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DEGLYCOSYLATED MAMMALIAN β_2-ADRENERGIC RECEPTORS ARE STILL ABLE TO UNDERGO FUNCTIONAL COUPLING TO Ns

Y. SEVERNE,* R. JURSS† and G. VAUQUELIN*

*Department of Protein Chemistry, Institute of Molecular Biology, Vrije Universiteit Brussel, 65 Paardenstraat, B-1640 St. Genesius Rode, Belgium, and †Department of Physiological Chemistry, University of Würzburg Medical School, D-8700 Würzburg, Federal Republic of Germany

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Abstract—Mammalian β_2-adrenergic receptors (R) have been shown to be structurally heterogenous with respect to glycosylation (Stiles *et al. J. biol. Chem.* **259**, 8655 (1984)). They are also heterogenous with respect to functional coupling to Ns. The ternary H.R.Ns complex can be frozen in the presence of the alkylating reagent *N*-ethylmaleimide. In hamster lung membranes 45% of the receptors are agonist/*N*-ethylmaleimide sensitive (i.e. coupling-prone receptors). β-Receptors in both native and isoproterenol/N-ethylmaleimide pretreated membrane preparations are retained by affinity chromatography on concanavalin A and wheat germ agglutinin and are equally sensitive to neuraminidase treatment. This is exhibited by the increase in mobility of the ^{125}I-iodocyanopindolol-azide photoaffinity labeled receptor peptide in SDS-polyacrylamide gel electrophoresis. These observations suggest that there is no link between the structural and functional heterogeneity of the receptors. Moreover, both partial (using neuraminidase) and near total (using endoglycosidase F) deglycosylation of membrane-bound receptors does not affect the H.R.–Ns coupling capacity as compared to native receptors.

The recent characterization of β-adrenergic receptors as glycoproteins was greatly facilitated by the availability of photoaffinity probes for the receptors allowing their direct identification and visualization on SDS-PAGE [1]. More detailed information concerning the composition of the sugar residues was obtained by the assessment of the receptors' susceptibility to different endo- and exoglycosidases as well as of their specific adsorption and elution from immobilized lectins [1, 2].

Using the exoglycosidases neuraminidase (specific for terminal sialic acid) and α-mannosidase (specific for terminal mannose), Stiles *et al.* [1] were able to distinguish both high mannose and complex type N-linked sugar chains in hamster lung β-adrenergic receptors. Moreover, on the basis of sequential exoglycosidase treatments, they suggested that both types of chains reside on discrete receptor populations. Separation of the two populations could be achieved by lectin affinity chromatography on concanavalin A- and wheat germ agglutinin-Sepharose [1]. The β-adrenergic receptors of hamster lung seem thus to be characterized by a structural heterogeneity.

The β-adrenergic receptors are also characterized by a functional heterogeneity [3, 4]. Since little is known about the functional significance of the heterogeneity in receptor glycosylation, we investigated whether there was a possible relationship with the ability of the agonist-bound receptor (H.R.) to undergo functional coupling to the adenylate cyclase stimulatory component Ns. This first step in β-receptor activation brings about a conformational modification of both H.R. and Ns, the receptor adopting a slow-agonist dissociating conformation [5, 6]. Addition of the reagent *N*-ethylmaleimide (NEM)‡ provokes the freezing of the H.R.Ns complex, possibly by alkylation of a sulfydryl group of Ns. This prevents subsequent receptor detection by radioligand binding [5, 7]. Only part of the β-adrenergic receptors are agonist/NEM sensitive and it follows that only this receptor subpopulation is capable of undergoing complex formation with Ns.

Using hamster lung membranes, we compared the glycosylation patterns of the total receptor population with that of the uncoupled receptors remaining after agonist/NEM treatment. We show that the putative structural heterogeneity of the hamster lung β-adrenergic receptor is not linked to its functional heterogeneity. Moreover, the modification of the oligosaccharide chains with exoglycosidases, and the near total deglycosylation using endoglycosidase F, do not alter receptor–Ns coupling.

MATERIALS AND METHODS

Materials. (−)-^{3}H-dihydroalprenolol (^{3}H-DHA) (92.4 Ci/mmol) and endoglycosidase F were supplied by New England Nuclear. ^{125}I-CYP-azide-2 (2.2 Ci/μmol) was synthetized according to Burgermeister *et al.* [8]. (−)-Isoproterenol bitartrate, *N*-ethylmaleimide, p-nitrophenyl-α-D-mannopyranoside, neuraminidase (from *Clostridium perfringens* type X), α-D-mannosidase, aldolase as well as the pro-

‡ Abbreviations used: ^{3}H-DHA, (−)-^{3}H-dihydroalprenolol; EDTA, ethylenediaminetetraacetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Con A, concanavalin A; WGA, wheat germ agglutinin; endo F, endoglycosidase F; GlcNAc, *N*-acetylglucosamine; ^{125}ICYP, ^{125}I-iodocyanopindolol; NEM, N-ethylmaleimide.

tease inhibitors leupeptin, pepstatin, phenylmethylsulfonylfluoride (PMSF) were from Sigma Chemical Co. Electrophoresis reagents were from Serva. Sephadex G-25, concanavalin A-Sepharose 4B and the premixed low molecular weight protein standard kit, phosphorylase b (94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000) and soybean trypsin inhibitor (20,000) were from Pharmacia. Ethylenediaminetetraacetic acid (EDTA) and Nonidet P-40 were from Fluka AG. *N*-acetyl-α-D-glucosamine and α-methyl-D-mannopyranoside were from Janssen Chimica. Lectin from wheat germ (WGA) was purchased from Boehringer Mannheim. WGA-Affigel 10 was prepared according to the instructions supplied by the manufacturer (Bio-Rad). Centricon 30 cones were from Amicon. Male Gold hamsters were obtained at the Katholieke Universiteit Leuven.

Membrane preparation. Hamster lung membranes were prepared from fresh lungs, directly upon excision as described by Benovic *et al.* [9]. The following protease inhibitors were added, when indicated, at the following concentrations: 5 mM EDTA, 100 μM PMSF, 5 μg/ml leupeptin, 3 μg/ml pepstatin. Protein determinations were performed by the method of Lowry *et al.* [10], using bovine serum albumin as standard.

Isoproterenol/NEM assay and radioligand binding. The preincubations with isoproterenol/NEM occurred as follows: 400 μl of membrane suspension in buffer A (Tris 50 mM, pH 7.4, $MgCl_2$ 10 mM) were incubated in Eppendorf tubes with 50 μl of (−)-isoproterenol (final concentration $5\ 10^{-7}$ M) and 50 μl of NEM (final concentration $2\ 10^{-4}$ M) for 10 min at 30°. Preincubation was ended by the addition of 1 vol. ice-cold buffer A and pelleting the membranes in an Eppendorf centrifuge. The membranes were resuspended in 1 ml fresh buffer and washed three times before incubation with the radioligand. ^{3}H-DHA (final concentration $2\ 10^{-9}$ M) binding assay was performed as described in [7].

Photoaffinity labeling. Hamster lung membranes were labeled with ^{125}I-CYP azide as described by Jurss *et al.* [11]. Briefly, 5 μl of a methanolic solution of ^{125}I-CYP azide (final concentration 200 pM) was added in dim light to 500 μl of membrane suspension in buffer B (Tris 20 mM, pH 7.4, with the protease inhibitors as described above). The samples were incubated in the dark at 30° for 30 min, then diluted with 1.5 ml buffer B and irradiated on ice for 12 min at 254 nm.

NP-40 solubilization and lectin affinity chromatography. Following photoaffinity labeling, the membranes were washed once with 50 mM Tris (pH 7.4), to remove the EDTA. The sedimented membranes (2–4 mg) were solubilized in NP-40 buffer (0.8% NP-40, 10 mM Tris, 150 mM NaCl, pH 7.3) for 1 hr with constant gentle stirring at room temperature. The preparation was then centrifuged at 48,000 *g* for 20 min and the supernatant of solubilized membranes was diluted 5 times with 10 mM Tris (pH 7.4), 150 mM NaCl to obtain a final concentration of NP-40 of 0.16% as described by Stiles *et al.* [1].

Lectin columns (3 ml syringe) with bed volume of 1 ml were washed before use with at least 30 ml of buffer C (10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.1% NP-40 and 0.1% aldolase to saturate the non-specific binding sites). One millimolar $CaCl_2$ and 1 mM $MnCl_2$ was included for washing of the Con A column. The solubilized membrane preparation (3 ml) was loaded at room temperature on the lectin column and slowly recycled 3 times through the column. Unbound material was collected in the flow-through, and the column was then washed with 25 ml buffer C; 0.6 ml fractions were collected. Elution of the bound material occurred with buffer C containing 0.3 M of the specific sugar (α-methyl-D-mannopyranoside for the Con A column and *N*-acetyl-glucosamine for the WGA). The fractions of interest were desalted using either Sephadex G-25 columns or Centricon 30 cones: the buffer exchange occurred then by repeated centrifugation and dilution with 0.2% SDS, 20 mM Tris–HCl (pH 6.8). The desalted samples were evaporated with a speed vac concentrator for 1 hr and resuspended in SDS-electrophoresis buffer (final concentration of 5% β-mercaptoethanol, 10% glycerol, 4.5% SDS and 100 mM Tris). These aliquots were kept at room temperature for 1 hr, centrifuged in an Eppendorf centrifuge for 4 min and the supernatants subjected to SDS-PAGE.

Exo- and endoglycosidase treatments. The treatments with α-mannosidase occurred as described in Stiles *et al.* [1]. Neuraminidase treatment (0.5 units/ml) occurred for 50 min at 30° in 10 mM sodium-phosphate buffered saline (PBS) pH 7, supplemented with the different protease inhibitors. For endoglycosidase F treatment, the membranes were incubated in buffer B with 5 units/ml of the enzyme with the protease inhibitors, for 2 hr at 30°. After enzymatic treatments, the membranes were washed once before further experimentation.

SDS-polyacrylamide gel eelctrophoresis. SDS-PAGE of the membranes was performed according to Laemmli [12] using homogenous slab gels containing 9% acrylamide in the separating gel. Following electrophoresis, the gels were dried prior to autoradiography at −70° on Kodak X-Omat film in Dupont Cronex cassettes with intensifying screens.

RESULTS

Hamster lung membranes are a rich source of β-adrenergic receptors; the total number of binding sites determined by Scatchard plot analysis [13] equals 1300 fmoles/mg protein and the equilibrium dissociation constant (K_D) for ^{3}H-DHA binding is 0.85 nM (data not shown). When these membranes are treated with 0.5 μM isoproterenol and 0.2 mM *N*-ethylmaleimide (NEM) for 10 min at 30°, the apparent number of ^{3}H-DHA binding sites is decreased by 45 ± 7% (Table 1).

The β-receptors are specifically photoaffinity labeled with ^{125}ICYP-azide [8]. A major peptide with a molecular weight corresponding to 63 KD in SDS-PAGE is detected in hamster lung membranes. Two other bands are also visible but their intensity is decreased upon addition of the adequate protease inhibitors (5 mM EDTA, 0.1 mM PMSF, 5 μg/ml leupeptin and 3 μg/ml pepstatin). Hence, as described by Benovic *et al.* [9], they are formed by proteolytic degradation of the major peptide. The

Table 1. Isoproterenol/NEM sensitive sites after pretreatments with neuraminidase and endoglycosidase F.

Pretreatment	None	Buffer 1 hr 30°	Neuraminidase	Buffer 2 hr 30°	Endo F
% Isoproterenol/NEM sensitive sites	45%	32%	35%	38%	34%

Hamster lung membranes were incubated with neuraminidase (0.5 units/ml), endo F (5 units/ml) or buffer only for the indicated time periods at 30°. The membranes were then washed, treated with isoproterenol/NEM and assayed for ^{3}H-DHA (2 nM) binding as described in Materials and Methods. The results shown are the means of two to four experiments.

coupling capability of the receptors (i.e. their capacity to react with isoproterenol/NEM) is unaffected by the relative proportion of the different bands (data not shown). Photoaffinity labeling of isoproterenol/NEM pretreated membranes provides for the specific tagging of the uncoupled receptor population. This population shows no difference in mobility in SDS-PAGE when compared to the total receptor population (Fig. 1, lanes 1 and 3).

To investigate a possible link between the presence of distinct sugars on the receptors and their capacity to undergo coupling with Ns we first examined the effects of exo- and endoglycosidase treatments on ^{125}ICYP-azide photolabeled control (buffer pretreated) and non-coupled (isoproterenol/NEM pretreated) receptor populations and whether the exo- and endoglycosidase treatments affected the isoproterenol/NEM reaction. As shown in Fig. 1,

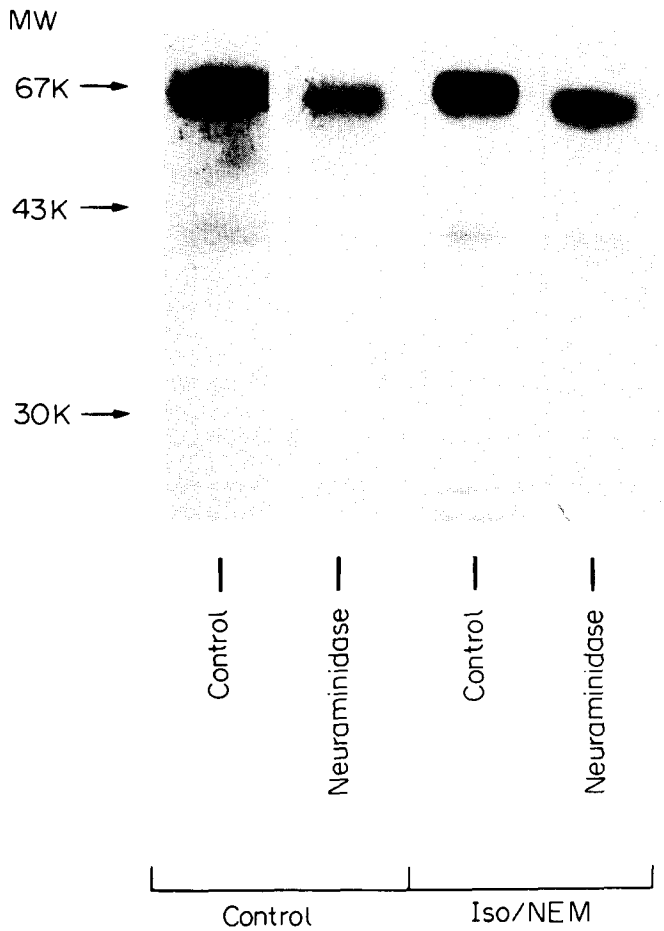

Fig. 1. Effect of neuraminidase on both control and isoproterenol/NEM pretreated membranes. Membranes were first incubated with buffer or isoproterenol/NEM as described in Materials and Methods. This caused a decrease of 38% in radioligand binding. Membranes were subsequently washed, labeled with ^{125}I-CYP azide, washed again and resuspended in buffer. The membrane suspensions were separated in two, one of them being treated with neuraminidase, the other with buffer. Incubation occurred at 30° for 1 hr, after which the membranes were precipitated by centrifugation and prepared for SDS-PAGE as indicated in Materials and Methods. The experiment shown is representative of three similar experiments.

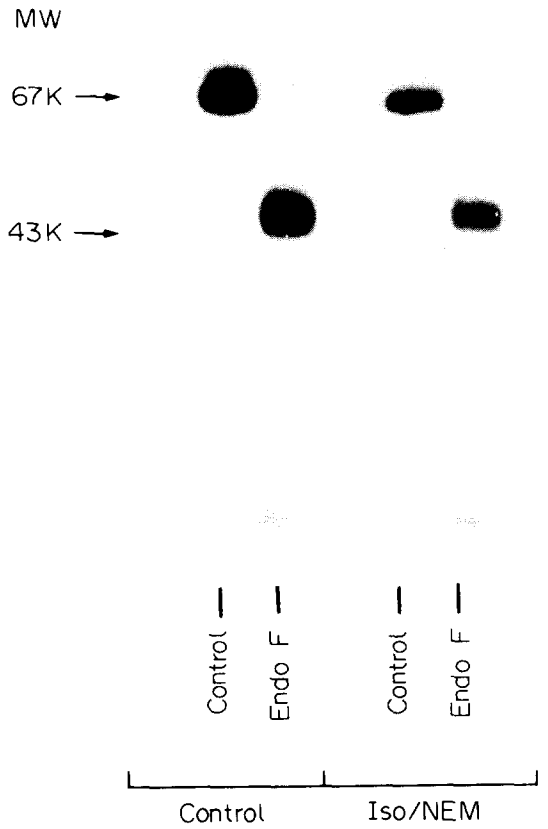

Fig. 2. Effect of endo F on both control and isoproterenol/NEM pretreated membranes. Membranes were first incubated with buffer or isoproterenol/NEM as described in Materials and Methods. This caused a decrease of 40% in radioligand binding. Membranes were subsequently washed, labeled with ^{125}I-CYP azide, washed again and resuspended in buffer. The membrane suspensions were separated in two, one of them being treated with endo F, the other with buffer. Incubation occurred at 30° for 2 hr, after which the membranes were precipitated by centrifugation and prepared for SDS-PAGE as indicated in Materials and Methods. The experiment shown is representative of two similar experiments.

neuraminidase affects similarly the control (lane 2) and the isoproterenol/NEM pretreated membranes (lane 4); in both cases a small increase in the mobility of the receptors is observed. Similarly, we wanted to assess the effects of α-mannosidase. Under similar assay conditions, α-mannosidase treatment did not produce the changes in receptor peptide mobility that are described by Stiles *et al.* [1] for the hamster lung β-adrenergic receptors. Nevertheless, the enzyme was shown to be active under our incubation conditions (by colorimetric measurement [14] of the hydrolysis product of the enzyme substrate *p*-nitrophenyl α-mannoside). The treatment of hamster lung β-receptors with endoglycosidase F (endo F), resulted in its deglycosylation (Fig. 2, lanes 1 and 2) and is accompanied by an increased mobility of the receptor (48 KD). As shown by Stiles [15], deglycosylation does not affect the ability of the receptor to recognize or bind either agonists or antagonists. Moreover, we found that the isoproterenol/NEM resistant receptors demonstrate the same sensitivity towards endo F as the total population (Fig. 2, lanes 3 and 4).

To test further if the oligosaccharide moiety does affect the receptor coupling process, we carried out isoproterenol/NEM treatments on neuraminidase pretreated and endo F-deglycosylated receptor preparations. Isoproterenol/NEM treatment resulted in loss of 35% and 34% of binding sites respectively for neuraminidase- and endo F-pretreated membranes (Table 1). Our results indicate that the partially or totally deglycosylated hamster lung β-adrenergic receptors are not impaired in their coupling capability to Ns since they are still isoproterenol/NEM sensitive.

Lectin affinity chromatography was used by Stiles *et al.* [1] to confirm the existence of two distinct sugar-linked receptor populations. Likewise, membranes pretreated with buffer (control) or with isoproterenol/NEM, then labeled with ^{125}ICYP-azide, solubilized with NP-40 were fractionated by chromatography on concanavalin A-Sepharose 4B (Con A) and on wheat germ agglutinin linked to Affigel 10 (WGA). Figure 3 compares representative elution profiles of receptor from control and isoproterenol/NEM treated membranes. After extensive washing, the columns were eluted with 0.3 M of the competing sugars, respectively α-methyl-D-mannopyranoside and *N*-acetylglucosamine. From both gels a peak of ^{125}ICYP-azide labeled peptide could be specifically eluted, whether control or isoproterenol/NEM treated membranes were used. Autoradiography after SDS-PAGE of the indicated fractions from the lectin columns are shown in Fig. 3, inset. These data show that there is no difference in electrophoretic mobility of the total and isoproterenol/NEM resistant β-adrenergic receptors in the fractions eluted after WGA or Con A chromatography. This confirms the presence of high mannose and complex type of sugars linked to the β-adrenergic receptors, regardless of their ability to undergo coupling to Ns.

DISCUSSION

It has recently been shown that the sugar moiety of both β_1- and β_2-adrenergic receptors is not important for the receptor–ligand interaction [2, 15]. However, the functional role of the sugars bound to the β-adrenergic receptors is still unknown. The results presented in this study suggest that there is no relation between the glycosylation state of the membrane-bound β-receptor and its ability to interact with the regulatory component Ns.

We have used the agonist/NEM reaction to assess the extent of H.R–Ns coupling. Based on this probe, neuraminidase treated or endo F deglycosylated hamster lung membranes exhibited unchanged coupling ability as compared to control membranes. Comparable conclusions also hold for the turkey erythrocyte system. SDS-PAGE of photoaffinity labeled turkey erythrocyte membrane preparations allows for the detection of two peptides of 50 KD and 40 KD. The conversion of the larger peptide to 40 KD is produced by the proteolytic trimming of a fragment containing most or all of the sugar chains

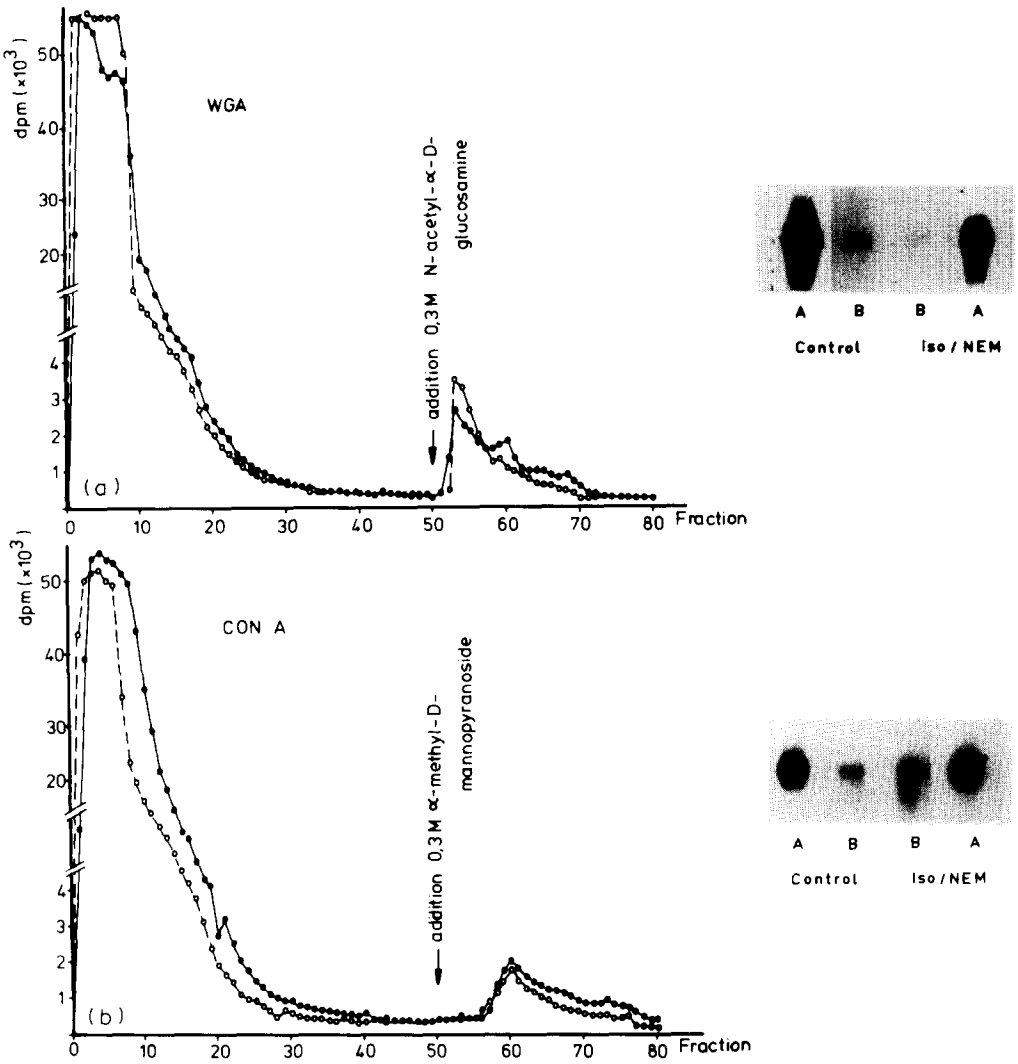

Fig. 3. Lectin affinity chromatography of control and isoproterenol/NEM pretreated β-receptors from hamster lung membranes. Membranes were labeled with ^{125}I-CYP azide, solubilized with NP-40 and eluted from WGA-affigel and Con A-sepharose columns as described in Materials and Methods. The first peak of radioactivity represents the flow-through and after the radioactivity has returned to its baseline level, specific elution of the columns is shown by the second peak. Top: elution profile on WGA-affigel: ○—○, control; ●—●, preincubated for 10 min at 30° with isoproterenol and NEM prior to affinity labeling with ^{125}I-CYP azide: 39% of the receptors were isoproterenol/NEM sensitive in this experiment. Bottom: elution profile on Con A-sepharose columns. Legend as above. 48% of the receptors were isoproterenol/NEM sensitive in this experiment. Data are representative of two similar experiments. Insets: autoradiograms of SDS-PAGE of representative fractions from the lectin columns. In WGA chromatography fraction 4 (a) and fraction 55 to 58 (b) and from Con A chromatography fraction 4 (a) and fraction 59 to 62 (b) were desalted in Centricon cones, lyophilized and prepared for SDS-PAGE.

of the β-adrenergic receptor [11]. These two receptor forms show the same ligand binding properties and are capable of stimulating the adenylate cyclase in reconstituted membrane systems [17–19]. Both forms are isoproterenol/NEM sensitive and undergo coupling to Ns (Severne *et al.*, unpublished results). Moreover, preliminary work of Benovic *et al.* [20] indicates that endo F-deglycosylated hamster lung receptors can interact with Ns when inserted into phospholipid vesicles. This phenomenon was monitored by the restoration of β-adrenergic agonist mediated stimulation of the GTPase activity of Ns.

A structural heterogeneity of the hamster lung β-adrenergic receptors was suggested by Stiles *et al.* [1] on the basis of the receptor sensitivity towards α-mannosidase and neuraminidase as well as their binding capacity to Con A- and WGA-sepharose. As demonstrated by Figs. 1 and 3, the functional heterogeneity of the receptors is independent of any oligosaccharide structural heterogeneity. Indeed, the

same percentage of coupling-prone receptors are found in the deglycosylated and in the control receptor populations. Moreover, the comparison of the total with the non-coupled (isoproterenol/NEM resistant) receptor populations shows that both are neuraminidase sensitive and both are specifically eluted from a WGA column, indicating the presence of terminal sialic acid. Although we could not demonstrate any change in the band pattern on SDS-PAGE after α-mannosidase treatment, both the total receptor and the uncoupled receptor populations showed the same elution profile from Con A-sepharose chromatography. This demonstrates the presence of mannose or glucose in the sugar chain, although not specifically in a terminal position [21].

A possible role of the oligosaccharide moiety could be related to the translocation of the receptor to specific membrane domains. The translocation to the cell membrane or to a proper lipid environment is indeed crucial for receptor activity since delipidated β-receptors are totally inactive [22]. In this context, Meier *et al.* [16] reported that a canine kidney epithelial cell line (MDCK cells) has a high density of WGA binding sites on their basolateral surface. The authors suggested that the presence of neuraminic acid bound to α_1- and β_2-adrenergic receptors in these cells may be related to their localization on the basolateral surface.

It has been shown that glycosylation is necessary for the binding of EFG and insulin to their receptors [23, 24]. In addition, Doss *et al.* [25] have reported that β-receptors accumulation is blocked in human astrocytoma cells when cultured in the presence of tunicamycin, an antibiotic that inhibits the formation of Asn-N-linked glycoproteins. Tunicamycin could indirectly interfere with N-linked glycosylation of other cellular glycoproteins, including any glycosylated protein involved in protein transport or cellular processing of the receptors. Recently, by *in vitro* mutagenesis, Guan *et al.* [26] demonstrated that N-linked glycosylation allowed for the transport to the cell membrane of a hybrid protein consisting of portion of the rat growth hormone and of a membrane protein. This novel approach to the functional role of oligosaccharides bound to proteins avoids the problems of interpretation linked to the use of general inhibitors of the glycosylation.

In conclusion, differences in glycosylation do not explain the functional heterogeneity of the β-adrenergic receptors and could relate to the vectoriality or efficacy of intracellular transport of newly synthesized β-receptor molecules. Once the receptor is inserted in the membrane, the oligosaccharide moiety does not seem to be important for its ability to undergo coupling with Ns.

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