosin (*curve b*), 0.1 μM yohimbine (*curve c*), and 100 μM (−)-epinephrine (*curve d*); washed and allowed to react with SANAH. Following photolysis, SDS-PAGE was performed, and the autoradiogram of the dried gel was scanned as described under Methods. Molecular weights of markers are shown × 1000 (*K*), and results are representative of three experiments.

are shown in Table 2. The results show that identical peptides were labeled by both techniques and the relative proportions of the peptides in each tissue were very similar. Of the systems examined, membranes of rabbit aortic smooth muscle cells exhibited the cleanest labeling pattern with a single $alpha_1$-adrenergic receptor peptide of M_r = 85,000–88,000 which represented >90% of the total protectable radioactivity. Porcine spleen membranes showed a labeling pattern with the greatest degree of heterogeneity, perhaps reflecting more significant amounts of postmortem proteolysis.

DISCUSSION

A new radioligand ^{125}I-A55453 has been used to identify binding sites in rat hepatic and splenic membranes

TABLE 2

Comparison of molecular weight estimates of peptides "specifically" labeled with ^{125}I-A55453/SANAH and ^{125}I-APDQ using SDS-PAGE

Identical membranes were covalently labeled with the above ligands using techniques described under Methods in ref. 27, and SDS-PAGE was performed. Determinations of the relative mobilities and percentage of radioactivity incorporated into protectable peptides were made by densitometric scanning of the autoradiograms.

Tissue	^{125}I-A55453/SANAH	^{125}I-APDQ
Rat liver	82K (76%); 50K (20%); 40K (4%)	80K (72%); 48K (19%); 38K (9%)
Rat spleen	82K* (>90%)	79K* (87%); 42K (9%); 35K (4%)
Pig spleen	78K* (36%); 63K (32%); 54K (17%); 40K (15%)	78K* (36%); 64K (35%); 56K (15%); 42K (14%)
Rabbit vascular smooth muscle cells	88K (>90%)	85K (>90%)

* Denotes maxima of a broad peak.

which have the pharmacological characteristics of $alpha_1$-adrenergic receptors. This radioiodinated ligand, which is based on the prazosin structure, binds reversibly to the same number of hepatic *alpha*-adrenergic receptors and with similar affinities as the established $alpha_1$-selective ligand ^{125}I-HEAT. The binding is stereoselective and inhibited by a variety of adrenergic agents with affinities similar to those determined using [^{3}H]dihydroergocryptine or [^{3}H]prazosin (28). The competition curves of subtype-selective adrenergic receptor agents had slope factors which were not significantly different from unity. Collectively, these data indicate that ^{125}I-A55453 selectively labels $alpha_1$-adrenergic receptors which are the predominant *alpha*-adrenergic receptors in liver membranes (29). Direct evidence that this ligand has substantial selectivity was provided by data showing that A55453 had an affinity for hepatic $alpha_1$-adrenergic receptors which was 1000-fold greater than its affinity for human platelet $alpha_2$-adrenergic receptors.

The pharmacological characteristics of rat liver $alpha_1$-adrenergic receptors are identical using ^{125}I-A55453 or ^{125}I-HEAT as the radioligand. The data obtained in this system were similar to those reported for rat lung and cerebral cortex (30) and rat vas deferens (31) $alpha_1$-adrenergic receptors using ^{125}I-HEAT. These results underline the identity of $alpha_1$-adrenergic receptors independently of the specific radioligand used.

An additional advantage of the ligand A55453 is its potential for covalent incorporation into $alpha_1$-adrenergic receptors. The design of this compound was based upon experience gained with photoaffinity labels for *beta*-adrenergic receptors (9, 25). We have previously shown that ^{125}I-labeled *p*-aminobenzylcarazolol can be covalently photoincorporated into membrane-bound and partially purified frog erythrocyte *beta*-adrenergic receptors following a reaction with SANAH (26). The use of photoaffinity cross-linking agents to characterize ligand binding sites of receptors has generally been applied to large polypeptide hormones (32–35). The problems associated with this technique have been discussed in a number of reviews (36, 37), but the most important

include (a) the potential labeling of nonreceptor proteins following diffusion of the derivatized ligand from the ligand binding site; (b) "exo" labeling of adjacent nonreceptor proteins because of the long sidearm of the derivatized ligand; (c) the potential cross-linking of receptor peptides to other membrane-bound proteins; and (d) the low yield of photochemical labeling.

Some of these problems are common to all photoaffinity labeling techniques, including the use of photoaffinity probes already possessing an azide functional group. Recently, radioiodinated azidobenzyl substituents have been incorporated into the *beta*-receptor antagonists carazolol (9), pindolol (10), and cyanopindolol (11), and the radioiodinated products have been used to label *beta*-adrenergic receptors from a number of mammalian tissues (9). A major advantage of these probes is their higher efficiency of photoincorporation (generally 10–15% of specifically bound ligand) and their more direct method of insertion. In the present studies, 1–1.5% of specifically bound ^{125}I-A55453 was covalently incorporated into hepatic *alpha*$_1$-adrenergic receptors. This efficiency of photoincorporation is similar to that determined for ^{125}I-labeled *p*-aminobenzylcarazolol cross-linked into frog erythrocyte *beta*-adrenergic receptors (26). In view of the low efficiency of the cross-linking reaction, it becomes important to establish that the labeling pattern which is obtained reflects that of the entire receptor population. The demonstration that different affinity probes, especially those which do not have the problems inherent in the cross-linking technique, label the same membrane peptides is good evidence that the labeled peptides are representative.

The results of this study indicate that the major peptide "specifically" labeled in rat hepatic membranes by ^{125}I-A55453 exhibited M_r = 82,000 with minor peptides at 50,000 and 40,000. These peptides are similar to those labeled in the same membrane preparations by ^{125}I-APDQ (27); see Table 2. The similarity of the labeling pattern in this and other systems suggests that the bands labeled by SANAH/^{125}I-A55453 do not represent falsely generated bands due to cross-linking of receptor peptides to other membrane components nor exo-labeling of adjacent nonreceptor peptides. Moreover, the major labeled peptides represent minor protein components of the membrane as assessed by Coomassie blue staining. Thus the peptides present in hepatic membranes are not indiscriminately labeled by SANAH/^{125}I-A55453. Indeed, the protection experiments clearly showed that covalent incorporation of ^{125}I-A55453 was into receptor-related peptides. Thus incorporation of label into hepatic and splenic membranes exhibited an *alpha*$_1$-adrenergic receptor specificity which exactly mirrored the reversible binding data, both qualitatively and quantitatively.

Similar 82,000-dalton peptides were labeled in both rat spleen and liver by SANAH/^{125}I-A55453. However, in contrast to liver membranes, where the labeled band was discrete, labeling of rat spleen membranes resulted in a rather broad band of photoincorporated radioactivity. This may relate to some further degree of heterogeneity, perhaps related to different carbohydrate content of splenic *alpha*$_1$-adrenergic receptor peptides. It is pertinent to note, however, that a similar broad band was demonstrated using ^{125}I-APDQ (Table 2 and ref. 27).

Further evidence that SANAH/^{125}I-A55453 specifically labels *alpha*$_1$-adrenergic receptor peptides is shown by the results of using membranes derived from vascular smooth muscle cells in culture, a tissue which has a single M_r = 85,000 *alpha*$_1$-adrenergic receptor subunit (27). Membranes labeled with SANAH/^{125}I-A55453 and ^{125}I-APDQ produce identical labeling patterns (see Table 2), which indicates that some of the potential problems associated with the cross-linking technique are not manifest in the systems studied. However, some cross-linking of receptor peptides to other components clearly occurs, as shown by the high molecular weight radiolabeled material that is found at the interface of the running and stacking gels. In some hepatic preparations, radiolabeled peptides at M_r = 160,000 were observed which may represent cross-linked dimers of 80,000 subunits. Whether these products represent functional *alpha*$_1$-adrenergic receptors, however, as is suggested from radiation inactivation experiments (38), must await further studies.

A number of other workers have attempted to identify the hormone-binding subunit of hepatic *alpha*$_1$-adrenergic receptors (6, 39, 40). Most investigators have utilized the irreversible *alpha*-antagonist [^{3}H]phenoxybenzamine, which has low specific activities varying from 3.3 to 45 Ci/mmol. Phenoxybenzamine is a notoriously poor *alpha*-adrenergic receptor ligand, since it also interacts covalently with dopamine, muscarinic cholinergic receptors, and human platelet *alpha*$_2$-adrenergic receptors and with nonspecific sites in rat liver membranes (7, 41). In general, the pharmacological specificity of [^{3}H]phenoxybenzamine binding to hepatic membranes has been reported to correlate with [^{3}H]dihydroergocryptine (6) or [^{3}H]prazosin (41) binding. However, its use to covalently label hepatic *alpha*-adrenergic receptors has resulted in reports of "specifically" labeled peptides of M_r = 45,000 (40), 80,000, and 58,000 (39) after SDS-PAGE. Another recent study suggested that the ligand binding component of *alpha*$_1$-adrenergic receptors purified from rat liver by affinity chromatography exhibited an apparent M_r upon SDS-PAGE of 59,000 (41). Previous work with *beta*-adrenergic receptors (42) and with nicotinic (43) and muscarinic cholinergic (44) receptors has suggested that the presence of multiple receptor peptides may occur as a result of proteolysis. Thus the relative stoichiometry of the labeled peptides from rat liver may depend upon the precautions taken to inhibit proteases. Indeed the M_r = 82,000 peptide found in this study in which proteolysis was controlled by inhibitors and that in the study by Kunos *et al.* (39) may represent the native hormone-binding subunit which is susceptible to proteolytic conversion to smaller peptides (27).

The availability of the high-affinity radioiodinated ligand ^{125}I-A55453, which has excellent characteristics as a reversible ligand and which can be derivatized and covalently incorporated into *alpha*$_1$-adrenergic receptors, should provide a useful tool for the molecular characterization of *alpha*$_1$-adrenergic receptors in tissues. In particular, this ligand may lend itself to the development of

fluorescent or electron-dense probes for the localization of *alpha*$_1$-adrenergic receptors in tissues.

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