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The Mammalian β_2-Adrenergic Receptor: Purification and Characterization†

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ABSTRACT: The β_2-adrenergic receptors from hamster, guinea pig, and rat lungs have been solubilized with digitonin and purified by sequential Sepharose–alprenolol affinity and high-performance steric-exclusion liquid chromatography. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and autoradiography of iodinated purified receptor preparations reveal a peptide with an apparent M_r of 64 000 in all three systems that coincides with the peptide labeled by the specific β-adrenergic photoaffinity probe (*p*-azido-*m*-[^{125}I]iodobenzyl)carazolol. A single polypeptide was observed in all three systems, suggesting that lower molecular weight peptides identified previously by affinity labeling or purification in mammalian systems may represent proteolyzed forms of the receptor. Purification of the β-adrenergic receptor has also been assessed by silver staining, iodinated lectin binding, and measurement of the specific activity (~15 000 pmol of [^{3}H]dihydroalprenolol bound/mg of protein). Overall yields approximate 10% of the initial crude particulate binding, with 1–3 pmol of purified receptor obtained/g of tissue. The purified receptor preparations bind agonist and antagonist ligands with the expected β_2-adrenergic specificity and stereoselectivity. Peptide mapping and lectin binding studies of the hamster, guinea pig, and rat lung β_2-adrenergic receptors reveal significant similarities suggestive of evolutionary homology.

Previous studies of the β-adrenergic receptor from this laboratory have documented development of procedures for solubilization (Caron & Lefkowitz, 1976), affinity chromatography (Caron et al., 1979), and total purification of the receptor from both amphibian (Shorr et al., 1981, 1982a) and avian erythrocytes (Shorr et al., 1982b). While these sources have proven to be valuable model systems for study of the β-adrenergic receptor, the purification and characterization of mammalian β-adrenergic receptors is of potentially greater interest. Recently, the purification of the β_2-adrenergic receptor from canine lung has been described (Homcy et al., 1983). However, as judged by sodium dodecyl sulfate (SDS) gel electrophoresis, the polypeptide isolated by Homcy et al. (1983) was apparently smaller (i.e., M_r = 52 000–53 000) than that of the β_2-adrenergic receptor in several other mammalian systems as determined by photoaffinity labeling in membrane preparations (Lavin et al., 1982; Benovic et al., 1983; Stiles

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et al., 1983a). One potential explanation for this discrepancy is that the purified canine lung peptide may represent a proteolyzed form of the β-adrenergic receptor. We have shown recently that proteolysis appears to be a major contributing factor to the structural heterogeneity of the β-adrenergic receptor in several systems (Benovic et al., 1983; Stiles et al., 1983a,b). Purification of a mammalian β_1-adrenergic receptor has also recently been reported (Cubero & Malbon, 1984). That study documents the purification of a major M_r 67 000 peptide; however, Cubero and Malbon were unable to demonstrate labeling of this peptide with a β-adrenergic-specific photoaffinity probe.

Although the studies described above have provided initial information concerning the size of the β-adrenergic receptor from lower vertebrates and more recently from mammalian sources, the procedures utilized were tedious and time consuming, and the amounts of receptor available have been insufficient for detailed biochemical or functional characterization. Accordingly, the studies described here were undertaken to develop procedures for the rapid and high-yield purification of mammalian β-adrenergic receptors in quantities sufficient for rigorous investigation of structural and functional properties. In this paper we describe the successful purification, characterization, and comparison of the mammalian β-adrenergic receptor from several species. In the accompanying paper (Cerione et al., 1984), we directly document the functionality of the pure receptor by reconstitution of its hormone-promoted interactions with the pure guanine nucleotide regulatory protein of the adenylate cyclase system.

Experimental Procedures

Materials

Alprenolol hydrochloride was generously supplied by Hassle Pharmaceuticals. ICI 118,551 was a generous gift from Imperial Chemical Industries, while atenolol was from Stuart Pharmaceuticals. All other drugs were from sources previously described (Caron & Lefkowitz, 1976). Digitonin was obtained from Gallard-Schlesinger and prepared as described by Shorr et al. (1981). (−)-[^{3}H]Dihydroalprenolol ([^{3}H]DHA), (*p*-azido-*m*-[^{125}I]iodobenzyl)carazolol([^{125}I]pABC), (−)-[^{125}I]-iodocyanopindolol ([^{125}I]CYP), and carrier-free Na^{125}I were from New England Nuclear Corp. Hamster (*Cricetus auratus*, Golden Syrian), guinea pig (*Cavia porcellus*, mixed breed), and rat (*Rattus rattus*, Sprague-Dawley) lungs, which were immediately frozen in liquid nitrogen upon excision, were obtained from Pel-Freeze Biologicals. Sepharose CL-4B–alprenolol was prepared as previously described (Caron et al., 1979). Premixed electrophoresis standards (phosphorylase *b*, M_r 94 000; albumin, M_r 67 000; ovalbumin, M_r 45 000; carbonic anhydrase, M_r 30 000; soybean trypsin inhibitor, M_r 20 100; α-lactalbumin, M_r 14 400) were from Pharmacia and were iodinated by the chloramine T method (Greenwood et al., 1963). Electrophoresis reagents were from Bio-Rad Laboratories. X-ray film (XAR-5) and developing solutions were from Kodak while intensifying screens (Cronex Lightning Plus) were from Du Pont. Agarose-coupled lectins were from E-Y Laboratories. *Staphylococcus aureus* strain V-8 protease was from Miles Laboratories while trypsin, chymotrypsin, and papain were from Sigma. Ethylenediaminetetraacetic acid (EDTA) was from Mallinckrodt while other biochemical reagents were usually from Sigma.

Methods

Membrane Preparation. Hamster, guinea pig, and rat lung membranes were prepared from frozen lungs, which were thawed, dissected, and minced in 10 volumes of ice-cold buffer A [50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), 5 mM EDTA, 2 μg/mL leupeptin, 5 μg/mL soybean trypsin inhibitor, 15 μg/mL benzamidine, pH 7.2 at 22 °C]. Fifty-milliliter aliquots of the suspension were homogenized with three 10-s bursts of a tissue disrupter (Brinkmann PT 10/35 homogenizer with a PTA 20TS probe) at a setting of 10 (crude homogenate). The homogenate was then centrifuged at 200*g* (1000 rpm; Sorvall RT6000 centrifuge) for 10 min. The pellet from this low-speed centrifugation was homogenized in 10 volumes of buffer A in a motor-driven Teflon–glass homogenizer (24 × 200 mm grinding chamber, 0.1-mm clearance) before being recentrifuged at 200*g* for 10 min. The supernatants from these two spins were pooled and centrifuged at 48000*g* (19 000 rpm; Sorvall RC-5B centrifuge with a SS-34 rotor) for 20 min. The pelleted membranes were then washed twice before being resuspended in 5 volumes of buffer A. Membranes were used immediately or were frozen in liquid nitrogen and stored at −90 °C.

Solubilization Procedures. Typically, 200 mL of membrane suspension were pelleted by centrifugation, homogenized with a Dounce homogenizer (10 strokes) in 40 mL of ice-cold "low-digitonin" buffer (0.2–0.3% digitonin, 100 mM NaCl, 10 mM Tris-HCl, 5 mM EDTA, 2 μg/mL leupeptin, 5 μg/mL soybean trypsin inhibitor, 15 μg/mL benzamidine, pH 7.2), and brought to a final volume of 200 mL with the same buffer. The suspension was stirred for 20 min at 0–4 °C and was then centrifuged at 48000*g* for 20 min. The pellets were resuspended in 80 mL of ice-cold "high-digitonin" buffer (as above except contains 1.2% digitonin), Dounce homogenized, and centrifuged at 48000*g* for 10 min. Pellets were resuspended in 80 mL of buffer, again Dounce homogenized, and added to the supernatant from the first extraction. Final volumes were then adjusted to 200 mL with the high-digitonin buffer, and the suspension was stirred at 0–4 °C for 30 min. Particulate material was then removed by centrifugation at 48000*g* for 20 min. Centrifugation of the solubilized receptor preparation at 350000*g* for 90 min resulted in only a slight loss of [^{3}H]DHA binding sites (<2%), indicating that the receptor is indeed soluble.

Sepharose–Alprenolol Chromatography. Solubilized β-adrenergic receptor activity (200–300 mL) was loaded at 25 °C onto a 200-mL column of Sepharose CL-4B–alprenolol at 120–150 mL/h. The column was washed at 0–4 °C with 1 column volume of 500 mM NaCl, 50 mM Tris-HCl, 0.5% digitonin, and 2 mM EDTA, pH 7.2 (22 °C), followed by 1 column volume of 100 mM NaCl, 10 mM Tris-HCl, 0.05% digitonin, and 2 mM EDTA, pH 7.2 (22 °C). The column temperature was returned to 25 °C, and receptor activity was eluted with a 400-mL linear gradient of 0–40 μM (±)-alprenolol in the low salt wash buffer. Typically, the first 170–180 mL of the eluate was discarded while the next 180–220 mL was pooled and concentrated to 2–3 mL in an Amicon concentrator with a YM-30 membrane.

Gel Permeation High-Performance Liquid Chromatography. Concentrated receptor from affinity column eluates was chromatographed on steric-exclusion columns by using two 60-cm TSK-4000 and one 60-cm TSK-3000 columns tandem linked. The mobile phase contained 0.1% digitonin and 100 mM Tris–SO_4, pH 7.5 (22 °C), and was filtered through a 0.2-μm Millipore membrane before use. Samples (2 mL) were manually injected and chromatographed at a flow rate of 1 mL/min; 0.5-mL fractions were collected, assayed for receptor activity, and frozen in liquid nitrogen.

Receptor Assay. Soluble and particulate receptor assays using [^{3}H]DHA were as described previously (Caron &

Lefkowitz, 1976). Competition curves with [^{125}I]CYP were performed as described in Figure 4. Protein was determined by the amidoschwarz assay (Schaffner & Weissman, 1975) with bovine serum albumin in the range of 1–10 μg as standard. Typically, 0.5 mL of the concentrated Sepharose–alprenolol eluate (~3 μg) or 1 mL of the HPLC eluate (~2 μg) was concentrated to 0.25 mL (by lyophilization) before being assayed for protein.

Receptor Labeling. Purified receptor preparations (in 0.1% digitonin and 100 mM Tris–SO_4, pH 7.5 at 22 °C) at a concentration of 2–10 nM were incubated with 10 nM [^{125}I]pABC in the dark at 0–4 °C for 8–16 h or at 25 °C for 1–2 h. Free [^{125}I]pABC was removed by filtration over columns (0.7 × 12 cm) of Sephadex G-50. Labeled receptor was then photolyzed (in a 35 × 10 mm uncovered plastic Petri dish) for 60–90 s 12 cm from a Hanovia 450-W medium-pressure mercury arc lamp filtered with 5 mm of Pyrex glass.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE). Gel electrophoresis was performed on homogeneous slab gels (Laemmli, 1970). Sample buffer consisted of 8% SDS, 10% glycerol, 5% β-mercaptoethanol, 25 mM Tris-HCl, and 0.003% bromphenol blue, pH 6.5 (22 °C). After electrophoresis, gels were either stained as described below or were immediately dried with a Bio-Rad (Model 224) gel dryer prior to autoradiography at −90 °C.

Iodination of Purified Receptor Preparations. Aliquots of receptor purified by affinity chromatography were labeled with Na^{125}I by the chloramine T method (Greenwood et al., 1963). Tris-HCl (pH 7.2) at a final concentration of 75 mM served as buffer. After the labeling, unreacted Na^{125}I was removed by filtration over columns of Sephadex G-50, and the iodinated receptor was then separated from labeled detergent and other proteins by HPLC as described above. Alternatively, samples purified by affinity chromatography and HPLC could be iodinated directly followed by Sephadex G-50 filtration and SDS–PAGE.

Protein and Carbohydrate Staining. Sodium dodecyl sulfate–polyacrylamide gels were stained for protein by a silver staining method previously described (Merril et al., 1981). Polyacrylamide gels were also stained for carbohydrate by using iodinated lectin (Chu et al., 1981). Concanavalin A (Sigma) was iodinated to a specific activity of ~1 μCi/μg by the chloramine T method.

Enzymatic Digests. Aliquots (10–20 μL) of [^{125}I]pABC-labeled HPLC-purified rat, guinea pig, and hamster lung receptor (1–5 nM final concentration) were digested with 1.5 μg of *S. aureus* V-8 protease, 0.5 μg of chymotrypsin, 0.2 μg of trypsin, or 0.04 μg of papain for 15 min at 0–4 °C. Reactions were stopped by the addition of phenylmethanesulfonyl fluoride (PMSF) and *N*-ethylmaleimide to a final concentration of 0.1 mM. Electrophoresis sample buffer (20 μL) was added, and the digests were electrophoresed on 12% polyacrylamide gels.

Lectin Binding. Aliquots of iodinated purified receptor (400 μL in 75 mM NaCl, 32 mM Tris, and 0.025% digitonin, pH 7.4 at 22 °C) were incubated with 150 μL of agarose-coupled lectins for 60 min at 22 °C. The supernatants containing unadsorbed receptor were removed by centrifugation in a microfuge. The lectin gels were then washed 2 times with 1 mL of 0.01% digitonin, 100 mM NaCl, and 10 mM Tris-HCl, pH 7.2 (22 °C) buffer at 0–4 °C. Supernatants were counted for ^{125}I in an Auto 800 gamma counter. Bio-Gel A, containing no immobilized lectin, was used as a control for lectin binding. The lectins and their specificities are as follows: *Ricinus communis* (RCA-1), β-Gal; concanavalin A (Con A), α-D-Man > α-D-Glc > α-D-GlcNAc; *Limulus polyphemus* (LPA), sialic acid; *Ulex europaeus* (UEA-1), α-L-fucose; *Dolichos biflorus* (DBA), α-D-GalNAc; *Helix pomatia* (HPA), α-D-GlcNAc, α-D-GalNAc, α-D-Gal; *Arachis hypogaea* (PNA), D-Gal-β(1→3)-D-GalNAc > D-$GalNH_2$ = α-D-Gal; glycine max (SBA), α-D-GalNAc > β-D-GalNAc ≫ α-D-Gal; *Triticum vulgaris* (WGA), [β(1→4)-D-GlcNAc]$_2$, sialic acid.

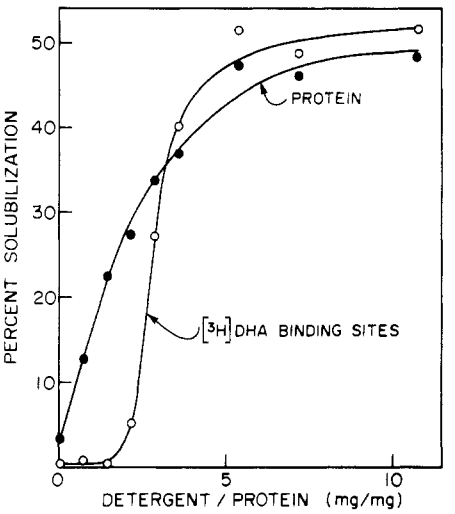

FIGURE 1: Solubilization of protein and [^{3}H]DHA binding sites as a function of the detergent to protein ratio. Hamster lung membranes (5 mL), prepared as described under Methods, were pelleted and resuspended in 5 mL of 100 mM NaCl and 10 mM Tris-HCl, pH 7.2 (22 °C), buffer containing varying levels of digitonin (0–1.5%). The suspensions were Dounce homogenized and stirred for 1 h at 4 °C. Following centrifugation, the supernatants were assayed for receptor by [^{3}H]DHA binding and for protein by the amidoschwarz method. The results are the mean of duplicate determinations and are representative of three separate experiments.

Results

Purification of the β_2-Adrenergic Receptor. Lung membranes (typically 200–300 mL prepared from 40–60 g of tissue) were solubilized with the detergent digitonin by utilizing differential solubilization at two digitonin concentrations. This procedure evolved from studies on β-adrenergic receptor solubilization as a function of the detergent to protein ratio. As shown in Figure 1, at detergent to protein ratios of 1–2, minimal amounts of [^{3}H]DHA binding sites are solubilized while significant levels of protein are solubilized. Increasing the detergent to protein ratio to 5–10 results in the solubilization of ~50% of the [^{3}H]DHA binding sites from the membrane. This differential solubilization procedure results in an approximate 2-fold purification of receptor (see Table I).

When the solubilized receptor preparations were chromatographed on Sepharose CL-4B–alprenolol columns, the bulk of the protein was unretarded while most of the receptor activity (typically >70%) was retained (Figure 2). Washing the column with a high-salt, high-digitonin buffer resulted in an ~5-fold improvement in the specific activity of the eluted receptor. Elution of the receptor with an alprenolol gradient vs. a front was also found to significantly improve the receptor specific activity (~8-fold). Protein determinations across the elution profile reveal that specific activities of the peak [^{3}H]DHA binding fractions are as high as 6000 pmol of binding sites/mg of protein. The corresponding SDS–PAGE profile of the iodinated affinity fractions (Figure 2, top inset) reveals major contaminating proteins at M_r ~45 000 and ~28 000 with the former appearing to coelute with the receptor. Yields at this step are typically 30–50% with an

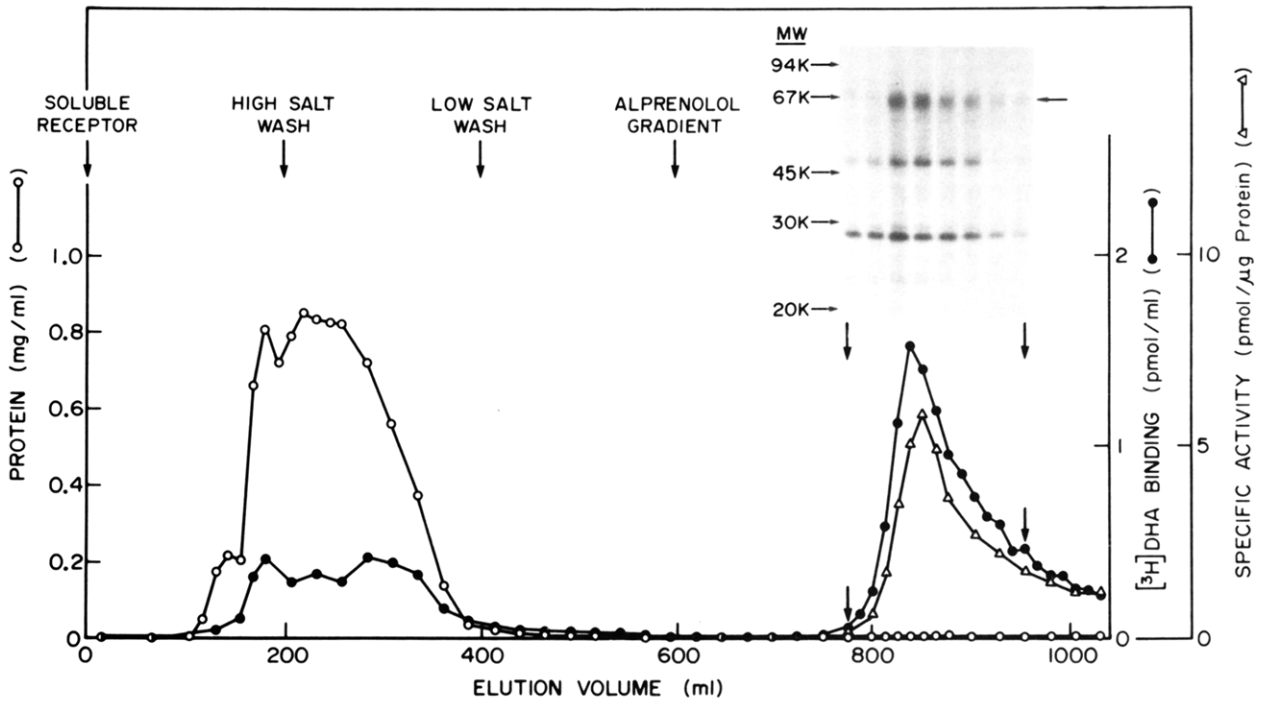

FIGURE 2: Sepharose–alprenolol chromatography of digitonin-solubilized hamster lung β-adrenergic receptor activity. Approximately 340 pmol of digitonin-solubilized hamster lung receptor (200 mL) was applied to a 200-mL column of Sepharose–alprenolol previously equilibrated with 100 mM NaCl, 10 mM Tris-HCl, 0.05% digitonin, and 2 mM EDTA, pH 7.2 (22 °C). The column was then washed and eluted as described under Methods. Individual fractions (12.9 mL) were assayed for receptor by [^{3}H]DHA binding (●) and for protein by the amidoschwarz assay (○). These results were used to calculate specific activities for the eluted receptor (△). Additionally, 300-μL aliquots of several alprenolol-eluted fractions were iodinated by the chloramine T method and electrophoresed on a 10% SDS–polyacrylamide gel. The autoradiogram of the dried gel is shown in the inset. The arrow indicates where [^{125}I]pABC-labeled hamster lung β-receptor electrophoresed in this experiment. The molecular weight standards (MW) are shown ×1000 (K). The alprenolol-eluted receptor activity in the experiment shown (160 pmol) represents a 47% recovery of the applied digitonin-solubilized receptor activity.

Table I: Purification of β-Adrenergic Receptor of Hamster Lung[a]

step	protein (mg)[b,c]	activity (pmol)[b,c]	yield (%) step	yield (%) overall	sp act. (pmol/mg of protein)[b]	x-fold purification step	x-fold purification overall
crude homogenate	2950	1300	100	100	0.44 ± 0.01	1	1
purified membranes	625	1065	82 ± 2	82	1.7 ± 0.1	3.8	3.8
digitonin extract	165	530	50 ± 2	41	3.2 ± 0.2	1.9	7.3
Sepharose–alprenolol eluate[d]	0.047	170	32 ± 2	13	3630 ± 710	1134	8250
HPLC pass	0.010	145	82 ± 4	11	14660 ± 2270	4.0	33320

[a] Typically, 40–60 g of tissue was homogenized yielding 26 ± 1 pmol of [^{3}H]DHA binding sites/g of hamster lung, 20 ± 2 pmol/g of guinea pig lung, and 13 ± 1 pmol/g of rat lung in the crude homogenate. After several centrifugation steps, purified membranes were solubilized with digitonin before undergoing successive Sepharose–alprenolol affinity and high-performance steric-exclusion chromatography as described under Methods. The results are for 50-g hamster lung preparations and are expressed as the means ± standard error as determined from a minimum of four separate experiments. [b] As measured by [^{3}H]DHA binding and amidoschwarz protein assay. [c] Average values, which varied ≤30% between preparations. [d] After ultrafiltration in an Amicon concentrator with a YM-30 membrane.

~1000-fold purification (see Table I).

As a final step of purification, concentrated eluates from the affinity chromatography gel were chromatographed on high-performance liquid chromatography steric-exclusion columns. Figure 3 shows a typical elution profile obtained when a hamster lung receptor preparation was chromatographed. Shown on the HPLC profile are prelabeling with the antagonist [^{125}I]CYP, postlabeling with the antagonist [^{3}H]DHA, and absorbance at 280 nm. Additionally, an aliquot (300 μL) of concentrated affinity-purified receptor was iodinated (see Methods) and chromatographed by HPLC. Subsequent SDS–PAGE of the iodinated HPLC fractions reveals that the peak receptor fractions are essentially homogeneous (Figure 3, top inset).

Table I summarizes the purification of hamster lung β_2-adrenergic receptor by these procedures. As shown, after complete purification a specific activity of ~15 000 pmol of binding sites/mg of protein is obtained. This represents an ~33 000-fold purification and an overall 11% recovery of binding sites from the crude hamster lung homogenates.

Identification of Purified Receptor Preparations as Containing β_2-Adrenergic Binding Sites. The specificity for binding ligands is a major criterion in establishing the functional integrity of purified receptor preparations. For the β-adrenergic receptor, this criterion has been very useful because of the availability of a wide variety of agonists and antagonists including several selective β_1 and β_2 antagonists (Barratt et al., 1973; Bilski et al., 1980). Thus, as shown in Figure 4, when the specificity of binding of [^{125}I]CYP to an HPLC-purified hamster lung receptor preparation was examined, competition of binding by agonists and antagonists revealed a typical β_2-adrenergic order of potency (isoproterenol > epinephrine > norepinephrine and ICI 118,551 ≫ atenolol). In addition, purified receptor preparations demonstrated binding characteristics similar to both the membrane and soluble preparations as shown in Table II. It is evident from these results that stereospecificity and rank order of potency are maintained throughout the purification procedures. This

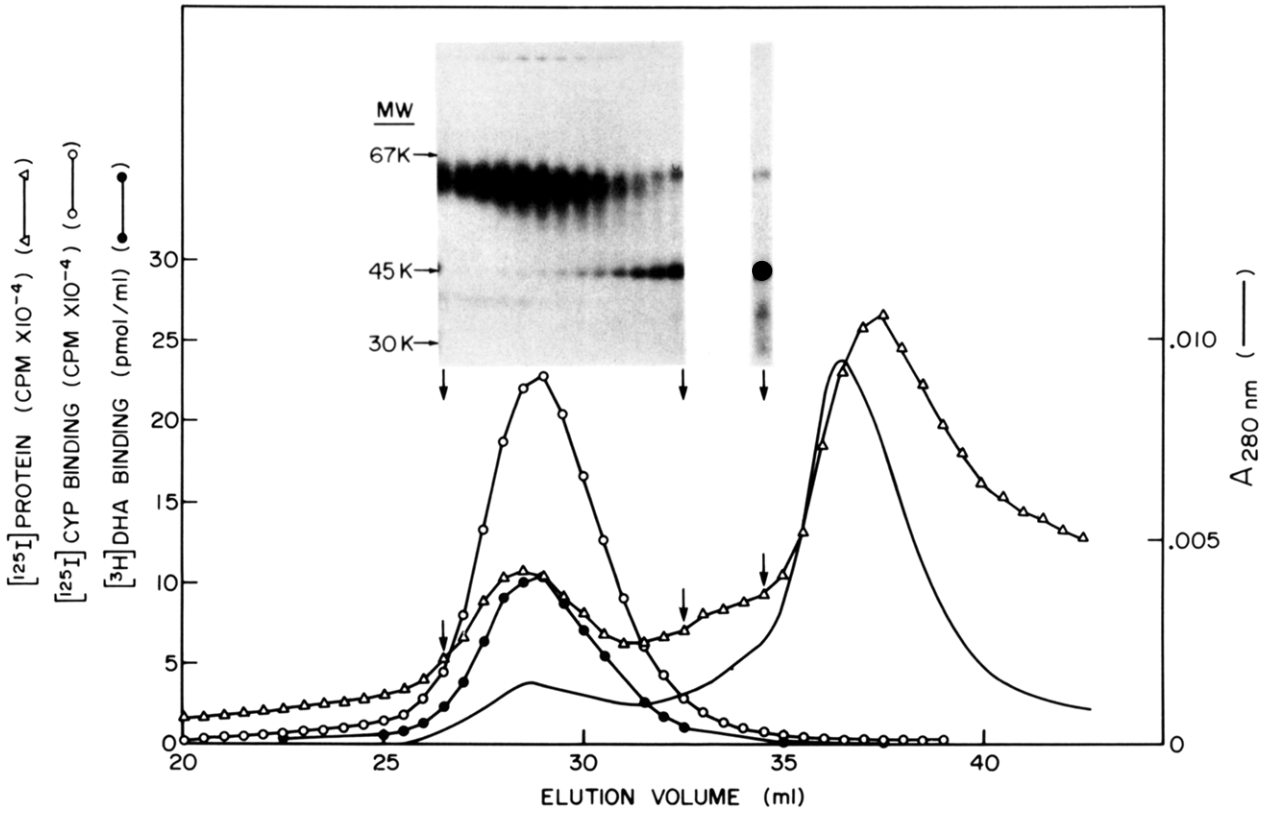

FIGURE 3: High-performance steric-exclusion chromatography of affinity-purified hamster lung receptor activity. Receptor-containing fractions after Sepharose–alprenolol chromatography were pooled and concentrated to 2 mL by ultrafiltration in an Amicon concentration cell with a YM-30 membrane. The concentrated receptor was chromatographed on two TSK-4000 and one TSK-3000 steric-exclusion columns tandem linked (total volume 72 mL). Receptor activity was located by [^{3}H]DHA binding (●) or by chromatography of an aliquot of the affinity column eluate that had been incubated with [^{125}I]CYP prior to chromatography (○). The absorbance at 280 nm (—) is also shown in this profile. Additionally, an aliquot of the affinity column eluate concentrate was iodinated as outlined under Methods prior to chromatography. After chromatography, fractions were counted (Δ), and 10-μL aliquots were electrophoresed on an 8% SDS–polyacrylamide gel. The resulting autoradiogram (top inset) is shown for the corresponding fractions. The molecular weight standards (MW) are shown ×1000 (K). Mobile-phase conditions were 0.1% digitonin and 100 mM Tris-SO_4, pH 7.5, at a flow rate of 1 mL/min with 0.5-mL fractions being collected 36 min after sample injection.

Table II: Comparison of Affinities of Membrane-Bound, Solubilized, and Purified Mammalian Lung β-Adrenergic Receptors for Various β-Adrenergic Ligands[a]

	K_D (nM) of β-adrenergic receptor prepn					
	membrane bound		solubilized	purified		
agents	hamster	rat	hamster	hamster	rat	guinea pig
(−)-alprenolol	4.7	1.1	2.4	1.7	0.8	1.2
(+)-alprenolol	47	27	75	34	30	35
ICI 118,551	10	4.0	4.4	3.8	3.0	5.9
atenolol	ND[b]	7340	42800	64000	33100	46900
(−)-isoproterenol	67	57	16	26	58	50
(+)-isoproterenol	7720	16700	4170	7840	5070	18100
(−)-epinephrine	403	275	68	100	172	244
(−)-norepinephrine	1880	1230	1280	1630	3110	3260

[a] K_D values for the various ligands were calculated from data similar to that in Figure 4 (Cheng & Prusoff, 1973). A K_D of 50 pM for [^{125}I]CYP, as assessed by saturation binding, was used in calculating the dissociation constants (data not shown; Engel et al., 1981). The values indicated for the membrane-bound and solubilized receptors are from single experiments while values for the purified preparations are averages of two to three separate experiments. [b] ND, not determined.

demonstrates that the hamster, guinea pig, and rat lung β_2-adrenergic receptor binding sites can be purified with complete retention of their binding characteristics.

Characterization of Purified Receptor Preparations. To demonstrate that the purified hamster lung β_2-adrenergic receptor is indeed homogeneous, SDS–PAGE was performed as shown in Figure 5. In lane 2, iodinated purified receptor shows a single band of radioactive material after autoradiography. To demonstrate that this same peptide also contains the β-adrenergic binding site, purified receptor was labeled with [^{125}I]pABC, photolyzed, and run on SDS–PAGE (lane 1). Lane 3 shows that the hamster lung receptor is also pure as judged by protein staining; however, a minor contaminant at M_r ~28 000 is evident in this particular preparation. It can also be shown that the receptor is a glycoprotein as evidenced by its ability to bind ^{125}I-labeled concanavalin A (lane 4). The apparent broadness of the radiolabeled bands in lanes 1 and 2 is most likely due to overexposure of the film during autoradiography.

Figure 6 demonstrates that the purified β_2-adrenergic receptors from guinea pig, rat, and hamster lung identified by iodination or [^{125}I]pABC-labeling all migrate at M_r ~64 000

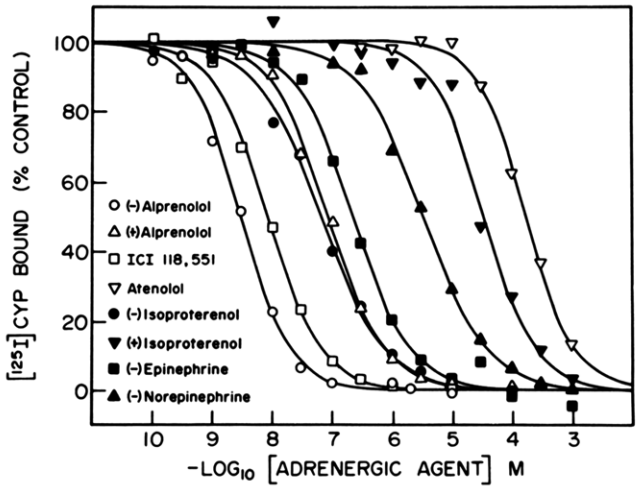

FIGURE 4: Binding of adrenergic ligands to an HPLC-purified hamster lung preparation. Purified hamster lung receptor was prepared as described under Methods. The receptor preparation was incubated in 100 mM NaCl, 10 mM Tris-HCl, and 0.1% digitonin, pH 7.4 (22 °C), in the presence of 100 pM [^{125}I]CYP and various concentrations of adrenergic agents for 4 h at 4 °C. Bound ligand was determined by the Sephadex G-50 chromatography method with 100% [^{125}I]CYP bound equal to 7 pM. Data were analyzed by computer modeling methods previously validated in this laboratory (De Lean et al., 1982).

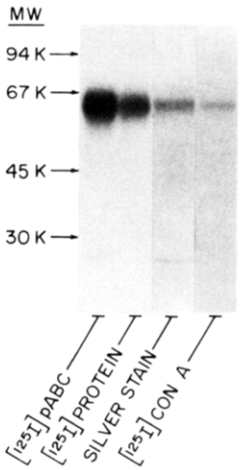

FIGURE 5: SDS–PAGE of HPLC-purified hamster lung receptor activity. An aliquot (3 pmol) of HPLC-purified hamster lung receptor was incubated with [^{125}I]pABC for 12 h at 4 °C and photolyzed as outlined under Methods. A total of 20 μL of the labeled receptor was combined with 40 μL of SDS sample buffer and electrophoresed on a 10% SDS–polyacrylamide gel. The resulting autoradiogram is shown in lane 1. Iodinated HPLC-purified hamster lung receptor, prepared as described in Figure 3, was also electrophoresed on a 10% gel and is shown in lane 2. Additionally, a 10-pmol aliquot of HPLC-purified hamster lung receptor was lyophilized and taken up in 100 μL of SDS sample buffer. After electrophoresis, as described above, the gel was silver stained and photographed (lane 3). After being destained with Farmer's reducer (Kodak), the gel was overlayed with 4 mL of ^{125}I-labeled Con A and after a 12-h incubation was washed for 24 h, dried, and exposed to Kodak XAR-5 film. The resulting autoradiogram is shown in lane 4. The molecular weight standards (MW) are shown ×1000 (K).

on SDS–PAGE. In some HPLC-purified preparations of the guinea pig, rat, and hamster lung, a minor contaminating band at M_r ~45 000 is observed. In the purified rat lung receptor, a minor band at M_r ~28 000, which increases on prolonged storage at −90 °C, is also sometimes observed. The M_r ~ 45 000 band can be removed by an additional chromatography step on HPLC while the M_r ~28 000 peptide appears to represent a degradation product of the M_r ~64 000 rat lung receptor. While these peptides are present in some purified

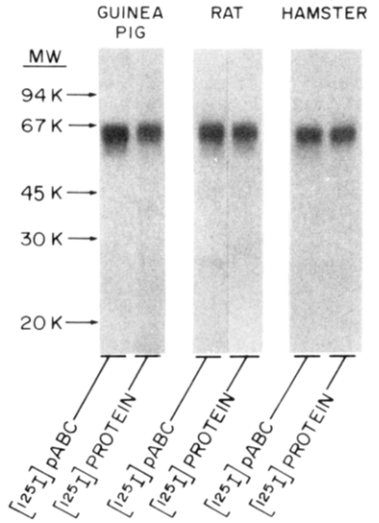

FIGURE 6: SDS–PAGE of [^{125}I]pABC-labeled and iodinated HPLC-purified guinea pig, rat, and hamster lung β_2-adrenergic receptors. [^{125}I]pABC-labeled and iodinated HPLC-purified receptors were prepared as described in Figures 3 and 5. The rat lung receptor was subjected to two HPLC runs with the front half of the second chromatography run being pooled and labeled as described under Methods. The guinea pig and hamster lung receptors were pooled after one HPLC run. The first lane in each set corresponds to the [^{125}I]pABC-labeled receptors while the iodinated receptors are shown in lane 2. Samples were electrophoresed on a 10% polyacrylamide gel. The molecular weight standards (MW) are shown ×1000 (K).

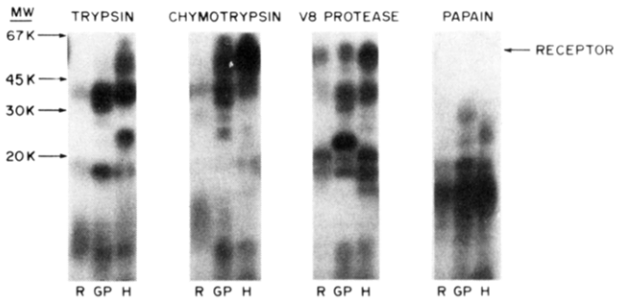

FIGURE 7: Proteolytic digestion of the [^{125}I]pABC-labeled rat, guinea pig, and hamster lung β_2-adrenergic receptors. [^{125}I]pABC-labeled receptors were prepared as described in Figures 5 and 6. Aliquots (10 μL) of each labeled receptor were digested with 0.2 μg of trypsin, 0.5 μg of chymotrypsin, 1.5 μg of *S. aureus* V-8 protease, or 0.04 μg of papain for 15 min at 0–4 °C. A 1-μL aliquot of 1 mM PMSF and 1 mM *N*-ethylmaleimide was then added to each sample, followed by the addition of 20 μL of SDS sample buffer. The samples were then electrophoresed on 12% polyacrylamide gels as described under Methods. The arrow indicates where nonproteolyzed receptors electrophoresed in this experiment. The molecular weight standards (MW) are shown ×1000 (K). The abbreviations used are R (rat lung), GP (guinea pig lung), and H (hamster lung). This experiment is representative of several similar experiments.

preparations, they typically constitute <5% of the total protein.

These results demonstrate that the binding sites of the β-adrenergic receptor from the hamster, guinea pig, and rat lung are all present on peptides of M_r ~64 000 and that these peptides can be purified to apparent homogeneity.

Comparison of the Hamster, Guinea Pig, and Rat Lung β_2-Adrenergic Receptors. Similarities and differences between the three isolated β_2-adrenergic receptor peptides were assessed by peptide mapping and lectin binding. Aliquots of [^{125}I]-pABC-labeled purified receptor preparations were subjected to partial proteolytic degradation with the proteases trypsin, chymotrypsin, V-8 protease, and papain followed by SDS–PAGE of the fragments. The results of these procedures should reflect not only potential similarities and differences in sequence but also those factors such as glycosylation and

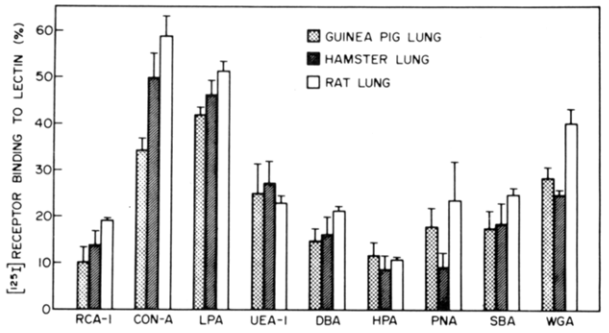

FIGURE 8: Binding of iodinated HPLC-purified guinea pig, hamster, and rat lung β_2-adrenergic receptors to agarose-coupled lectins. Affinity chromatography purified receptor was iodinated and chromatographed on steric-exclusion columns as described in Figure 3. Aliquots of iodinated receptor (100 μL) were diluted to 400 μL with 100 mM NaCl and 10 mM Tris-HCl, pH 7.2 (22 °C), and incubated with 150 μL of agarose-coupled lectin for 1 h at 22 °C. After centrifugation and removal of the supernatant, the gel was washed 2 times with 1 mL of 0.01% digitonin, 100 mM NaCl, and 10 mM Tris-HCl, pH 7.2 (22 °C). The graph represents the percent of iodinated receptor that remained bound to the agarose-coupled lectin after the two washes. The error bars indicate standard errors derived from two to four separate experiments.

conformation that may affect the accessibility of sites for proteolytic cleavage. In Figure 7, the proteolytic degradation of the purified rat, guinea pig, and hamster lung [^{125}I]-pABC-labeled receptors is shown. It is evident that while many of the peptides generated by proteolysis of the receptor peptides of the three species are similar, there are several significant differences in the peptides in both occurence and relative intensity. After trypsin treatment, the data reveal that the guinea pig lung β-receptor has a peptide at M_r ~30 000 that is not present in the rat or hamster preparations while only the hamster has a peptide at M_r ~23 000. Upon chymotrypsin treatment, a peptide of M_r ~30 000 appears in the guinea pig that is not present in the other systems. The hamster lung β-receptor appears particularly resistant to chymotrypsin treatment as it is degraded to a major peptide of M_r ~55 000 and undergoes little additional proteolysis. Treatment of the samples with *S. aureus* V-8 protease reveals peptides at M_r ~29 000 and 21 000 in the guinea pig that are not present in the hamster or rat. Papain treatment of these receptor peptides indicate that all three preparations appear to have a similar susceptibility to papain since peptides of similar molecular weight were generated. The rat lung data are somewhat difficult to assess because of the lower amount of labeling; however, it appears that all of the peptides derived from the rat lung β-receptor are also present in both the guinea pig and hamster lung peptide data. These data provide evidence for some sequence similarity between the β_2-receptors from these mammalian species and suggest that the proteins are evolutionary homologues.

Lectin binding has been used extensively as a tool for studying the carbohydrate nature of glycopeptides (Dulaney, 1979). Such techniques can yield valuable information not only about the nature of the sugars present but also about their sequence. Figure 8 demonstrates binding of iodinated purified guinea pig, hamster, and rat lung receptor to a battery of nine different agarose-coupled lectins. Qualitatively, it is apparent that the binding of the iodinated purified receptors to the majority of the lectins appears very similar. Thus, binding to RCA-1, LPA, UEA-1, DBA, HPA, and SBA is very similar among the three species. (The reported specificity of these lectins is given under Methods.) The carbohydrates sialic acid and α-L-fucose appear to be present on the β-receptors from all three species due to the significant levels of binding to, respectively, LPA and UEA-1. These data suggest the presence of complex-type carbohydrate chains on the mammalian β-receptor. The PNA data suggest that the hamster lung β-receptor might contain lower levels of D-Gal-β(1→3)-D-GalNAc since it appears to bind only ~50% as well as the rat or guinea pig lung β-receptors. The binding of the iodinated receptors to PNA, as well as to DBA, HPA, and SBA, suggests the presence of O-linked carbohydrate chains (through serine or threonine).

The most interesting findings were with the Con A and wheat germ agglutinins. Con A specifically binds to mannose-containing carbohydrates in which more than one mannose has positions C3, C4, and C6 unsubstituted (Lotan et al., 1977). The binding to Con A most likely demonstrates the presence of high-mannose chains in the β-receptors of all three species. The data suggest, however, that the guinea pig lung β-receptor probably contains significantly lower levels of the high-mannose chains. Wheat germ agglutinin binds to *N*-acetylglucosamine and probably more likely to sialic acid (Monsigny et al., 1980). The WGA data reveal higher levels of binding of the rat lung β-receptor, possibly indicating higher levels of complex-type chains in the rat if one assumes binding to sialic acid. Alternatively, the increased binding in the rat could be due to more accessible *N*-acetylglucosamine, which would be found in high-mannose chains that typically contain less fucose than complex-type chains.

Discussion

Considerable progress has previously been made in the purification and characterization of the β-adrenergic receptor from both frog and turkey erythrocytes (Shorr et al., 1981, 1982a,b). Some progress has also recently been made in the purification of mammalian β-adrenergic receptors (Homcy et al., 1983; Cubero & Malbon, 1984). In this study, we have shown that the techniques of digitonin solubilization, affinity chromatography, and high-performance liquid chromatography, which we have previously applied to the purification of the β-adrenergic receptors of the avian and amphibian erythrocytes, can also be used to purify the β_2-adrenergic receptors from the hamster, guinea pig, and rat lung. This procedure allows the preparation of substantial quantities of receptor protein, which can then be used in the biochemical or functional characterization of the receptor.

The modifications incorporated into this purification scheme, such as the differential solubilization, the high-salt wash, and the gradient alprenolol elution, result in the need for only one HPLC step as compared to the two HPLC steps used in the frog and turkey purification procedures (Shorr et al., 1982a,b). Additionally, the rapidity of the procedure (~30 h) along with the use of protease inhibitors significantly reduces proteolytic degradation of the receptors. These procedures result in the purification of a single receptor peptide for both the hamster and guinea pig lung while only a few minor degradation products are observed with the rat lung. These proteolytic products include a M_r ~28 000 fragment, which likely corresponds to the M_r ~30 000 peptide observed by [^{125}I]pABC labeling of rat lung membranes (Lavin et al., 1982). Two other minor degradation products, the M_r ~53 000 and 44 000 peptides also previously seen by photoaffinity labeling (Lavin et al., 1982; Benovic et al., 1983), can be separated from the M_r ~64 000 rat lung receptor peptide by selectively pooling the front half of the receptor peak from the HPLC elution (cf. Figure 3). This procedure, however, reduces the overall yield of the preparation by ~50%. These lower molecular weight peptides likely represent fragments of the M_r ~64 000 peptide that are generated by endogenous proteolysis and cannot be

totally eradicated. These lower molecular weight peptides become the predominant photoaffinity-labeled peptides in the absence of protease inhibitors in rat lung preparations (Benovic et al., 1983).

The measured specific activity of the purified hamster lung β-adrenergic receptor averaged 14 700 pmol/mg of protein from four separate preparations (Table I). This value compares favorably with the theoretical specific activity of 15 625 pmol/mg calculated for a single binding site per M_r ~64 000 subunit, although since the receptor is a glycoprotein, its true molecular weight may be less.

The purified hamster, guinea pig, and rat lung receptors all appear to be predominantly of the β_2 subtype (Figure 4, Table II). This is evidenced by the one-site fits obtained from computer modeling of the ICI 118,551 and atenolol competition curves. These data contrast with the results obtained by ligand binding in membranes where it has been observed that the rat and guinea pig lungs contain 20% β_1:80% β_2 (Rugg et al., 1978; Dickinson et al., 1981; Engel et al., 1981), while hamster lung contains >95% β_2 receptors (Benovic et al., 1983). The loss of the minor fraction of β_1 receptors during the purification is most likely due to their inherent instability (Strauss et al., 1979).

The functional integrity of the purified receptor peptides prepared by these procedures is supported by two main lines of evidence. (1) As shown in Figure 4 and Table II, agonists and antagonists bind to the isolated peptides with an appropriate β_2-adrenergic specificity and order of potency. (2) Recently, in reconstitution/fusion experiments we have demonstrated that these isolated peptides can confer β-adrenergic responsiveness to an acceptor cell that is devoid of β-adrenergic receptors but contains the other elements of the adenylate cyclase system (Cerione et al., 1983a,b). These results clearly suggest that the purified receptor peptides are functionally intact (i.e., they can bind and discriminate ligands and elicit a biochemical signal in the cell). The availability of the larger quantities of β-adrenergic receptor afforded by the procedures described here has now made possible the examination of the interaction of the pure receptor protein with other pure components of the adenylate cyclase system such as the stimulatory guanine nucleotide binding protein (N_s). In the accompanying paper (Cerione et al., 1984), we demonstrate that the co-reconstitution of pure mammalian β_2-adrenergic receptor and pure N_s results in their functional coupling. This coupling can be assessed by measuring the formation of a high-affinity agonist-binding state of the receptor or by monitoring GTPase activity of N_s (Cerione et al., 1984).

Comparison of the hamster, guinea pig, and rat lung β-adrenergic receptors by peptide mapping and lectin binding reveals significant similarities among the three receptors. The receptors each appear to be glycoproteins with N-linked (both high-mannose and complex) and possibly O-linked carbohydrate chains present (Figure 8). The only significant differences among the receptors seem to reside in their ability to interact with agarose-immobilized Con A and WGA. These data suggest that the rat lung β-receptor contains a higher overall level of carbohydrate than either the hamster of guinea pig lung β-receptor. A more definitive result, however, will have to await the direct measurement of the total percent of carbohydrate and the determination of the oligosaccharide sequences on each receptor.

Limited enzymatic digestion and comparison show that there is significant structural similarity between the three receptors since many common peptides were observed after such partial proteolytic digestion (Figure 7). Distinct peptides were also observed, however, indicating that differences in structure may also exist.

The finding that the hamster, guinea pig, and rat lungs all contain a M_r ~64 000 β-adrenergic peptide lends additional credence to previous reports based on photoaffinity labeling in membrane preparations that the mammalian β-adrenergic receptor is a M_r 62 000–65 000 peptide (Lavin et al., 1982; Benovic et al., 1983; Stiles et al., 1983). This is in contrast to the results of Homcy et al., however, where a β-adrenergic peptide of M_r 52 000–53 000 was identified by purification and photoaffinity labeling in the canine lung (Homcy et al., 1983). The likelihood that this lower molecular weight peptide might be a result of proteolytic degradation of a larger β-adrenergic receptor peptide, as previously observed in several other mammalian systems (Benovic et al., 1983; Stiles et al., 1983a), should be considered. Attempts in our laboratory at photoaffinity labeling crude dog lung membranes with [^{125}I]pABC in the presence of protease inhibitors have resulted in the labeling of a major M_r ~56 000 peptide (data not shown). However, a minor (~7%) specifically protected M_r ~64 000 peptide is also observed, strongly suggesting that the M_r 52 000–56 000 peptide observed by Homcy et al. and by us is indeed a degradation product of the M_r ~64 000 peptide.

It is also of interest to compare the β-adrenergic receptor from other more diverse species and to compare the mammalian β_1 and β_2 receptors by these techniques. Recently, Stiles et al. have demonstrated some structural differences in β_1 and β_2 mammalian β-adrenergic receptors as revealed by peptide mapping of [^{125}I]pABC-labeled membranes (Stiles et al., 1983b). Their data suggest that alterations in the primary structure of the receptor may be responsible for the unique pharmacological specificities of the β_1 and β_2 subtypes. Other recent work on several mammalian systems utilizing photoaffinity labeling of membranes has demonstrated that the lung β_2-receptor has a M_r 62 000–64 000 peptide (Lavin et al., 1982; Benovic et al., 1983) while the heart β_1 receptor resides on a M_r ~62 000 peptide (Stiles et al., 1983a). That mammalian β receptors reside on peptides of M_r 62 000–65 000 has also been demonstrated in S-49 cells (Rashidbaigi et al., 1983). Their studies reveal β-adrenergic peptides of M_r ~65 000 and 55 000 as assessed by ([^{125}I]iodoazidobenzyl)pindolol photoaffinity labeling in membrane preparations. The present work, however, is the first to report the purification and characterization of the M_r ~64 000 peptide as containing the β-adrenergic receptor binding site. Previous studies have also demonstrated that the frog erythrocyte β_2-adrenergic receptor is contained on a M_r ~58 000 peptide (Shorr et al., 1981, 1982a) while the turkey erythrocyte β_1-adrenergic receptor resides on two peptides of M_r ~45 000 and 40 000 (Shorr et al., 1982b; Burgermeister et al., 1982; Rashidbaigi et al., 1982). While the turkey erythrocyte β_1-receptor, as well as the receptor from two other avian erythrocyte species (Rashidbaigi et al., 1981, 1982), appears to reside on a distinctly different peptide from the other systems, proteolytic modification of these avian β-adrenergic receptors cannot be conclusively ruled out.

In summary, the β_2-adrenergic receptors from hamster, guinea pig, and rat lung have been purified to apparent homogeneity by affinity and high-performance liquid chromatography. The purified receptors appear to be structurally very similar and can be purified in amounts sufficient for further biochemical characterization of these proteins. The hamster and guinea pig lungs should prove to be excellent model systems for study of mammalian β-adrenergic receptors owing to their commercial availability, high receptor density, limited

endogenous proteolysis, and relative ease of receptor purification.

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Registry No. (−)-Alprenolol, 23846-71-1; (+)-alprenolol, 23846-72-2; IC 118,551, 72795-19-8; atenolol, 29122-68-7; (−)-isoproterenol, 51-31-0; (+)-isoproterenol, 2964-04-7; (−)-epinephrine, 51-43-4; (−)-norepinephrine, 51-41-2.

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