Identification of Structurally Distinct \( \alpha_2 \)-Adrenergic Receptors*

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Recent studies involving a variety of membrane receptors and ion channels indicate that diversity exists among these proteins as evidenced by tissue-specific and developmentally related expression of different isomers. \( \alpha_2 \)-Adrenergic receptors, plasma membrane proteins involved in sympathetic neurotransmission, may similarly represent a nonhomogeneous class of binding sites based on the following observations. First, their activation can elicit a wide variety of effector cell responses, which are apparently triggered by at least three different signal transduction mechanisms. Second, \( \alpha_2 \)-adrenergic receptors in various tissues and species exhibit marked differences in their ligand recognition properties. To determine if heterogeneity of the receptor protein itself is involved in generating this diversity, we structurally characterized the \( \alpha_2 \)-adrenergic receptor in two tissues that exhibit the greatest differences in ligand recognition properties, neonatal rat lung and human platelet. We report here that these differences in ligand recognition are maintained after partial receptor purification (50–100-fold) and are associated with distinct differences in the physical and structural properties of the receptor protein. The human platelet and neonatal rat lung receptor differ in the apparent molecular weight of their hormone-binding subunits (human platelet, \( M_r \approx 64,000 \) versus neonatal rat lung, \( M_r \approx 44,000 \)) as well as in the number or type of their associated oligosaccharide moieties. The observed diversity is consistent with expression of isomers of the \( \alpha_2 \)-adrenergic receptor and suggests the presence of more than one gene encoding similar but distinct receptor proteins.

\( \alpha_2 \)-Adrenergic receptor (\( \alpha_2 \)AR) activation results in a variety of tissue-specific effects including smooth muscle contraction, epithelial cell chloride secretion, and platelet aggregation/secretion, as well as inhibition of insulin release, lipolysis, and depolarization-induced neurotransmitter release (1). Recent studies suggest that these effects may involve different mechanisms of signal transduction (1–3). Thus, many effects elicited by \( \alpha_2 \)AR activation are apparently unrelated to inhibition of adenylate cyclase and may involve coupling to a Na+/H+ antiporter or to ion channels. In addition to these differences in effector cell responses, \( \alpha_2 \)AR in various tissues and species exhibit differing affinities for a number of ligands including clonidine, idazoxan, prazosin, and oxymetazoline, but maintain similar high affinity for the \( \alpha_2 \)-selective antagonist rauwolscine (1, 4–6). The present study was undertaken to determine if the observed differences in ligand recognition properties, which range from 10- to 100-fold, are associated with distinct structural differences in the \( \alpha_2 \)AR expressed in these tissues.

**EXPERIMENTAL PROCEDURES**

Materials—[\( ^{3} \text{H} \)]Rauwolscine (80 Ci/mmol) was obtained from Du Pont-New England Nuclear. Heparin-agarose (Type II) was purchased from Sigma. Wheat germ lectin agarose was from Vector Laboratories (Burlingame, CA). N-Glucosamine was obtained from Genzyme Corp. (Boston, MA). Prazosin was a gift from Pfizer, and oxymetazoline was provided by Schering Corp. (Bloomfield, NJ). Rats were obtained from Charles River Breeding Laboratories (Wilmington, MA). Outdated human platelets were purchased from the Massachusetts General Hospital blood bank. All other chemicals and materials were obtained or synthesized as described previously (7, 8).

Membrane Preparation and Partial Receptor Purification—Rat lung and brain tissue were removed from 1-day-old rat pups and immediately frozen in liquid nitrogen. Frozen rat lung tissue obtained from 50–75 1-day-old pups was thawed and minced in ice-cold buffer containing 250 mM sucrose, 10 mM Tris, 2 mM EGTA, pH 7.4. Tissue was disrupted with a Polytron tissue homogenizer (2 × 10 s, setting 8; Brinkmann Instruments) and then with a P200 homogenizer after which membrane aggregates and nuclear material were pelleted by centrifugation at 2,400 g for 10 min and resuspended in buffer containing 50 mM Hepes, 5 mM EDTA, 2 mM EGTA, pH 7.4. The column was then washed with 50 mM Tris, 5 mM EDTA, 2 mM EGTA, pH 7.4, followed by disruption with a P200 homogenizer. The disrupted tissue was pelleted by centrifugation at 39,000 g for 10 min and rehomogenized in buffer containing 500 mM KCl, 20 mM imidazole, 5 mM EDTA, 2 mM EGTA, pH 7.4. The homogenate was then pelleted and washed twice with buffer containing 50 mM Hepes, 5 mM EDTA, 2 mM EGTA, 100 mM NaCl, pH 7.5, prior to final resuspension in the same buffer. Platelet membranes were prepared from outdated platelets (1-day postexpiration date) as described previously (8), except that the crude membrane fraction was not further purified by sucrose gradient centrifugation.

Membranes were solubilized at 4 °C for 15 min in buffer containing 50 mM Hepes, 5 mM EDTA, 2 mM EGTA, 100 mM NaCl, 1% digitonin, pH 7.4, at a detergent to protein ratio of 3:1. The soluble fraction was isolated by centrifugation (100,000 × g) and subjected to heparin-agarose affinity chromatography (10 volumes of detergent-solubilized preparation to 1 volume of heparin-agarose; flow rate, 2.5 cm/min; column volumes h-1). The column was then washed with 50 column volumes of 50 mM Hepes, 5 mM EDTA, 2 mM EGTA, 150 mM NaCl, 0.2% digitonin, pH 7.4, after which the absorbance of the flow-through was 0 at 280 nm. The receptor protein was then eluted with wash buffer
containing 700 mM NaCl at a flow rate of 2 column volumes h\(^{-1}\), and the collected fractions were assayed with \(^{3}H\)rauwolscine for receptor binding activity. This technique resulted in approximately a 50-fold purification of the receptor protein (peak specific activity, 1.5-3 pmol mg\(^{-1}\) of protein) with 75% recovery, based on receptor binding in the crude solubilized preparation. All procedures were conducted at 4°C, and all buffers contained EDTA (5 mM), EGTA (2 mM), and the following protease inhibitors: pepstatin (2 \(\mu\)g ml\(^{-1}\)), leupeptin (2 \(\mu\)g ml\(^{-1}\)), antipain (2 \(\mu\)g ml\(^{-1}\)), soybean trypsin inhibitor (5 \(\mu\)g ml\(^{-1}\)), benzamidine (10 \(\mu\)M), phenylmethylsulfonyl fluoride (100 \(\mu\)M).

In some experiments, human platelet membranes (80 ml, 560 mg of protein, \(\sim100\) pmol of receptor) were mixed with frozen intact rat lung membranes. The mixed tissue preparation was then processed as described above for rat lung membranes. In these experiments the amount of platelet tissue utilized was approximately equal to the calculated yield of rat lung membranes. The mixed membrane preparation was then solubilized and subjected to heparin-agarose affinity chromatography.

For lectin affinity chromatography the elutions from the heparinagarose resin were pooled (10 ml), and magnesium was added to 10 mM prior to wheat germ-lectin affinity chromatography. One ml of resin was initially equilibrated with 20 column volumes of 50 mM Hepes, 5 mM EDTA, 2 mM EGTA, 700 mM NaCl, 0.2% digitonin, pH 7.4 (Buffer A) followed by 5 column volumes of the same buffer containing 10 mM MgCl\(_2\). The solubilized receptor preparation was then loaded onto the resin by gravity flow and the resin subsequently washed with 10 column volumes of Buffer A containing 10 mM MgCl\(_2\) followed by 5 column volumes of Buffer A containing 100 mM NaCl without MgCl\(_2\). Bound glycoproteins were then eluted by batch technique with 3 column volumes of the second wash buffer containing 300 mM N-acetylglucosamine.

Binding Studies—Membrane binding assays were conducted at 24°C as described previously (8). For solubilized receptor binding assays, increasing concentrations of competing ligand were incubated in duplicate with receptor (10-20 fmol) and \(^{3}H\)rauwolscine (10 nM) for 2 h at 4°C, and bound ligand was separated by precipitation with bovine \(\gamma\)-globulin/polyethylene glycol followed by vacuum filtration. The filters were placed in 10 ml of Hydrofluor scintillation fluid and counted in a liquid scintillation spectrometer (Beckman, model LS 1800) with a counting efficiency of 50%. Nonspecific binding was determined in the presence of 10 \(\mu\)M yohimbine. IC\(_{50}\) values are defined as the concentration of competing ligand inhibiting 50% of specific \(^{3}H\)rauwolscine binding and are presented as the mean \pm S.E.

Photoaffinity Labeling and SDS-PAGE—Aliquots of receptor (~100 fmol) eluted from heparin-agarose or wheat germ-agarose chromatographic steps were photolabeled with \(^{125}\)I-rau-AZPC as described previously (7). The photolyzed samples were then desalted, lyophilized, and subsequently solubilized in sample buffer prior to SDS-PAGE (7). Gels were dried, and autoradiographs were obtained by exposing the dried gels to Kodak XAR-5 film at ~70°C in a cassette containing Du Pont Cronex Lightning Plus intensifying screens.

RESULTS

Ligand Recognition Properties of the Partially Purified Receptor Protein—In membranes prepared from human platelets and neonatal rat lung, the \(\alpha_2\)AR exhibits markedly different affinity for the \(\alpha\)-adrenergic receptor for ligands, prazosin and oxymetazoline, as previously observed by Bylund (6). To characterize the binding properties of the receptor in these two tissues in more detail and to eliminate potential differences due to membrane environment, the \(\alpha_2\)AR from these two tissues was solubilized and the receptor protein purified ~50-fold by heparin-agarose affinity chromatography. As shown in Table I, the differences in ligand recognition properties observed in membrane preparations were maintained after solubilization and partial purification of the receptor protein. Thus, the human platelet and neonatal rat lung \(\alpha_2\)AR exhibited similar high affinity for rauwolscine but differed by ~100-fold in their affinity for prazosin and oxymetazoline. In similar preparations of rat brain cortex, the \(\alpha_2\)AR exhibited affinities for oxymetazoline and prazosin, which were intermediate between those observed in human platelet and neonatal rat lung receptor preparations (Table I). Attempts to characterize the binding properties of the solubilized \(\alpha_2\)AR from adult rat lung were unsuccessful due to low levels of specific \(^{3}H\)rauwolscine binding.

Identification of the Hormone Binding Subunit—To determine if the differences in ligand recognition properties of the human platelet and neonatal rat lung \(\alpha_2\)AR are associated with structural variations of the receptor protein, a photoaffinity probe specific for the \(\alpha_2\)AR, \(^{125}\)I-hydroxy-20e-yohimb-an-16\'\-[N-(4-azido-3-\([^{125}\)Iodophenyl] carbamoyl (\(^{125}\)I-rau-AZPC) (7-10), was used to label covalently the receptor’s hormone-binding subunit. In neonatal rat lung preparations, \(^{125}\)I-rau-AZPC labels a major peptide species with an apparent molecular weight of ~44,000, and ~64,000 in rat lung and human platelet \(\alpha_2\)AR, respectively.

![Fig. 1. Autoradiogram of photolabeled neonatal rat lung (A) and human platelet (B) \(\alpha_2\)-adrenergic receptor. The solubilized receptor protein was purified ~50-fold by heparin-agarose affinity chromatography and photolabeled with \(^{125}\)I-rau-AZPC in the presence and absence of competing ligands. Lanes 1, 7, and 10, no competing ligand; lanes 2 and 8, rauwolscine (1 \(\mu\)M); lanes 3 and 9, and 11, (+)-epinephrine (100 \(\mu\)M); lane 4, (-)-epinephrine (10 \(\mu\)M); lanes 5 and 12, (+)-epinephrine (100 \(\mu\)M); lane 6, (+)-epinephrine (10 \(\mu\)M). Numbers at the left indicate the migration of nonglycosylated molecular weight standards (Bethesda Research Laboratories) (Mr, \(\times 10^3\)). The arrows indicate specifically labeled peptides with apparent Mr, the values of ~44,000 and ~64,000 in rat lung and human platelet \(\alpha_2\)AR, respectively.](image-url)
(M_r \sim 49,000 \text{ and } \sim 44,000) \text{ (Fig. 2A). It is not known if the two labeled species are distinct proteins or merely reflect minor differences in post-translational processing (e.g. glycosylation). A similar finding has been observed for other adrenergic receptor subtypes identified by photolabeling (13, 14).}

In additional experiments, we have shown that photolabeling of both the human platelet and neonatal rat lung \( \alpha_2 \text{AR} \) is inhibited by adrenergic agonists and antagonists with a rank order of potency consistent with an \( \alpha_2 \text{AR} \) binding site. However, labeling in the two tissues is differentially inhibited by prazosin and oxymetazoline as predicted by competition binding studies. Thus, in neonatal rat lung prazosin inhibits photoincorporation of \(^{125}\text{I}-\text{rau-AZPC} \) with approximately 100-fold greater potency than in human platelet, while the converse is true for oxymetazoline (Fig. 2).

Taken together, these experiments demonstrate that in addition to the differences in ligand-binding properties between human platelets and neonatal rat lung, the \( \alpha_2 \text{AR} \) isolated from these two tissues also exhibit distinct biophysical properties. This may be due to differences in their primary structure or to differences in post-translational processing, or both. A trivial explanation for these findings is that the lower molecular weight species identified in neonatal rat lung is the result of tissue-specific proteolysis. However, the following considerations apparently exclude this possibility. All procedures (membrane preparations, receptor solubilization, and purification) were carried out at 4°C, and all buffers contained several protease inhibitors. In addition, a specifically labeled \( M_r \sim 44,000 \) species was observed for neonatal rat lung when the concentration of protase inhibitors was increased 10-fold and additional protease inhibitors added (aprotinin, 20 milliunits/ml; 1,10-phenanthroline, 0.1 mM).

To address the further issue of proteolysis, platelet membranes were mixed with either intact rat lung tissue or neonatal rat lung membranes and were then processed as described under “Experimental Procedures.” Competition binding studies using the resulting membrane preparation indicated that the mixed \( \alpha_2 \text{AR} \) population recognized oxymetazoline and prazosin with affinities intermediate between those observed in either tissue alone. Furthermore, photoaffinity labeling of the receptor proteins purified after solubilization of the mixed membrane preparation revealed both a \( M_r \sim 64,000 \) (platelet) and the \( M_r \sim 44,000 \) species characteristic of neonatal rat lung (Fig. 3, lanes 1 and 2). These experiments indicate that \( M_r \sim 64,000 \) species has not been observed in neonatal rat lung preparations under any conditions strongly suggest that proteolysis does not account for the heterogeneity revealed by photoaffinity labeling.

**Analysis of Receptor Glycosylation**—The human platelet and porcine brain \( \alpha_2 \text{AR} \) are apparently glycosylated proteins as they readily bind to lectin affinity resins and migrate as broad diffuse bands on SDS-PAGE (7, 9–12), a finding that is characteristic of several glycosylated membrane-bound hormone receptors. The differences in \( M_r \) of the human platelet and neonatal rat lung receptor protein may reflect differential glycosylation as the number or type of oligosaccharide chains often influences the migration of glycoproteins in polyacrylamide gels. To address this issue, we determined the ability of the human platelet and neonatal rat lung \( \alpha_2 \text{AR} \) to adsorb to a wheat germ-lectin resin. Whereas greater than 75% of the solubilized human platelet receptor adsorbed to the lectin resin, 80–85% of the neonatal rat lung \( \alpha_2 \text{AR} \) was found in the fall-through (Fig. 4). The human platelet \( \alpha_2 \text{AR} \) could be eluted with N-acetylglucosamine, and subsequent photolabeling revealed the expected \( M_r \sim 64,000 \) protein. In contrast, with neonatal rat lung preparations a \( M_r \sim 44,000 \) protein was visualized by photoaffinity labeling of the fall-through fractions from the lectin resin. Similarly, when mixed platelet and neonatal rat lung preparations were processed and subsequently adsorbed to and eluted from the lectin resin, only the \( M_r \sim 64,000 \) peptide was visualized in the eluted fractions (Fig. 3, lanes 3 and 4).

**Fig. 2.** Inhibition of receptor photolabeling by prazosin and oxymetazoline in neonatal rat lung (A) and human platelet (B) receptor preparations. The solubilized receptor protein was purified 50-fold by heparin-agarose affinity chromatography and photolabeled with \(^{125}\text{I}-\text{rau-AZPC} \). The numbers to the left of the autoradiograms represent the migration of nonglycosylated molecular weight standards (\( M_r \times 10^6 \)). Lanes 1, 6, and 8, no competing ligand; lanes 2 and 7, prazosin (1 \( \mu \text{M} \)); lane 3, prazosin (0.1 \( \mu \text{M} \)); lanes 4 and 10, oxymetazoline (0.1 \( \mu \text{M} \)); lanes 5 and 11, oxymetazoline (1 \( \mu \text{M} \)); lane 9, oxymetazoline (0.01 \( \mu \text{M} \)).

**Fig. 3.** Photolabeling of a mixed \( \alpha_2 \text{-adrenergic receptor population obtained by coprocessing of human platelet and neonatal rat lung tissue.** Photolabeling of the heparin-agarose affinity-purified preparation is shown in lanes 1 and 2. Lanes 3 and 4 represent the mixed receptor preparation after a second purification step utilizing a wheat germ-agarose affinity resin. Note that whereas both the platelet (\( M_r \sim 64,000 \)) and rat lung (\( M_r \sim 44,000 \)) \( \alpha_2 \text{AR} \) were visualized in the elution from the heparin-agarose resin, only the platelet \( \alpha_2 \text{AR} \) (\( M_r \sim 64,000 \)) was apparent after lectin affinity chromatography. Lanes 1 and 3, no competing ligand; lanes 2 and 4, rauwolscine (1 \( \mu \text{M} \)).**
not altered by enzyme treatment. Furthermore, the shift in the $M_r$ of the photolabeled human platelet $\alpha_2$AR following treatment with N-glycanase was prevented (Fig. 5A) by $(\text{Man})_3(\text{GlcNAc})_2$-$\text{Asn}$-peptide, which is thought to act as a competitive inhibitor of the enzyme (16).

To determine if the presence of N-linked oligosaccharides accounts for the observed differences in ligand recognition, the binding properties of the human platelet $\alpha_2$AR were re-examined after treatment with N-glycanase. As shown in Fig. 5B after deglycosylation of the human platelet $\alpha_2$AR, high affinity oxymetazoline and low affinity prazosin binding were still observed.

The difference in the glycosylation state of the neonatal rat lung and human platelet $\alpha_2$AR is not due to a deficiency of the glycosylation machinery in the neonatal animal itself, since photolabeling of the $\alpha_2$AR from a different tissue of the neonatal rat (cerebral cortex) revealed a $M_r \sim 61,000$ protein, which also migrated as a broad species characteristic of heavily glycosylated proteins. Similar results were obtained in cerebral cortex membranes isolated from adult rats (Fig. 6).

**DISCUSSION**

The $\alpha_2$AR is a member of a class of membrane-bound receptors which utilize guanine nucleotide binding proteins in their signal transduction pathway and share several structural features as determined by molecular cloning. Members of this receptor group include the muscarinic and $\beta$-adrenergic receptors as well as the visual pigment rhodopsin and the substance K receptor (17–24). These receptor proteins consist of seven stretches of hydrophobic amino acids, which are presumed to span the membrane bilayer with intervening hydrophilic sequences forming intracellular and extracellular loops. Glycosylation likely occurs at the extracellular amino terminus where a variable number of sites for N-linked glycosylation are found. Potential sites for phosphorylation by cAMP-dependent protein kinase or receptor-specific protein kinases are found in the carboxyl terminus and/or in the third intracellular loop.

Photoaffinity labeling and mutagenesis studies suggest that the ligand binding domain actually exists in a hydrophobic pocket formed by the membrane-spanning sequences rather

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2 B. I. Terman, R. M. Graham, and S. M. Lanier, unpublished data. These data indicate that the photolabeled $\alpha_2$-adrenergic receptor in neonatal rat lung exhibits a $M_r$ of $\sim 77,000$ as expected for the glycosylated receptor protein (39). This observation suggests that the glycosylation machinery is intact in the neonatal rat lung.
Fig. 6. Autoradiogram of photolabeled $\alpha_2$-adrenergic receptor isolated from rat cerebral cortex. The solubilized receptor protein from either neonatal (lanes 1 and 2) or adult rats (lanes 3 and 4) was purified 50-fold by heparin-agarose affinity chromatography and photolabeled with $^{125}$I-raft-AZPC. The numbers to the left of the autoradiogram represent the migration of nonglycosylated molecular weight standards ($M_r \times 10^3$). Lanes 1 and 3, no competing ligand; lanes 2 and 4, rauwolscine (1 $\mu$M).

The data presented in this paper suggest that $\alpha_2$-adrenergic receptors also consist of a family of related but distinct proteins that are expressed in a tissue-specific manner. The differences in ligand recognition properties among human platelet, neonatal rat lung, and rat brain cortex $\alpha_2$AR are maintained after solubilization and partial purification. This suggests that their binding properties are specific for the particular receptor protein and unrelated to the membrane environment. The human platelet and neonatal rat lung $\alpha_2$AR also exhibit distinct biophysical properties which are due to differences in glycosylation. Our observation that in contrast to the human platelet $\alpha_2$AR, the neonatal rat lung $\alpha_2$AR does not adsorb to a wheat germ-lectin resin and that its $M_r$ is unaffected by N-glycanase suggests that the receptor protein is devoid of N-linked oligosaccharides. The differential glycosylation of the human platelet and rat lung $\alpha_2$AR is not due to a deficiency of the glycosylation pathway in the neonatal animal but may reflect differences in the primary structure of the receptor protein in the two tissues. The human platelet and rat neonatal lung $\alpha_2$AR may differ in the number of potential sites for N-linked glycosylation in a manner analogous to the four muscarinic subtypes recently identified by molecular cloning (18). Alternatively, differences in the primary sequence at the amino terminus may result in an altered secondary structure, which could preclude glycosylation of the rat lung $\alpha_2$AR despite the presence of an appropriate consensus sequence.

It is clear from the present study that variability in glycosylation does not account for the observed differences in ligand recognition properties of the human platelet and neonatal rat lung $\alpha_2$AR. Thus, the high affinity for oxymetazoline and low affinity for prazosin exhibited by the human platelet $\alpha_2$AR is maintained after removal of N-linked oligosaccharides. Therefore, the differences in ligand recognition between the two receptor proteins must reflect differences in the amino acid composition of the ligand-binding pocket.

The apparent $\alpha_2$AR subtypes are likely products of different genes and not the result of alternative splicing since the gene encoding the human platelet $\alpha_2$AR lacks introns in the coding region as do the genes encoding muscarinic and $\beta$-adrenergic receptors (18, 19). The existence of different genes that could give rise to related but distinct $\alpha_2$AR proteins is also supported by the demonstration that at least two hybridizing species are observed after Southern analysis of a human genomic library with a restriction fragment of the human platelet $\alpha_2$AR gene (17). The $\alpha_2$AR isolated from rat cerebral cortex may represent yet another distinct receptor subtype as suggested by its apparent molecular weight and ligand recognition properties. This possibility is currently under investigation. Although the rat brain $\alpha_2$AR exhibits affinity for prazosin and oxymetazoline, which is intermediate between that observed in neonatal rat lung and human platelet $\alpha_2$AR, the rat brain $\alpha_2$AR does not appear to be a mixture of the two proteins since only a higher molecular weight species is observed by photolabeling using a photoaduct that binds to both isoforms with equal affinity.

Although $\alpha_2$-adrenergic receptors have been subclassified as $\alpha_2A$ and $\alpha_2B$ based on their relative affinities for prazosin and oxymetazoline (6), the wide variation in ligand recognition properties of the receptor protein in different tissues and species suggests that additional receptor subclasses exist (Refs. 1, 4–6, and present study), the pharmacological classification of which may require the use of as yet undeveloped ligands. This hypothesis is supported by the recent development of ligands capable of distinguishing between the pre- and postsynaptic $\alpha_2$AR (36–38). Determination of the precise differences between human platelet and rat lung $\alpha_2$AR and identification of additional subclasses will require isolation of the cDNA encoding the particular subtype. Such studies will be greatly facilitated by the availability of the recently described genomic clone for the human platelet $\alpha_2$AR (17). The characterization and localization of $\alpha_2$AR subtypes, as well as apparent isoforms of other G-protein-coupled receptors and ion channels, may eventually allow the tissue-specific targeting of novel therapeutic agents.

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