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Photoaffinity Labeling of the α_1-Adrenergic Receptor Using an ^{125}I-Labeled Aryl Azide Analogue of Prazosin†

Christine E. Seidman, Hans-Jürgen Hess, Charles J. Homcy,* and Robert M. Graham

ABSTRACT: α_1-Adrenergic receptor probes, which can be radioiodinated to yield high specific activity radioligands, have been synthesized and characterized. 2-[4-(4-Aminobenzoyl)piperazin-1-yl]-4-amino-6,7-dimethoxyquinazoline (CP63,155), an arylamine analogue of the selective α_1-adrenergic antagonist prazosin, and its iodinated derivative, 2-[4-(4-amino-3-[^{125}I]iodobenzoyl)piperazin-1-yl]-4-amino-6,7-dimethoxyquinazoline ([^{125}I]CP63,789), bind reversibly and with high affinity (K_D = 1 nM and 0.6 nM, respectively) to rat hepatic membrane α_1-adrenergic receptors. Conversion of [^{125}I]CP63,789 to the aryl azide yields a photolabile derivative, 2-[4-(4-azido-3-[^{125}I]iodobenzoyl)piperazin-1-yl]-4-amino-6,7-dimethoxyquinazoline ([^{125}I]CP65,526), which prior to photolysis binds competitively and with high affinity (K_D = 0.3 nM). Binding of [^{125}I]CP63,789 and [^{125}I]CP65,526 (prior to photolysis) is rapid and saturable. Both ligands identify similar α_1-adrenergic receptor binding site concentrations as the parent probe, [^{3}H]prazosin. Specific binding by these iodinated ligands is stereoselective and inhibited by a variety of adrenergic agents with a specificity typical of the α_1-adrenergic receptor. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and autoradiography of [^{125}I]CP65,526-labeled rat hepatic membranes reveal major protein species with molecular weights of 77K, 68K and 59K. Each protein binds adrenergic ligands with stereoselectivity and with a specificity typical of the α_1-adrenergic receptor. Inclusion of multiple protease inhibitors during membrane preparation prior to SDS–PAGE does not alter the labeling of these peptides. Smaller peptides with molecular weights of 42K and 31K display prazosin-inhibitable [^{125}I]CP65,526 binding. Labeling of these protein species with [^{125}I]CP65,526 is not inhibitable by other adrenergic agonists or antagonists. They are thus unlikely to represent subunits of the receptor. These findings confirm and extend our observations on the subunit composition of the receptor determined with the purified protein and indicate the utility of these novel high-affinity radioiodinated probes as tools for more detailed elucidation and comparison of the molecular properties of the receptor in a variety of tissues.

Considerable progress has been made in the isolation, purification, and molecular characterization of a number of hormone and drug receptors (Homcy et al., 1983; Momoi & Lennon, 1982; Schneider et al., 1982) including the α_1-adrenergic receptor (Graham et al., 1982a,b). To further aid in the molecular characterization of this subtype of the α-adrenergic receptor, we synthesized and characterized a photoaffinity label, which upon photolysis covalently and specifically incorporates into the receptor binding site (Hess et al., 1983).

We here report on the development of a high-affinity α_1-selective probe, which can be radioiodinated and purified by high-performance liquid chromatography (HPLC) to yield a compound of high specific activity (2175 Ci/mmol, assumed). Conversion of this radioiodinated derivative to the aryl azide yields a radiolabeled photoaffinity probe, selective for the α_1-adrenergic receptor. This probe can be used to readily identify and characterize the minute quantities of receptor present in most tissues and cells.

Materials and Methods

Carrier free Na^{125}I was purchased from Amersham, Arlington Heights, IL. Premixed sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) standards were purchased from Pharmacia and iodinated according to the method of Hunter & Greenwood (1962). Phenylmethanesulfonyl fluoride (PMSF), sodium azide (NaN_3), bacitracin, and soybean trypsin inhibitor (STI) were from Sigma. Sodium nitrite ($NaNO_2$) was obtained from Fisher, acetonitrile (CH_3CN) was from Baker, and 24-mm filters (no. 32 glass) were from Schleicher & Schuell. X-ray film (XAR-5) was from Kodak and was developed in an X-omat M20 processor (Kodak). Image-intensifying screens were from Du Pont. The

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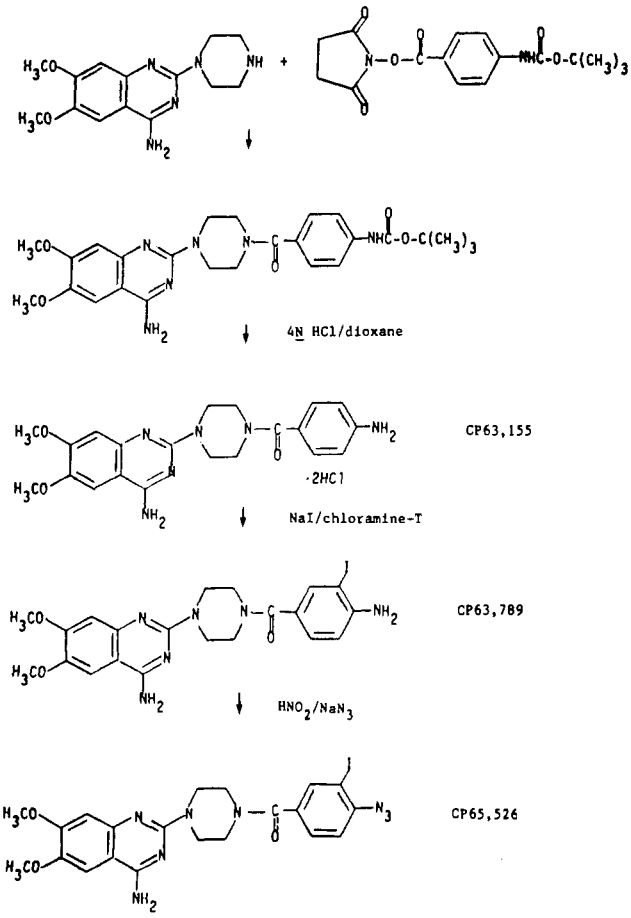

FIGURE 1: Reaction sequence leading to 2-[4-(4-azido-3-iodobenzoyl)piperazin-1-yl]-4-amino-6,7-dimethoxyquinazoline (CP65,526).

source of all other reagents and compounds were as previously described (Graham et al., 1982a,b; Hess et al., 1983).

Synthesis of CP63,155, CP63,789, and CP65,526. The reaction sequence leading to CP63,155, CP63,789, and CP65,526 is shown in Figure 1.

2-[4-(4-Aminobenzoyl)piperazin-1-yl]-4-amino-6,7-dimethoxyquinazoline (CP63,155). CP63,155 was prepared by a two-step reaction. Initially, a Boc (*N-tert*-butyloxycarbonyl) derivative [2-[4-Boc-*p*-aminobenzoyl)piperazin-1-yl]-4-amino-6,7-dimethoxyquinazoline] was prepared as follows. A solution of 2-(1-piperazinyl)-4-amino-6,7-dimethoxyquinazoline (8.0 g, 27.7 mmol) and the *N*-hydroxysuccinimide ester of *N-tert*-butyloxycarbonyl-*p*-aminobenzoic acid (24 g, 27.7 mmol) in 75 mL of dimethylformamide (DMF) was stirred at room temperature for 12 h and then concentrated to yield a precipitate which was filtered (Althuis & Hess, 1977; Hoffman et al., 1978). This precipitate was suspended in 55 mL of methanol/DMF (10:1), heated on a steam bath, and filtered to give 4.4 g of product. The mother liquor was concentrated, and the solid which remained was dissolved in DMF and purified by flash column chromatography on silica gel, eluting successively with ethyl acetate (EtOAc), 5% methanol/EtOAc, and 10% methanol/EtOAc, to give an additional 3.0 g of product. The combined batches (7.4 g, 52.6%) were suspended in 220 mL of methanol/DMF (10:1), heated on a steam bath, and filtered to yield 5.11 g of pure colorless product, mp 235–237 °C. Anal. Calcd for $C_{26}H_{32}N_6O_6 \cdot 0.5H_2O$: C, 59.40; H, 6.51; N, 15.96. Found: C, 59.70; H, 6.17; N, 15.86.

CP63,155 was then prepared by deprotection of the *p*-amino moiety as follows: A suspension of 2-[4-(Boc-*p*-aminobenzoyl)piperazin-1-yl]-4-amino-6,7-dimethoxyquinazoline (800 mg, 1.57 mmol) in 30 mL of 4 N HCl/dioxane was stirred at 0 °C for 2 h. The reaction was concentrated in vacuo to give a residue which was washed successively with EtOAc, ether, and ethanol. The resulting solids were suspended in ethanol, and the suspension was heated on a steam bath, cooled to room temperature, and filtered to give 621 mg of cream-colored product (80%), the dichloride salt of CP63,155, mp 237–241 °C. Anal. Calcd for $C_{21}H_{24}N_6O_3 \cdot 2HCl \cdot 0.5H_2O$: C, 51.43; H, 5.55; N, 17.17. Found: C, 51.05; H, 5.64; N, 16.88.

2-[4-(4-Amino-3-iodobenzoyl)piperazin-1-yl]-4-amino-6,7-dimethoxyquinazoline (CP63,789). To a solution of CP63,155 dihydrochloride hemihydrate (258 mg, 0.526 mmol) in 19 mL of 0.1 N HCl was added 53 mL of 0.5 M NaOAc buffer, and the pH of the resulting cloudy suspension was adjusted to 5.6 with 1 N NaOH. To this suspension was added concurrently a solution of Chloramine-T (343 mg, 1.22 mmol) in 50 mL of H_2O and a solution of NaI (161 mg, 1.07 mmol) in 10 mL of H_2O. The red-brown cloudy suspension was stirred for 2 h. The reaction was then quenched with sodium metabisulfite (335 mg, 1.76 mmol) and the pH adjusted to 8.5 with 1 N NaOH. The resulting mixture was extracted with $CHCl_3$/ethanol (5:3, 3 × 50 mL), and the combined extracts were dried over anhydrous $MgSO_4$, filtered, and evaporated to give 546 mg of a solid residue. These solids were purified by flash column chromatography on silica gel (20 g, Brinkmann 230–400 mesh) and eluted with a gradient of 2%, 5%, 10%, and 20% $CHCl_3$ in ethanol. The fractions containing product were collected and evaporated to give 103 mg (36.6%) of a tan solid. A portion of this solid (39 mg) was recrystallized from ethanol/H_2O to yield 20 mg of tan crystals, mp 279–281 °C: thin-layer chromatography (TLC) ($CHCl_3$/ethanol, 5:1) CP63,789 R_f 0.43, CP63,155 R_f 0.30, (CH_2Cl_2/methanol, 9:1) CP63,789 R_f 0.36, CP63,155 R_f 0.26; NMR ($CDCl_3$) 3.7–3.91 (8 H, multiplet, aliphatic), 3.94 (3 H, singlet, OCH_3), 3.98 (3 H, singlet, OCH_3) 4.36 (2 H, singlet, NH_2), 5.12 (2 H, singlet, NH_2), 6.75 (1 H, doublet, aromatic), 6.78 (1 H, singlet, aromatic), 6.92 (1 H, singlet, aromatic), 7.31 (1 H, multiplet, aromatic), 7.81 (1 H, doublet, aromatic). Anal. Calcd for $C_{21}H_{23}O_3N_6I \cdot 0.5C_2H_5OH$: C, 47.47; H, 4.70; N, 15.08. Found: C, 47.46; H, 4.92; N, 15.16. High-Resolution Mass Spectrum Calcd for $C_{21}H_{23}O_3N_6I$: 534.0877. Found: 534.0867.

2-[4-(4-Azido-3-iodobenzoyl)piperazin-1-yl]-4-amino-6,7-dimethoxyquinazoline (CP65,526). To a suspension of CP63,789 (53.4 mg, 0.1 mmol) in 2 mL of H_2O cooled to 0–5 °C with an ice bath was added 1 N HCl (0.8 mL, 0.8 mmol). A freshly prepared 0.5 M $NaNO_2$ solution (0.4 mL, 0.2 mmol) was added dropwise and the resulting yellow suspension stirred for 7 min. A freshly prepared 0.5 M NaN_3 solution (0.4 mL, 0.2 mmol) was then added dropwise. The resulting creamy white suspension was stirred for 5 min and then quenched by the dropwise addition of 1 N NH_4OH (0.8 mL, 0.8 mmol) and extracted with $CHCl_3$/ethanol (5:3), 3 × 5 mL. The combined extracts were dried over anhydrous $MgSO_4$, filtered, and evaporated to give 47.9 mg of an amorphous solid. This material was purified via flash column chromatography on silica gel (20 g, Brinkmann 230–400 mesh) and eluted with 2% ethanol in $CHCl_3$. The fractions containing product were collected and evaporated to give 19 mg (33.9%) of a tan solid, mp 250 °C dec. A sample was purified for analysis by HPLC on a Zorbax C-8 analytical column, using as eluant a 40:60 mixture of acetonitrile/0.1 M KH_2PO_4 buffer (pH 2.1): NMR ($CDCl_3$) 3.40–4.20 (8 H, multiplet, aliphatic), 3.95 (3 H, singlet, OCH_3), 3.99 (3 H, singlet, OCH_3), 5.16 (2 H,

singlet, NH_2), 6.79 (1 H, singlet, aromatic), 6.92 (1 H, singlet, aromatic), 7.19 (1 H, doublet, aromatic), 7.5 (1 H, multiplet, aromatic), 7.9 (1 H, doublet, aromatic); IR (KBr) 2100, 2140 cm^{-1} (N≡N); UV (MeOH) λ_{max} 253, 273, 340 (ϵ 66, 951; 31, 167; 6. 925); mass spectrum, *m/e* 560 (M+). Anal. Calcd for $C_{21}H_{21}O_3N_8I$: C, 45.01; H, 3.78; N, 20.00. Found: C, 44.96; H, 3.83; N, 19.64.

Instrumentation. NMR spectra were recorded on a Bruker WM250 spectrometer, IR spectra were recorded on a Perkin-Elmer 283B recording infrared spectrometer, and UV spectra were recorded on a Hewlett-Packard 8450A array spectrometer.

Radioiodination of CP63,155. Radioiodination of 2-[4-(4-aminobenzoyl)piperazin-1-yl]-4-amino-6,7-dimethoxyquinazoline (CP63,155) was performed by mixing 6 μL of CP63,155 (1 μg/μL of H_2O, 11.5 nmol) with 0.5 M sodium acetate buffer (30 μL, pH 5.6) and carrier-free $Na^{125}I$ (3 mCi in 0.1 N NaOH). The reaction was started by the addition of 6 μL of Chloramine-T (1 μg/μL of H_2O, 21 nmol). After 10 min, the reaction was stopped by the addition of 8 μL of sodium metabisulfite (1 μg/μL, 42 nmol). The resulting product, [^{125}I]CP63,789 [2-[4-(4-amino-3-[^{125}I]iodobenzoyl)piperazin-1-yl]-4-amino-6,7-dimethoxyquinazoline], was separated from unreacted CP63,155 and $Na^{125}I$ by HPLC on an ODS 5-μm reverse phase column (Altex) using a Beckman Model 420 system attached to a variable wavelength spectrophotometer (Hewlett-Packard Model 8450A), computer (Hewlett-Packard Model 85), and data plotter (Hewlett-Packard Model 7225 B) and a stepwise CH_3CN gradient at a flow rate of 2 mL/min (Figure 2). CP63,789 and the radioiodinated product, [^{125}I]CP63,789, moved with identical R_f values, as determined by radiochromatogram scanning and radioautography of the TLC plates.

Preparation of [^{125}I]CP65,526. [^{125}I]CP63,789 (0.5–1.0 mCi, 0.23–0.45 nmol) in CH_3CN/H_2O was evaporated to dryness under N_2. The compound was resuspended in 20 μL of 1 N HCl and then diazotized on ice in the dark by reaction with 10 μL of 0.5 M $NaNO_2$ for 5 min. Conversion to the aryl azide was then achieved by addition of 10 μL of 0.5 M NaN_3 for 7 min. The reaction was then quenched with 15 μL of NH_4OH and the resulting product, 2-[4-(azido-3-[^{125}I]-iodobenzoyl)piperazin-1-yl)-4-amino-6,7-dimethoxyquinazoline ([^{125}I]CP65,526), purified by HPLC, as described above, in the absence of a UV monitor. A single peak of radioactivity was recovered by this procedure and was found to elute in an identical volume to that determined by UV monitoring of [^{125}I]CP65,526 (Figure 2) prepared as described above, but with NaI rather than Na[^{125}I].

Membrane Preparation. Membranes were prepared by a modification (Wolfe et al., 1976) of the Neville procedure (Neville, 1968). Briefly, the livers from young (170–200 g) female Sprague-Dawley (Charles River) rats were excised and placed in cold 1 mM $NaHCO_3$ buffer containing the protease inhibitors 5 mM ethylenediaminetetraacetic acid (EDTA), 0.02% NaN_3, 1 mM PMSF, 10 μg/mL bacitracin, and soybean trypsin inhibitor, 1 μg/mL. The tissue was minced, homogenized with a Dounce tissue grinder, and filtered twice through eight layers of cheesecloth. The protease inhibitors were maintained in all subsequent steps. The filtrate was diluted with buffer (15 mL/g weight of liver) and stirred in the cold for 5 min. Membranes were pelleted by centrifugation at 4000*g* and resuspended in 69% (w/w) sucrose to produce a final sucrose concentration of 47.5% (w/w). The slurry was well stirred, and 22-mL aliquots were transferred to Beckman ultraclear centrifuge tubes. Sixteen milliliters of 42.5% sucrose was layered on top, and the tubes were spun at 100000*g* for 2 h in an SW 28 rotor (Beckman). Membranes were extracted from the upper surface into 0.9% NaCl, washed twice, and stored frozen (liquid N_2) at a concentration of 0.7 mg/mL in 100 mM Tris, pH 7.4, 5 mM EDTA, and 1 mM $MgCl_2$ containing the protease inhibitors (buffer B). Prior to use, the membranes were washed and homogenized in buffer B. Protein concentrations were determined by the method of Lowry (1951).

Binding Studies. Typical binding experiments employed 25–50 μg of protein per assay in 100 μL of buffer B. Membranes were incubated with shaking, in test tubes containing 25 μL of [^{125}I]CP63,789, [^{125}I]CP65,526, or [^{3}H]prazosin and either 25 μL of buffer B or 25 μL of competing ligand. After 30 min at room temperature, the reaction mixture was washed with 3 × 4 mL aliquots of buffer B onto glass 24-mm filters and bound ligand separated by vacuum filtration as previously described (Hess et al., 1983). Filters were counted directly for ^{125}I in a γ spectrometer (Micromedic) at 80% efficiency or dissolved in 10 mL of Hydrofluor scintillation fluid for the ^{3}H samples and counted in a liquid scintillation spectrometer (Packard) with a counting efficiency of 50%. Specific binding was determined as previously described (Graham et al., 1982b) with 10^{-5} M prazosin. Data derived from these binding studies were analyzed by a computerized-iterative nonlinear curve fitting program (Munson et al., 1980). Association and dissociation rate constants were determined by graphical analysis of data derived from kinetic studies using published methods (Schmitz et al., 1981).

Covalent Labeling of α_1 Receptors and SDS–PAGE. Membranes (300 μg in 100 μL of buffer B) were incubated with 25 μL of [^{125}]CP65,526 in the presence or absence of 25 μL of various concentrations of competing ligand. The mixture was incubated with shaking in the dark at room temperature for 45 min and then diluted to 1.5 mL with cold buffer B containing a competing ligand. Samples were photolyzed on ice for 15 s, 10 cm from a Hanovia mercury vapor lamp (450 W). Membranes were pelleted in an Eppendorf microfuge and solubilized in sample buffer: 1 g of SDS dissolved in 24.75% 1 M Tris (pH 6.8), 50% glycerol, 25% β-mercaptoethanol, and 0.25% bromphenol blue. Samples were solubilized with or without boiling and applied to a 10% acrylamide gel. Gels were run in Tris–glycine–SDS buffer according to the Laemmli protocol (Laemmli, 1970) in a Hoeffer apparatus at a constant current of 25 mA/gel. Gels were dried on a Bio-Rad slab Drier, and autoradiography was performed by exposing Kodak XAR-5 film on the dried gels at −70 °C for 3–9 days.

Results

Purification of the ^{125}-labeled compounds could be achieved by TLC using the solvent systems described above. When larger quantities (25 μg) of the nonradioactive CP63,789 compound were separated by TLC, following iodination of CP63,155 with NaI, and then eluted and subjected to subsequent HPLC purification, contaminating CP63,155 could still be detected. Moreover, [^{125}I]CP63,789, prepared by TLC separation, identified one-sixth of the binding sites observed with [^{3}H]prazosin, whereas comparable numbers of binding sites per milligram of protein were observed with [^{125}I]-CP63,789 prepared by HPLC purification (Figure 3). As shown in Figure 2, HPLC permitted complete resolution of the iodinated derivatives from the parent compounds. For this reason, and as carrier-free $Na^{125}I$ was used in the radioiodination procedure, a specific activity of 2175 Ci/mmol was assumed for the resulting ^{125}I-labeled compounds.

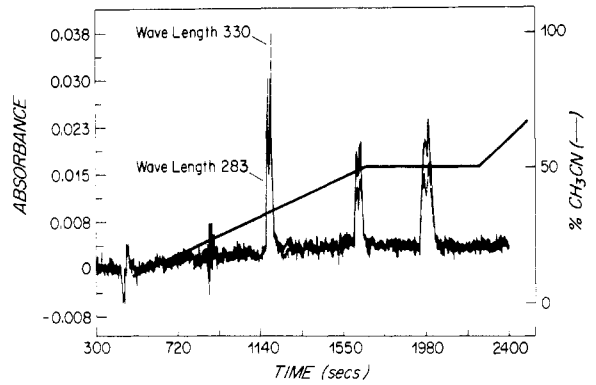

FIGURE 2: HPLC (from left to right) separation of amino (CP63,155), iodoamino (CP63,789), and iodoazido (CP65,526) analogues of prazosin. Separation was achieved by using an ODS 5-μm reverse-phase column and a stepwise CH_3CN gradient as indicated at a flow rate of 2 mL/min.

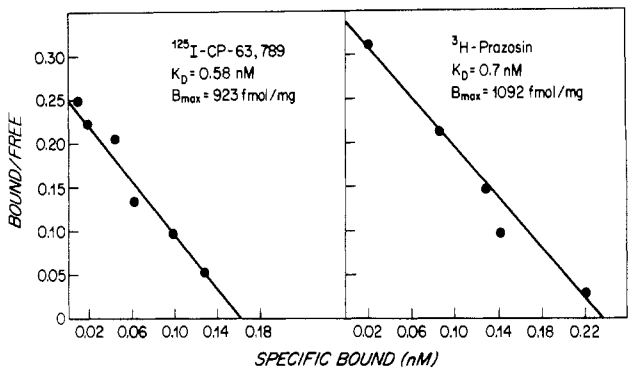

FIGURE 3: Scatchard plots comparing [^{125}I]CP63,789 and [^{3}H]prazosin: 25 μg of rat hepatic membranes were incubated with multiple concentrations of [^{125}I]CP63,789 in the presence or absence of excess cold prazosin (10^{-5} M) as described under Materials and Methods. Similar binding studies were performed with [^{3}H]prazosin and 32 μg of rat hepatic membranes. Reaction mixtures were incubated for 45 min at 21 °C and terminated by vacuum filtration. Points are mean from triplicate determinations.

Scatchard plots of equilibrium binding studies with [^{125}I]-CP63,789 or [^{125}I]CP65,526 revealed saturable high-affinity binding to a single class of sites in rat hepatic membranes as illustrated for the former in Figure 3. The K_D values determined from these studies were 0.6 nM and 0.3 nM, respectively. In parallel studies using the same membrane preparations, similar binding site concentrations were observed with the parent radioligand [^{3}H]prazosin (K_D = 0.7 nM). The kinetics of [^{125}I]CP63,789 binding to α_1 receptors is shown in Figure 4. Analysis of these studies revealed an association rate constant k_1 of 2.44 ± 0.25 (SE) × 10^8 M^{-1} min^{-1} and a dissociation constant (k_2) of 0.0415 ± 0.0075 min^{-1}, giving a $K_D = k_2/k_1$ = 0.17 nM, which is in agreement with that obtained from the equilibrium binding studies.

The specificity of [^{125}I]CP63,789 and [^{125}I]CP65,526 binding to the hepatic membrane was examined by competitive inhibition studies with a variety of adrenergic agents. As shown in Table I, these studies indicate that [^{125}I]CP63,789 binds to sites with an α_1-adrenergic specificity as the alpha-1 α_1-selective antagonist, prazosin, competes with higher affinity than the nonselective antagonist phentolamine or the α_2-selective antagonist rauwolscine (Starke & Docherty, 1982; Timmermans & Zwieten, 1982). Additionally the α agonist (−)- and (+)-epinephrine compete with higher affinity than the β-agonist (−)-isoproterenol. Stereospecificity is also confirmed by the finding of a higher IC_{50} for (+)-epinephrine than for the (−) stereoisomer. A similar rank order of potency (IC_{50}

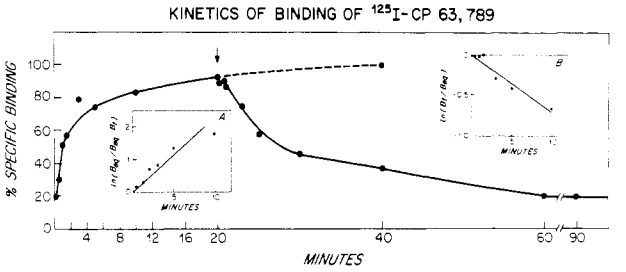

FIGURE 4: Kinetics of [^{125}]CP63,789 binding. (A) Association: 100 μg of rat hepatic membranes were added to test tubes containing 0.3 nM [^{125}I]CP63,789 and either 10^{-5} M prazosin or buffer B. The reaction mixture was incubated and terminated by vacuum filtration, at the time points indicated. (B) Dissociation: The reaction mixture defined above was preincubated for 45 min, then diluted 1000-fold with buffer as indicated by the arrow, and terminated by vacuum filtration at the time point indicated. Association and dissociation rate constants were calculated as previously described (Schmitz et al., 1981). The apparent rate constant (k_{ap}) for the pseudo-first-order association reaction was calculated from the slope of the line (insert A) relating ln $[B_{eq}/(B_{eq} - B_t)]$ and time (determined by linear regression analysis), where B_{eq} is the amount of [^{125}I]CP63,789 bound at equilibrium and B_t is the amount bound at each time t. The second-order rate constant, k_1, was calculated according to the equation $k_1 = (k_{ap} - k_2)/[[^{125}I]CP63,789]$, where the denominator is the concentration of iodinated ligand used in the assay and k_2 is the dissociation rate constant. This latter constant was determined from the dissociation reaction, where k_2 is the slope of the line (insert B) relating ln (B_t/B_0) and time (determined by linear regression analysis), where B_t is the amount of specific binding at each time t and B_0 is the amount of binding immediately prior to the 1000-fold dilution.

Table I: Competition by Adrenergic Ligands for the Binding of [^{125}I]CP63,789 to Rat Hepatic Membranes[a]

ligand	IC_{50}[b] (M)	ligand	IC_{50}[b] (M)
prazosin	10^{-9}	(+)-epinephrine	5×10^{-5}
phentolamine	8×10^{-7}	rauwolscine	4×10^{-4}
(−)-epinephrine	5×10^{-6}	isoproterenol	5×10^{-3}

[a] A total of 100 μg of rat hepatic membranes was incubated with 25 μL of 1.8 nM (0.3 nM final) [^{125}I]CP63,789 and 25 μL of buffer B or competing ligand, yielding a final volume of 150 μL. Reaction mixture was incubated at 21 °C for 45 min and terminated by vacuum filtration. [b] Fifty percent inhibitory concentration.

antagonists prazosin, 8×10^{-10} M; phentolamine, 3×10^{-7} M; rauwolscine, 10^{-4} M; agonists (−)-epinephrine, 2×10^{-6} M; (+)-epinephrine, 6×10^{-5} M; (−)-isoproterenol, 3×10^{-3} M) was observed in studies using [^{125}I]CP65,526 (0.3 nM) as the radioligand.

To further investigate the α_1-adrenergic specificity of the binding by the ^{125}I-labeled compounds to hepatic membranes, additional competitive inhibition studies were performed with the dopamine antagonist (−)-haloperidol, the serotonin antagonist methylsergide, and the calcium blocker nitrendipine. None of these compounds significantly inhibited binding of the ^{125}I analogues when used in concentrations less than 10^{-5} M. Verapamil, a calcium antagonist structurally different from nitrendipine, was also investigated for its ability to inhibit [^{125}]CP65,526 binding to hepatic membranes. This agent, in addition to being a calcium channel antagonist, has previously been shown to bind at reasonably low concentrations (1 μM) to both α_1- and α_2-adrenergic receptors (Motulsky et al., 1983). In keeping with these observations, we found that verapamil inhibited [^{125}]CP65,526 binding with an IC_{50} of 2.5×10^{-6} M. In additional studies, see below, we investigated the ability of this compound to inhibit covalent labeling of the membrane receptors by the photoaffinity probe [^{125}]CP63,789. In these studies, the inhibition of [^{125}]CP63,789 labeling by verapamil at concentrations of 10^{-4}–10^{-6} M was similar to that seen with prazosin.

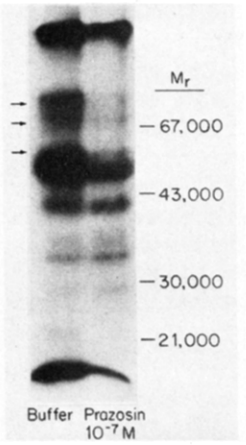

FIGURE 5: Autoradiograph from SDS–PAGE of [^{125}I]CP65,526-labeled rat hepatic membranes: Membranes were reacted with [^{125}I]CP65,526 in the presence or absence of 10^{-7} M prazosin as described under Materials and Methods. Arrows indicate specifically labeled peptides at 77, 68, and 59 kdaltons.

Photoaffinity Labeling of the α_1 Receptor. Conversion of [^{125}I]CP63,789 to the aryl azide permitted direct labeling of the α_1-adrenergic receptor. Figure 5 shows an autoradiograph of an SDS–PAGE of hepatic membranes labeled with [^{125}I]CP65,526 in the presence and absence of prazosin. While multiple protein species are labeled, three principal bands are inhibited by 10^{-7} M prazosin. These hormone binding protein species have molecular weights of 77K, 68K, and 59K. To further substantiate that these proteins represent α_1 hormone binding subunits, SDS–PAGE analyses of membranes labeled with [^{125}]CP65,526 in the presence of a variety of competing agonists and antagonists were performed. Figure 6 shows the autoradiograph of such an experiment. Only the 77K, 68K, and 59K peptides are specifically labeled, as indicated by appropriate inhibition by a variety of α-adrenergic ligands. Prazosin inhibits labeling of these proteins in a dose-related fashion typical of the α_1 receptor. Covalent incorporation of [^{125}I]CP65,526 into these bands is also attenuated by phentolamine and epinephrine, but not by the β-adrenergic antagonist, (−)-alprenolol. Stereospecificity of labeling is apparent by the more marked inhibition with (−)-epinephrine than with its (+) stereoisomer. A band at approximately 52K is consistently labeled by [^{125}I]CP65,526. However, specific inhibition of this labeling by adrenergic ligands was inconsistently achieved and typically incomplete. The use of lower percentage acrylamide (7%) or gradient (3–20%) gels did not result in better resolution of the species labeled in this molecular weight range.

Additionally, it is evident (Figure 6) that certain bands such as that at 33K are not inhibited by any of the ligands. This peptide, which clearly binds [^{125}I]CP65,526 but not other α-adrenergic agents, serves as an internal control to indicate that the samples applied to the gels contained similar protein and ^{125}I-labeled azide concentrations. Different protocols for solubilizing the photolabeled membranes, prior to electrophoresis, were employed with SDS or urea. Labeling was also performed in the presence or absence of β-mercaptoethanol. In these studies, the molecular weights of the peptide species identified by the ^{125}I-labeled aryl azide were unchanged.

Three smaller molecular weight species (42K, 31K, and 26K; Figure 6) are also labeled by [^{125}I]CP65,526, and inhibition of labeling by prazosin is observed. The band at 26K is not as clearly evident in Figure 5 as in Figure 6. This may be due to stronger autoradiographic exposure of the latter. Nevertheless, it is clear that incorporation of [^{125}I]CP65,526

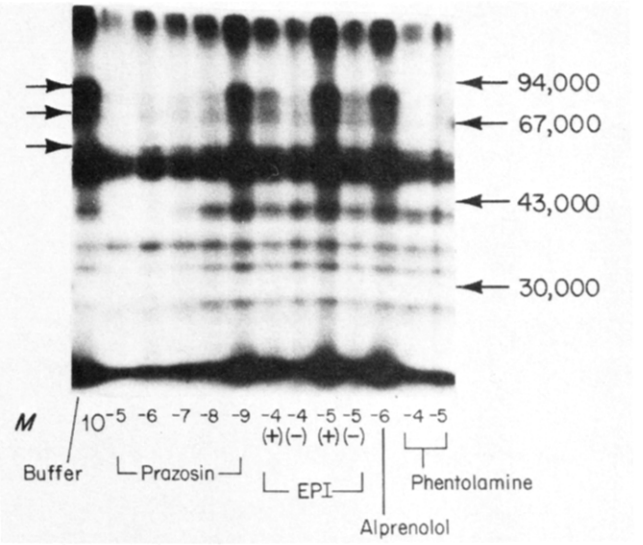

FIGURE 6: Autoradiograph from SDS–PAGE of α_1 receptor labeled with [^{125}I]CP65,526. Membranes prepared with protease inhibitors and labeled as described under Materials and Methods. Competing ligands used per lane as follows: lane 1, no competing ligand; lane 2, prazosin, 10^{-5} M; lane 3, prazosin, 10^{-6} M; lane 4, prazosin, 10^{-7} M; lane 5, prazosin, 10^{-8} M; lane 6, prazosin, 10^{-9} M; lane 7, (+)-epinephrine, 10^{-4} M; lane 8, (−)-epinephrine, 10^{-4} M; lane 9, (+)-epinephrine, 10^{-5} M; lane 10, (−)-epinephrine, 10^{-5} M; lane 11, alprenolol, 10^{-6} M; lane 12, phentolamine, 10^{-4} M; lane 13, phentolamine, 10^{-5} M. Arrows indicate specifically labeled peptides at 77, 68, and 59 kdaltons.

into this peptide species, as well as those at 42 and 31 kdalton is not inhibitable by other α-adrenergic specific ligands. Despite the use of protease inhibitors during the membrane preparation, it is possible that the appearance of the 68 and 59 kdalton peptides was the result of proteolytic degradation of a larger species. To examine this possibility, the following experiments were performed. Prior to excision, rat livers were perfused in situ with buffers containing multiple protease inhibitors, to eliminate plasma containing proteolytic enzymes. Membranes were then prepared as described under Materials and Methods. In another preparation, rat livers were perfused and membranes were made with buffers, excluding protease inhibitors. If proteolytic degradation was promoting the appearance of the 68 and 59 kdalton peptides, then in this latter preparation the visualization of these species would be more apparent. Figure 7 shows membranes prepared following perfusion with buffers containing no protease inhibitors. The 77, 68, and 59 kdalton species are clearly seen, with no alteration in the overall banding pattern, as compared to those gels using membranes prepared with protease inhibitors. The lower molecular weight proteins (42K, 31K, and 26K) are again noted to be inhibited by prazosin, but not by epinephrine or phentolamine. The similarity of labeling in membranes prepared with and without protease inhibitors is clearly apparent. It is unlikely, therefore, that the smaller molecular weight peptides (42K, 31K, and 26K) are generated by proteolysis.

Discussion

In the present study, the synthesis and characteristics of analogues of the highly selective α_1-adrenergic antagonist prazosin are described. These analogues after radioiodination and purification by HPLC, yield ligands of high specific radioactivity (2175 Ci/mmol, assumed). Competitive binding studies using well-defined adrenergic ligands demonstrate that [^{125}I]CP63,789 has a specificity and stereoselectivity typical of an α_1-adrenergic ligand. The conversion of ^{125}I-labeled

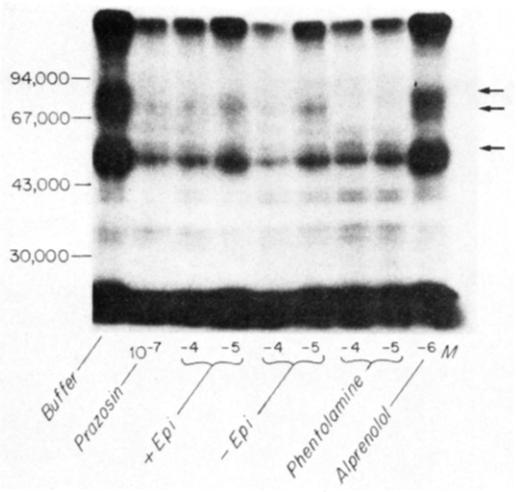

FIGURE 7: Autoradiograph from SDS–PAGE of [^{125}I]CP65,526-labeled hepatic membranes prepared without multiple protease inhibitors. Competing ligands used per lane as follows: lane 1, no competing ligands; lane 2, prazosin 10^{-7} M; lane 3, (+)-epinephrine, 10^{-4} M; lane 4, (+)-epinephrine, 10^{-5} M; lane 5, (−)-epinephrine, 10^{-4} M; lane 6, (−)-epinephrine, 10^{-5} M; lane 7, phentolamine, 10^{-4} M; lane 8, phentolamine, 10^{-5} M; lane 9, alprenolol, 10^{-6} M. Arrows indicate specifically labeled peptides at 77, 68, and 59 kdaltons.

arylamine to the ^{125}I-labeled aryl azide permitted covalent labeling of the receptor by photolysis with UV light. Three major hormone binding proteins with molecular weights of 77K, 68K, and 59K were detected which demonstrated an affinity and stereoselectivity for α_1 ligands comparable to that seen in direct binding studies. While hormone binding capacity typical of the α_1 receptor is retained, it is possible that the 59 and 68 kdalton species are proteolytic fragments. However, extensive use of proteolytic inhibitors and in situ perfusion of the rat livers with protease-containing buffers, prior to membrane preparation and photolabeling, did not prevent the identification of the lower molecular weight species.

Several smaller molecular weight peptides (M_r 42K, 33K, and 26K) bind [^{125}I]CP56,526, and this binding is inhibited by prazosin. However, the lack of inhibition by other α ligands including epinephrine and phentolamine indicates that these species are not subunits of the α_1 receptor protein.

Previous studies examining the molecular characteristics of the α_1-adrenergic receptor have yielded conflicting results. Guellaen et al. (1982) reported that rat hepatic α receptors have a molecular weight of 45K by SDS–PAGE. Kunos et al. (1983) reported an 85 kdalton species. Both groups labeled rat hepatic membranes with [^{3}H]phenoxybenzamine. However, this agent lacks selectivity for the α_1 receptor as it also binds to other monoaminergic receptors (Hess et al., 1983). The low specific activity of this ligand may also have prevented direct identification of the covalently labeled proteins by autoradiography.

We have previously reported the isolation and purification of the α_1-receptor hormone binding subunit. When purified to homogeneity by affinity and gel filtration chromatography, a single protein band of 59 kdalton was visualized by SDS–PAGE and Coomassie blue staining. Although a 59K protein has now been identified by both techniques as a component of the α_1 receptor, the heterogeneity of binding sites seen in our photoaffinity labeling experiments was not apparent in the purified preparation. One or more mechanisms may explain this finding. For example, proteolysis, which occurs despite the use of multiple inhibitors, may have taken place to a greater extent during the purification protocol, resulting in a predominance of the 59K protein. An additional possibility is that the small quantity of receptor purified precluded detection of other species. This discrepancy will require that the receptor protein(s) be purified in much larger quantities so that detailed structural analysis can be carried out.

The newly synthesized aryl azide analogue of prazosin, [^{125}I]CP65,526, should provide a facile approach for comparing the molecular properties of α_1 receptors from a wide variety of different tissues and species. It should also prove useful in elucidating the molecular mechanisms underlying the cellular regulation of α_1-receptor function.

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