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ALPHA$_1$- AND BETA$_2$-ADRENERGIC RECEPTORS CO-EXPRESSED ON CLONED MDCK CELLS ARE DISTINCT GLYCOPROTEINS

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SUMMARY: We have explored the molecular differences between α_1- and β_2-adrenergic receptors that are co-expressed by a clonally-derived cell line, Madin-Darby canine kidney clone D (MDCK-D). MDCK-D membranes were pre-labeled with selective α_1- and β-adrenergic radioligands and were then solubilized with the non-ionic detergent digitonin. Solubilized α_1- and β_2-adrenergic receptors were retained by immobilized wheat germ agglutinin and were eluted following addition of N-acetyl-D-glucosamine or sialic acid. Both receptors were also retained by immobilized Limax flavus lectin, a sialic acid-binding lectin. Lectins that were specific for N-acetyl-D-glucosamine residues did not bind to these receptors. These results indicate that both α_1 and β_2 receptors are sialylated glycoproteins. The solubilized α_1- and β_2-adrenergic receptors migrated with different elution profiles from an Ultragel AcA 34 column. The apparent molecular sizes of the digitonin-receptor complexes were 68Å for the α_1 receptor and 55Å for the β_2 receptor. These results show that α_1- and β_2-adrenergic receptors can be present on the same cell as distinct sialic acid-containing glycoproteins.

Biological responses to catecholamines are mediated by two principal classes of plasma membrane receptors, α- and β-adrenergic receptors (1), which have been subdivided into α_1, α_2, β_1 and β_2 subtypes (2). Although their binding site specificities have been clearly defined, the structural differences between these receptor types and subtypes have not yet been clearly established. Recent evidence has suggested that α- and β-adrenergic receptors may be separate protein complexes and that they may have different subunit molecular weights (3-9). However, these studies have used α and β receptors prepared from various species and from tissues containing heterogeneous

ABBREVIATIONS:
PMSF, phenylmethylsulfonylfluoride; [^{125}I]IHEAT, [^{125}I]iodo-2-[β(4-hydroxyphenyl)ethylaminomethyl]tetralone; [^{125}I]ICYP, [^{125}I]iodocyanopindolol; Con A, concanavalin A; WGA, Triticum vulgaris lectin; LFA, Limax flavus lectin; STA, Solanum tuberosum lectin; GSA II, Griffonia (bandeiraea) simplicifolia II lectin; RCA I, Ricinis communus I lectin; UEA I, Ulex europaeus I lectin.

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populations of cells. It is possible that differences in amino acid sequence or in post-translational modification of receptors may exist between different species or different cell types. In particular, differences in receptor glycosylation may affect the estimates of receptor molecular weight (10).

We have studied α- and β-adrenergic receptors using a unique model system, the Madin Darby Canine Kidney (MDCK) epithelial cell line. MDCK is a continuous renal cell line which retains features typical of the distal tubule and collecting duct (11-13) and which responds to both α- and β-adrenergic stimulation (14-16). We have shown that it is possible to isolate distinct MDCK clones that co-express α_1- and β_2-adrenergic receptors (17). We thus decided to use one of these cloned MDCK cells to determine whether the two adrenergic receptors are distinct molecular entities.

MATERIALS AND METHODS

Materials: Phenylmethylsulfonylfluoride (PMSF) was from CalBiochem and digitonin was from Sigma Chemical Co. (St. Louis, MO). [^{125}I]IHEAT and [^{125}I]ICYP were prepared in our laboratory as described previously (17); CYP and HEAT were gifts from Sandoz, Inc. (Basel, Switzerland) and Beiersdorf AG (Hamburg, FRG), respectively. Prazosin was a gift of Pfizer, Inc. (Groton, CT), and propranolol was a gift of Ayerst laboratories (New York, NY); Ultragel AcA 34 was obtained from LKB (Rockville, MD). Lectins and agarose-coupled lectins were purchased from E-Y Laboratories (Burlingame, CA). Carrier-free Na ^{125}I was from Amersham and [^{14}C]methylated protein standards were from New England Nuclear.

Receptor Solubilization: MDCK-D cells were grown as previously described (21). Confluent cells were lysed by incubation for 10 min with ice-cold hypotonic buffer (1 mM Tris-HCl, 2 mM $MgCl_2$, 0.2 mM EGTA, 1 mM PMSF, pH 7.5). All buffers used during cellular disruption and handling of solubilized receptors contained EGTA and PMSF to prevent proteolysis of receptors. Membrane fractions were prepared as previously described (21) and resuspended in incubation buffer (145 mM NaCl, 20 mM Tris-HCl, 2 mM $MgCl_2$, 0.2 mM EGTA, 0.1 mM PMSF, pH 7.5). Aliquots of the membrane suspension ($0.5\text{-}2 \times 10^7$ cells/ml) were incubated in a total volume of 1.5 ml for 60 min at 37° in the presence of the following ligands: 0.2-0.4 nM [^{125}I]IHEAT in the absence and presence (nonspecific binding) of 1 μM prazosin (to label the α_1-adrenergic receptors), or 0.2-0.3 nM [^{125}I]ICYP in the absence and presence (nonspecific binding) of 1 μM (±)propranolol (to label the β_2-adrenergic receptors). The receptor content of duplicate 150-μl aliquots of each sample was determined by a filtration binding assay (21).

Digitonin was freshly prepared as a 3.5% solution by heating digitonin in incubation buffer to 100°. The labelled membrane samples were cooled to 4°, and cooled digitonin solution was then added to give a final concentration of 0.7% digitonin. The samples were incubated for 30 min at 4° and were mixed once every 5-10 minutes. The samples were then centrifuged for 3 min in a refrigerated Beckman microfuge. The soluble labeled receptors were separated from free radioligand by loading 700 μl of the supernatant on a 1x20cm Sephadex G-50 column, eluting with incubation buffer containing 0.1% digito-

nin, and collecting the void volume (3.3 ml) containing the labeled receptors. The soluble receptor fractions were kept at 4° for all subsequent procedures. Non-specific binding in the solubilized preparations constituted 40-60% of the total radioactivity for the α_1-receptor and 10-50% for the β_2-receptor. Typically, the yield of solubilized receptor obtained in the void volume of the Sephadex G-50 column (used to separate bound and free radioligand), represented 30-50% of the membrane-bound α_1- and β_2-adrenergic receptors.

Lectin Affinity Chromatography: Lectin chromatography was performed by modification of a method described by Gioannini et al. (18). The incubation mixture consisted of 1.2 ml of soluble receptor-ligand complex, 1.8 ml of column buffer (incubation buffer plus 0.1% digitonin), and 1 ml of lectin-agarose (2-5 mg/ml) which had been equilibrated with column buffer. For Con A columns, the column buffer also contained 1 mM $CaCl_2$. Inclusion of 10 mM $CaCl_2$ did not alter receptor binding to LFA columns. The samples were incubated for 45 min at 4° in capped plastic columns, and the contents were mixed by shaking every 5-10 min. The mixture was allowed to flow through the gel and the eluate was collected. Following washing of the gel three times with 4 ml cold column buffer, column buffer (4 ml) containing 100 mM (50 mM for NeuAc) lectin-specific sugar was added to each column and the mixtures were incubated for 45 min at 4°. The eluate was collected and each column was washed four times with 4 ml of column buffer. The radioactivity contained in the four fractions eluted by buffer alone and in the four fractions eluted following the addition of sugar was determined.

Ultragel AcA 34 Chromatography: The soluble receptor fractions obtained from Sephadex G-50 chromatography were centrifuged at 100,000xg for 60 min. A 3-ml aliquot of each supernatant was loaded onto a 1.6 x 73 cm Ultragel AcA 34 column which had been equilibrated with cold incubation buffer containing 0.1% digitonin. The column was eluted at a flow rate of 15 ml/hr. Fractions (1.5ml) were collected and counted for radioactivity. The distribution coefficient (K_D) for eluted proteins was determined as $K_D=(V_e-V_o)/(V_t-V_o)$, where V_e is the elution volume, V_o is the void volume (60 ml as determined with blue dextran), and V_t is the total column volume (155 ml as determined with $[^3H]H_2O$).

RESULTS

Receptor Characterization. Previous experiments had shown that specific binding of the antagonist radioligands $[^{125}I]$IHEAT and $[^{125}I]$ICYP to MDCK-D membranes identified α_1- and β_2-adrenergic receptors, respectively (17). When membranes were labeled with radioligands in the presence of varying concentrations of the (-)- and (+)-enantiomers of epinephrine (α_1) or propranolol (β_2) and were then solubilized with digitonin, specific binding to the soluble receptors was stereoselective, as expected for adrenergic receptors, in that (-)-enantiomers were more potent than (+)-enantiomers (data not shown). The radioligands dissociated slowly from these solubilized receptors and the receptors were stable at 4°, since only 30% dissociation was observed for α-receptors and 15% for β-receptors after 26 hours at 4° (data not shown).

Lectin Affinity Chromatography: In order to determine whether the adrenergic receptors might be glycosylated, we incubated solubilized α_1 and β_2 receptors

with a series of agarose-coupled lectins having different sugar specificities. In initial lectin screening assays, the following lectins were used (sugar specificity in parentheses [19]): Con A (α-D-mannose, α-D-glucose), DBA (α-D-N-acetyl-D-galactosamine), RCA I (β-D-galactose), UEA I (α-L-fucose), and WGA (β-N-acetyl-D-glucosamine, sialic acid). Both α_1 and β_2 receptors bound to WGA but not to any of the other lectins in this initial series. The receptors retained and biospecifically eluted by WGA (corrected for non-specific binding) represented 42±24% (n = 6) and 27±10% (n = 10) (mean ± SEM) of the total α_1 and β_2 receptors, respectively. Receptors bound to WGA were biospecifically eluted by N-acetyl-D-glucosamine or sialic acid (Table 1).

The following experiments indicated that the radioactivity retained by WGA represented receptor-bound radioligand. First, when digitonin extracts of membranes labeled with [^{125}I]IHEAT plus prazosin or [^{125}I]ICYP plus propranolol (i.e., non-specific binding) were applied to WGA-agarose, less than 4% of the radioactivity was retained by the lectin. Second, α_1 receptors present in

TABLE I. Lectin Affinity Chromatography of Digitonin-solubilized MDCK Alpha- and Beta-receptors

		Receptor Recovery in Eluate Following Sugar Addition (% of control [WGA + GlcNAc])	
Lectin	Sugar	Alpha-adrenergic Receptor	Beta-adrenergic Receptor
WGA	GlcNAc	100	100
WGA	NeuAc	141.5 ± 21.9 (2)	119.5 ± 14.2 (5)
LFA	NeuAc	78.1 ± 22.3 (5)	39.6 ± 14.4 (8)
LFA	GlcNAc	38.8 ± 22.3 (2)	30.7 ± 6.5 (4)
STA	GlcNAc	20.3 ± 10.8 (4)	11.0 ± 5.2 (5)
GSA II	GlcNAc	27.5 ± 2.3 (2)	10.9 ± 6.2 (2)

Aliquots of digitonin-solubilized radioligand-receptor complexes were incubated with the indicated agarose-coupled lectins, eluted with buffer, and then eluted with sugar-containing buffer as described in the text. The data are expressed as receptor recovery from each column by addition of sugar as a percent of recovery from WGA + GlcNAc in the same experiment. These values are uncorrected for non-biospecific retention or elution of radioactivity (<5% of the total radioactivity on other lectin columns that failed to bind solubilized receptors). Relative to results for WGA + GlcNAC, these other "nonretaining" columns yielded values of 28.6±14.8% for α-adrenergic receptors and 14.4±7% for β-adrenergic receptors (n=8 for both types of receptors). Each value is shown as the mean ± S.E.M. for the number of experiments indicated in the parentheses.

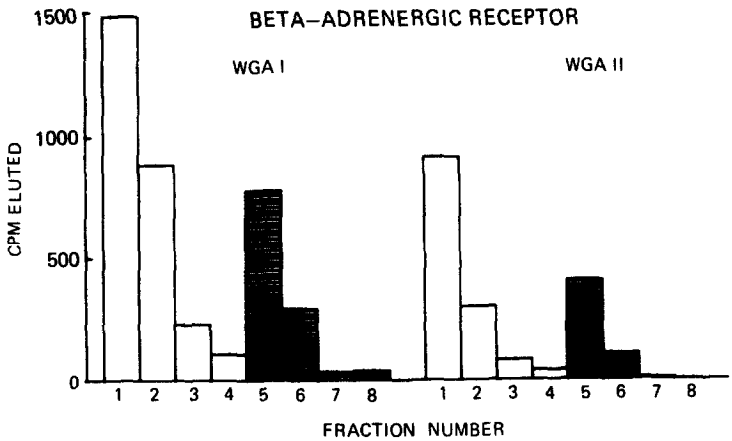

Figure 1. Retention of β_2 receptors on WGA-agarose and elution by GlcNAc. Digitonin-solubilized [^{125}I]ICYP-labeled β_2 receptors were applied to WGA-agarose as described in the text. Fractions 1-4 (open bars) were eluted with buffer and fractions 5-8 (shaded bars) were eluted with buffer following incubation of the gel with 100 mM GlcNAc. Fraction 1 from the first column (WGA I) was applied to a second fresh WGA column (WGA II) and was similarly eluted. The data shown represent "total" binding (specific + nonspecific); in this experiment nonspecific binding represented 18% of the total [^{125}I]ICYP present in the preparation.

specific binding fractions eluted from an Ultragel ACA 34 column (see Fig. 2) were retained and biospecifically eluted from WGA. Third, specific binding of radioligands to α_1 or β_2 receptors in MDCK-D membrane preparations was not inhibited by WGA at concentrations up to 100 µg/ml. To determine why only ~35% of the total α_1 or β_2 receptors bound to WGA, we incubated aliquots of the portions of the receptor preparations which were not retained on WGA (i.e., fractions eluted with buffer alone) with fresh WGA-agarose columns. We found that 37% of the β_2 receptors were retained (and eluted by GlcNAC) on the first column followed by a 40% yield from the second column (Fig. 1). Similar results (28% followed by 24%) were obtained for α_1 receptors, thus suggesting that the partial retention of receptors on WGA results from the inefficiency of the lectin columns rather than from receptor heterogeneity.

To determine whether WGA bound to N-acetyl-D-glucosamine or sialic acid residues on MDCK-D α_1 and β_2 receptors, we used three additional lectins with the following sugar specificities (Table 1): LFA (sialic acid) (20); STA (β-D(1 4)-linked oligomers of GlcNAC) (21), and GSA II (α- or β-N-acetyl-D-glucosamine) (19). Both α_1 and β_2 receptors were bound by LFA and WGA and not appreciably by STA or GSA II. Alpha$_1$ receptors bound to LFA were eluted more

effectively by NeuAc than by GlcNAc, confirming the sialic acid selectivity of this lectin. The lower extent of elution of β_2 receptors from LFA by NeuAc (40% of that from WGA + GlcNAC) resulted from a lower degree of receptor retention rather than incomplete elution of receptors by sugar (data not shown). The β_2 receptors were also eluted from LFA more efficiently by NeuAc than by GlcNAc ($p<.025$ by paired t-test for $n = 4$). These results imply that WGA binds to sialic acid residues present on α_1 and β_2 receptors, rather than to terminal or internal GlcNAc residues.

Gel Exclusion Chromatography: We evaluated the apparent molecular sizes of α_1 and β_2 receptors solubilized from MDCK cells. Digitonin extracts of membranes labeled in the absence and presence of prazosin (for α-receptors) or propranolol (for β-receptors) were chromatographed separately on Ultragel AcA34 (Figure 2). In several independent experiments, the α_1 and β_2 receptors consistently eluted as distinct entities. The apparent molecular (Stokes) radii were calculated to be 58 Å (6.8 nm) for the α receptor and 55 Å (5.5 nm) for the β receptor, yielding estimated molecular weights of the α_1 and β_2 receptors of 251,000 and 150,000 daltons, respectively.

DISCUSSION

From these observations, we conclude that both α_1- and β_2-adrenergic receptor complexes are sialylated glycoproteins. Although others have shown that WGA-Sepharose can bind solubilized adrenergic receptors (e.g., 22, 23) we believe that ours are the first data showing that these two adrenergic receptor subtypes possess terminal sialic acid. The presence of sialic acid-containing α_1 and β_2 receptors may relate to the localization and perhaps the insertion of the receptors in MDCK cells. MDCK cells appear to have a high density of WGA-binding sites on their basolateral surfaces (24), and α and β receptors appear to be located on the basolateral surface of these cells (15,16). Based on the different affinities of LFA for these receptors, α_1 and β_2 receptors may differ in their oligosaccharide residues.

The distinct elution profiles of α_1 and β_2 receptors from the Ultragel AcA 34 column indicate that the two receptors exist as separate detergent-

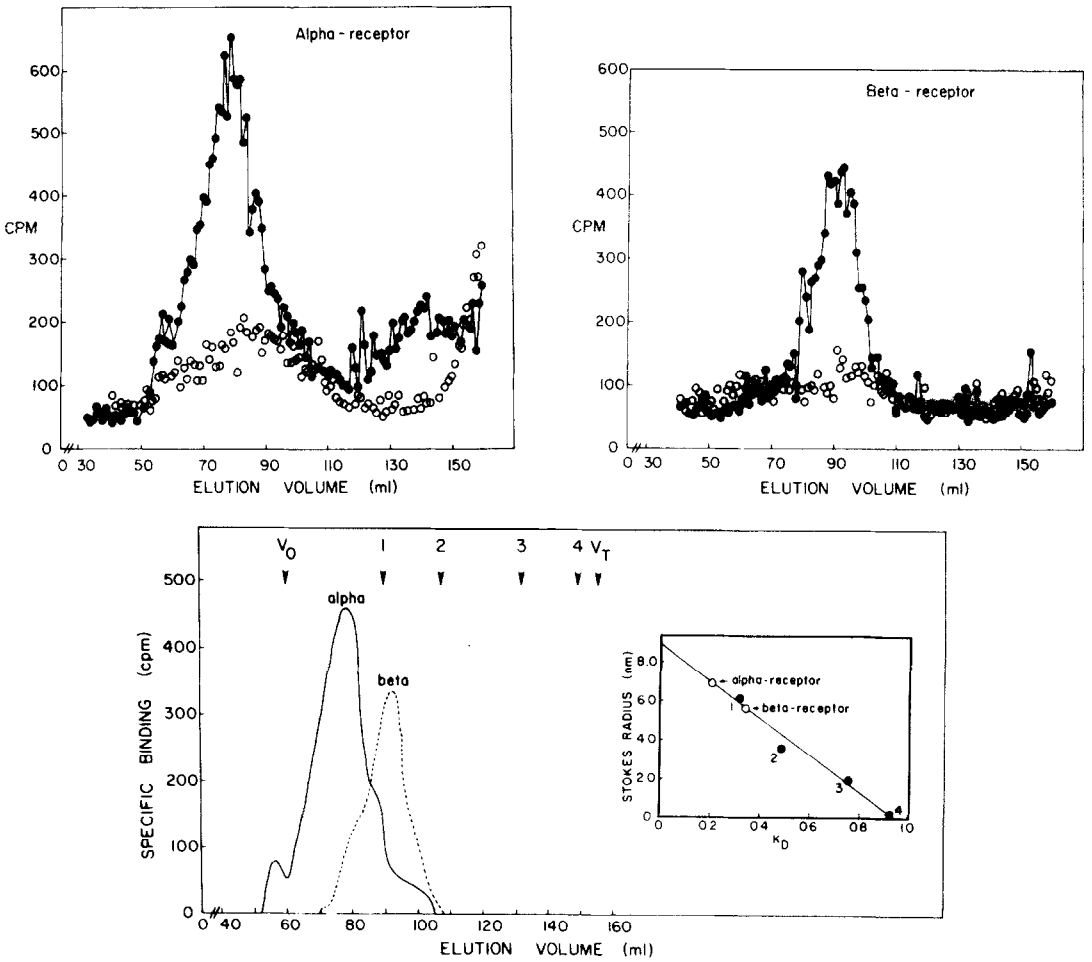

Figure 2. Ultragel AcA 34 Chromatography of Digitonin-solubilized MDCK α- and β-receptors.

The left panel shows the elution profile of soluble α-receptors labelled with [^{125}I]IHEAT in the absence (●) and presence (○) of 1 μM prazosin. The minor peak of specific binding eluting at 120-150 ml was not observed in other identical experiments; this experiment is shown because both α- and β-elution profiles were obtained using the same membrane preparation. The right panel shows the elution of β-receptors labelled with [^{125}I]ICYP in the absence (●) and presence (○) of 1 μM (±)-propranolol. The specific radioligand binding for α- (solid line) and β- (dashed line) receptors is shown in the bottom panel. The inset shows a plot of the distribution coefficient (K_D) for standard proteins versus the $\log_{10}$ of their molecular weights. The protein standards (New England Nuclear) are: 1) ferritin; 2) [^{14}C]methylated bovine serum albumin; 3) [^{14}C]methylated carbonic anhydrase; and 4) cytochrome C.

receptor complexes. Our estimate of the molecular weight of the digitonin-solubilized β_2-adrenergic receptor from MDCK cells is similar to a previously reported value (150,000 daltons) for the digitonin-solubilized frog erythrocyte β_2-adrenergic receptor (25). By contrast with our findings for α_1 receptors on MDCK cells, indicating a Stoke's radius of 78Å, work from other laboratories indicates that digitonin-solubilized (6), Lubrol PX-solubilized

(26) or Triton X-100-solubilized (8) α_1-adrenergic receptors from rat liver have Stokes radii of 49 Å, 57 Å, or 60 Å, respectively. Differences between our estimates for α_1 receptors and those of previous workers could relate to differences between species, tissues, or extent of proteolysis. The possibility that α_1- and β_2-adrenergic receptors might be similar in size but migrate differently on Ultragel AcA 34 because of association with other receptor or nonreceptor proteins is unlikely because previous data have shown that digitonin-solubilized adrenergic receptors prelabelled with antagonist radioligands migrate separately from receptor-linked guanine nucleotide-binding proteins that regulate adenylate cyclase (23,27). Alpha$_1$- and β_2-adrenergic receptors appear to be separate entities within the plasma membrane, and are not merely the same protein located in a different membrane environment.

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