The β_2 -adrenergic receptors of human epidermoid carcinoma cells bear two different types of oligosaccharides which influence expression on the cell surface

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The β_2 -adrenergic receptors of the human epidermoid carcinoma A431 cells reside on two polypeptide chains revealed by photoaffinity labelling with [125 I]iodocyanopindolol-diazirine. These proteins correspond to two distinct populations of *N*-asparagine-linked glycoproteins: the 55–52 kDa molecules are associated with complex carbohydrate chains(s), the 65–63 kDa component with polymannosidic carbohydrate chain(s). Both types of receptors are present in preconfluent cells, but only the polymannosidic type is found in the postconfluent cells. Moreover, complex chains appear to be associated with the receptors with the highest affinity for (–)-isoproterenol and polymannosidic chains with the receptors with the lowest affinity for this agonist. The carbohydrate moiety of the β_2 -adrenergic receptor is involved in the expression and function of the β_2 -adrenergic receptors at the surface of the A431 cells, since tunicamycin and monensin, complete and partial inhibitors of glycosylation respectively, diminish the number of binding sites at the cell surface and increase the total number of sites in the cell. In these conditions a diminution of cyclic AMP accumulation is also observed.

INTRODUCTION

Several membrane receptors are glycoproteins, including those for opiates (Gioannini *et al.*, 1982), insulin (Cuatrecasas, 1973; Hedo *et al.*, 1981; Cherqui *et al.*, 1981), nerve growth factor (Buxser *et al.*, 1983), muscarinic ligand (Shirakawa *et al.*, 1983), and epidermal growth factor (Carpenter & Cohen, 1977). More recently, hamster lung β_2 -adrenergic (Stiles *et al.*, 1984) and turkey erythrocyte β_1 -adrenergic receptors (Cervantes-Olivier *et al.*, 1985; Jürss *et al.*, 1985) were also shown to contain sugar moieties. Potential glycosylation sites have actually been localized (Dixon *et al.*, 1986; Yarden *et al.*, 1986).

The role of oligosaccharides in relation to biological properties of glycoproteins has been studied in several different systems with various results: in some cases the carbohydrate moiety is needed for the activity of the glycoprotein (Caro *et al.*, 1984; Mangelsdorf-Soderquist & Carpenter, 1984), in others it is needed for its secretion (Bartalena & Robbins, 1984; Rustan *et al.*, 1984) or for its recycling (Stein *et al.*, 1984). The absence of oligosaccharide chains on hepatoma fibrinogen does not interfere with its secretion, nor with its function (Gilman *et al.*, 1985). As for β_2 -adrenergic receptors, recent results (Stiles, 1985) suggest that enzymically deglycosylated mammalian β_2 -adrenergic receptors recognize both agonists and antagonists with the same potency order and affinities as the glycosylated form.

In the present work we discuss the role of the

carbohydrate moiety of β_2 -adrenergic receptors for expression and function at the cell surface in human A431 epidermoid carcinoma cells, which possess a large number of functionally active β_2 -adrenergic receptors (Delavie-Klutcho *et al.*, 1984). Using cells at pre- and post-confluent state, we established that the β_2 -adrenergic receptors of A431 cells are glycoproteins of the *N*-linked type which bear two different types of carbohydrate chains, complex and polymannosidic, corresponding to two discrete populations of receptors which differ by their affinity for concanavalin A and also by their molecular mass.

A431 cells were treated with tunicamycin or monensin, drugs which inhibit different steps in glycosylation of proteins and have proven to be valuable tools for investigating the function of carbohydrate chains (Ueda *et al.*, 1985; King, 1984; Pryor & Buse, 1984; Doss *et al.*, 1985). The effect of these treatments was evaluated on β -adrenergic ligand binding, agonist and antagonist, on expression of receptors and on amount of cyclic AMP accumulation induced by the agonist. The results show that the carbohydrate moiety is needed for translocation of receptors to the cell surface.

MATERIALS AND METHODS

Materials

Con-A-Sepharose was purchased from Pharmacia. (±)-Propranolol, (–)-isoproterenol, tunicamycin and

Abbreviations used: [125 I]ICYP, [125 I]iodocyanopindolol; [125 I]ICYP-diazirine, [125 I]iodocyanopindolol-diazirine; PMSF, phenylmethanesulphonyl fluoride; [3 H]CGP-12,177, 3 H-labelled Ciba-Geigy product 12,177; Con-A, concanavalin A; EGF, epidermal growth factor; IC₅₀, concentration of ligand producing 50% inhibition of specific binding; PEG, poly(ethylene glycol); PBS, phosphate (10 mM)-buffered (pH 7.4) saline (145 mM-NaCl).

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monensin were purchased from Sigma. [^3H]CGP 12,177 (40 Ci/mmol), [^{125}I]ICYP (2000 Ci/mmol), [^{125}I]ICYP-diazirine (2000 Ci/mmol) D-[2- ^3H]mannose (10 Ci/mmol), *N*-acetyl-D-[1- ^3H]glucosamine (2.8 Ci/mmol) and [^{35}S]methionine (1100 Ci/mmol) were obtained from Amersham International, α -methyl D-mannopyranoside was from Calbiochem. RPMI was purchased from Boehringer, and EDTA and Hanks' solution were obtained from Eurobio.

Cell culture

The human epidermoid carcinoma cell A431 line, from the American Type Culture Collection, was grown at 37 °C in RPMI 1640 supplemented with 10 % foetal calf serum, 200 mM-glutamine, 100 mM-sodium pyruvate, 0.05 g of streptomycin/ml and 50 000 i.u. of penicillin/ml in a humidified incubator under an atmosphere of air/CO₂ (9:1). The medium was replenished every 2 days. Cells were plated out into 135 mm diameter culture dishes in 20 ml medium, until confluence; then they were detached with a solution of 0.25 % trypsin in versene (1:5000) and subcultured into 35 mm diameter culture dishes (6-well plates) at a density of 1.5×10^5 cells/well. The cells were used on the third day (preconfluent cells) or the eighth day (postconfluent cells) following subculture.

Inhibition of glycosylation

A431 cells were treated with 2 μg of tunicamycin/ml or 0.5 μM -monensin, in the presence of 1 mM-PMSF, for 24 h prior to the binding assays. When indicated, D-[2- ^3H]mannose, *N*-acetyl-D-[1- ^3H]glucosamine, or L-[^{35}S]methionine was added to the medium 1 h after addition of these drugs.

Cell solubilization

A431 cells were washed twice with cold PBS and were solubilized with 10 mM-Tris/HCl (pH 7.4) containing 90 mM-NaCl, 2 mM-EDTA, 2 mM-EGTA, 1 mM-PMSF, 5 μg of leupeptin/ml, 7 μg of pepstatin/ml, 17 μg of aprotinin/ml, 100 μg of bacitracin/ml and 25 mM-benzamidine (buffer A) and 1.2 % digitonin for 20 min at 4 °C. The solubilized cells were centrifuged for 10 min at 40 000 *g*.

Membrane preparation

A431 cell membranes were prepared using the method described for fibroblast cells (Thom *et al.*, 1977), except that protease inhibitors (1 mM-PMSF, 5 mM-EGTA, 25 mM-benzamidine, 10 μg of leupeptin/ml, 10 μg of trypsin inhibitor/ml and 17 μg of aprotinin/ml) were added to all buffers. Membranes were stored at -80 °C in 20 mM-Hepes (pH 7.4) containing 20 % glycerol and protease inhibitors.

Photoaffinity labelling of A431 membranes

Membranes from preconfluent A431 cells (final concentration of receptors 60–70 pM) were incubated in the dark for 3 h with 150 pM-[^{125}I]ICYP-diazirine, in 20 mM-Tris/HCl (pH 7.4)/5 mM-MgCl₂ with protease inhibitors. After incubation, samples were diluted 2-fold with the same buffer at 4 °C. Membranes were then washed three times with 10 ml of the same buffer, centrifuged at 39 000 *g* for 10 min and then photolysed for 3 min at 10 cm from a 200 W super pressure mercury lamp HBO with a 310 nm filter.

These labelled membranes were solubilized with 0.05 % SDS in buffer A and incubated batch-wise with Con-A gel equilibrated with buffer A containing 0.05 % SDS. The gel was washed and the bound material eluted as described below. With such a low concentration of SDS, according to Lotan *et al.* (1977), the modification of the integrity (5 %) and of the binding efficiency (30 %) of the Con-A gel did not alter the ultimate purpose, which is the visualization of the elution profile of the Con-A gel.

Binding assays on intact cells

Adherent cells in 6-well plates were washed three times with Hanks'/Hepes buffer, pH 7.4, and then [^3H]CGP 12,177 and [^{125}I]ICYP binding were determined as described previously (Delavier-Klutchko *et al.*, 1984). In competition experiments, A431 cells were preincubated in Hanks'/Hepes buffer, pH 7.4, at 37 °C for 15 min. The binding of 2 nM-[^3H]CGP 12,177 was measured for 1 min in the presence of varying concentrations of (–)-isoproterenol, in a total volume of 1 ml/well.

Determination of cyclic AMP accumulation in intact cells

Adherent cells in 6-well plates were washed three times with Hanks'/Hepes buffer pH 7.4 devoid of Phenol Red and then maintained at 37 °C for 15 min in 1 ml of this buffer supplemented with 1 mM theophylline. Various concentrations of (–)-isoproterenol were added during the last 30 s.

Incubations were stopped by elimination of the medium and addition of 400 μl of 100 mM-propranolol. Cells were detached with a rubber spatula and centrifuged. Cyclic AMP was measured on aliquots of 50 μl of the supernatant with the Amersham kit, according to method of Tovey *et al.* (1974).

Binding assays with solubilized cells

Solubilized cells (100–200 μg of protein) were incubated with 5 nM-[^{125}I]ICYP for 30 min at 37 °C in 10 mM-Tris/HCl (pH 7.4)/90 mM-NaCl with and without 2 μM -($\pm$)-propranolol. At the end of the incubation, the assay mixture was treated with PEG 6000 as described by Vauquelin *et al.* (1979), except that 12 % instead of 8 % PEG was used.

Lectin affinity chromatographies

Solubilized pre- or post-confluent cells (2–5 ml) were incubated with 5 nM-[^{125}I]ICYP in the absence or in the presence of 2 μM -($\pm$)-propranolol for 30 min at 37 °C.

The suspensions were then incubated batchwise with 1 ml of Con-A gel equilibrated with buffer A containing 0.1 % digitonin at room temperature for 60 min while gently shaking. The free material was recovered by centrifugation (2000 *g*) for 5 min and its binding activity was determined on an aliquot by separating bound [^{125}I]ICYP from free ligand by PEG precipitation.

The gel was transferred into a column and washed successively with buffer A containing 0.1 % digitonin (3 column vol.) and with buffer B which differs from buffer A in that it contains 1 M-NaCl instead of 90 mM (3 column vol.).

Bound material was eluted with 7 portions of 1 ml of 10 mM- α -methyl D-mannopyranoside followed by 6 portions of 1 ml of 300 mM- α -methyl D-mannopyranoside. The binding activity in each fraction was then determined.

Aliquots from the desorbed fractions were incubated for 30 min at 37 °C with [125 I]ICYP and with or without 2 μ M-($\pm$)-propranolol. The protein-bound ligand was precipitated by PEG.

Electrophoresis

SDS/polyacrylamide-gel electrophoresis on slab gels was performed under reducing conditions (5% β -mercaptoethanol), according to the method of Laemmli & Favre (1973) with 4% stacking gel and 10% separating gel. The molecular weight markers used were lysozyme (14 kDa), soybean trypsin inhibitor (21 kDa), carbonic anhydrase (31 kDa), ovalbumin (45 kDa), serum albumin (67 kDa), and phosphorylase (92 kDa).

Immunoblotting of β_2 -adrenergic receptor

Immunoblotting was carried out as previously described (Towbin *et al.*, 1979). The solubilized cells and eluted fractions from the Con-A gel were electrophoresed and electrophoretically transferred onto a nitrocellulose membrane. The filter was then incubated overnight with PBS containing 1% powdered milk and 0.1% Tween 20.

Monoclonal antibodies were diluted 50-fold and incubated with the nitrocellulose strips overnight at 4 °C. The strips were thoroughly washed in PBS/milk/Tween and incubated with 125 I-labelled protein A (10^6 c.p.m./ml) for 1 h. Finally, the strips were washed five times in PBS, dried and autoradiographed.

Data analysis

Competitive binding experiments were achieved in non-equilibrium conditions (1 min); to interpret the competition curves, experimental data points were analysed using the procedure described by Betton *et al.* (1985). The quantity of radioligand bound to the receptors after 1 min of exposure to the cells was calculated using the Kubicek & Visnack procedure (1974) on the basis of the model proposed by Motulsky & Mahan (1983); the calculations were based on the following parameters:

(1) the binding reactions follow the law of mass action;

(2) radioligand ([3 H]CGP 12,177) and competitor [(–)-isoproterenol] are in large excess compared with the binding sites and are simultaneously exposed to the receptors;

(3) association and dissociation rate constants for [3 H]CGP 12,177 are known ($k_{+1} = 2 \times 10^7 \text{ M}^{-1} \cdot \text{min}^{-1}$; $k_{-1} = 7 \times 10^{-3} \text{ min}^{-1}$);

(4) at 1 min exposure, internalization is negligible;

(5) the receptor concentration is $25 \times 10^{-12} \text{ M}$;

(6) dissociation rate constants for (–)-isoproterenol are at least 10-fold higher than the rate dissociation constant for antagonist ($k_{-1} > 1 \text{ min}^{-1}$); they were adjusted in order to obtain the best fitted curve by the use of the simplex procedure (Nelder & Mead, 1965).

In some data, with postconfluent cells, the two ligands compete for binding to a single class of receptors according to the law of mass action.

In contrast, competition curves obtained with pre-confluent cells do not match the model predicted for simple competition interaction described by the law of mass action. To explain these data, we have developed another model incorporating the following assumptions:

(1) two classes of receptors bind the agonist reversibly with different K_d values;

(2) association and dissociation rate constants for antagonists are respectively identical for the two classes of binding sites, since only one K_d is determined for each antagonist;

(3) dissociation rate constants for (–)-isoproterenol is at least 100-fold higher than the dissociation rate constant for antagonists for the two classes of receptors.

In these conditions, simulation curves best fit the observed data, and we thus can determine the proportion of each class of agonist binding sites, the K_d for the lower affinity sites and the highest value of the K_d for the higher affinity sites.

Fig. 1. Scatchard analysis of the specific binding of antagonists to intact A431 cells

Specific [3 H]CGP 12,177 (0.06–2 nM) (a) and specific [125 I]-ICYP (0.018–0.5 nM) (b) binding were determined as described under 'Materials and methods'. B is the number of fmol of radioligand specifically bound/ 10^6 cells, and F is the varying concentration of antagonist ligand, in pre-confluent (●) and postconfluent (▲) cells. Points represent means of duplicate determinations. The data are representative of five experiments.

Fig. 2. Competition of (–)-isoproterenol for [3 H]CGP 12,177 binding sites on intact A431 cells

Cells were incubated with [3 H]CGP 12,177 and various concentrations of (–)-isoproterenol for 1 min. Ligand binding was determined as described under Materials and methods in pre-confluent (●) and post-confluent (■) cells. Results are the means of three independent experiments done in duplicate.

RESULTS

We studied the β -adrenergic ligand binding activity and the hormone responsiveness in A431 cells at two states of growth, pre-confluence and post-confluence. Indeed, in 1321 NI human astrocytoma (Harden *et al.*, 1979; Doss *et al.*, 1985) and C6 glioma cells (Morris & Makman, 1976; Mallorga *et al.*, 1981) β -adrenergic receptors accumulate under pre-confluent culture conditions and, as cells reach confluency, their number per cell declines. This change is accompanied by a diminution of the responsiveness to catecholamines. We verified whether this was also the case for β -adrenergic receptors in A431 cells.

To assess antagonist binding, we used two radioligands: [3 H]CGP 12,177, a hydrophilic compound which binds selectively to cell surface receptors, and [125 I]ICYP, a hydrophobic ligand which at 37 °C identifies intracellular as well surface receptors.

The number of β_2 -adrenergic receptors determined by [3 H]CGP 12,177 was approximately 49 ± 4.8 fmol/ 10^6 pre-confluent cells and only about 23 ± 2.9 fmol/ 10^6 post-confluent cells (Fig. 1a). The number of receptors measured with [125 I]ICYP was 72 ± 7 and 31 ± 5 fmol/ 10^6 cells respectively for pre- and post-confluent cells (Fig. 1b). The apparent dissociation constants for [3 H]CGP 12,177 (0.29 ± 0.11 nM) and for [125 I]ICYP (0.09 ± 0.05 nM) were the same for both types of cells.

To assess agonist binding, competition for [3 H]CGP 12,177 was evaluated by using various concentrations of (–)-isoproterenol. To prevent desensitization of the system, binding sites were measured in non-equilibrium conditions (1 min incubation). The results, analysed as described in the Materials and methods section, are illustrated in Fig. 2: the binding affinity for (–)-isoproterenol of receptors differed depending on whether pre- or post-confluent cells were used. In the case of pre-confluent cells, two types of sites were observed: 55 %

Fig. 3. Intracellular cyclic AMP accumulation in intact A431 cells

Intracellular cyclic AMP accumulation was measured in response to the indicated concentrations of (–)-isoproterenol in 30 s assays on pre-confluent (●) and post-confluent (■) cells. The Figure is representative of a typical experiment done in duplicate.

Fig. 4. Affinity chromatography of solubilized A431 cells on Con-A gel

The affinity chromatography of pre- (●) or post-confluent (■) solubilized cells on a Con-A column were performed as described under Materials and methods. Specific binding was measured on aliquots incubated for 30 min at 37 °C with 5 nM [125 I]ICYP, with or without ($\pm$)-propranolol.

exhibited high affinity for (–)-isoproterenol (higher limit of K_d , 1 nM), the remaining 45 % sites possessed a lower affinity ($K_d = 0.1$ μ M) (Fig. 2). On post-confluent cells, only one kind of binding sites was found with the same K_d (0.1 μ M) as that of the lowest affinity sites of pre-confluent cells (Fig. 2).

To determine the responsiveness of the pre- and post-confluent cells to the β -adrenergic agonist, intracellular cyclic AMP accumulation was measured in both cellular populations. As shown in Fig. 3, cyclic AMP accumulation was (–)-isoproterenol-dose-dependent in pre- and post-confluent cells and the maximum level of cyclic AMP occurred for 1 μ M-isoproterenol: about 6 pmol were formed per 10^5 pre-confluent cells, and about 4 pmol per 10^5 postconfluent cells.

These results indicate that, during the growth of A431 cells, there are changes in the receptor density per cell, in the characteristics of agonist binding and in adenylate cyclase activity.

Characterization of the carbohydrate moiety of the β_2 -adrenergic receptors of pre- and post-confluent A431 cells by Con-A chromatography

Pre- and post-confluent cells were solubilized in 1.2% digitonin and then subjected to chromatography on Con-A gel. The binding activity in all subsequent experiments was measured with [125 I]ICYP. The gel retained 90% and 80% of the binding activity of the solubilized pre- and post-confluent cells respectively,

indicating that the β_2 -adrenergic receptor of both types of cells is a glycoprotein containing N-linked carbohydrate. To define the nature of the carbohydrate chains, differential elution from Con-A gel was performed by 10 mM- α -methyl D-mannopyranoside, which elutes complex carbohydrate chains (low affinity for Con-A), and 300 mM- α -methyl D-mannopyranoside, which elutes polymannosidic chains (high affinity for Con-A) (Narasimham *et al.*, 1979).

The elution pattern is shown in Fig. 4: 68% of the loaded binding activity of the solubilized pre-confluent cells was eluted with 10 mM- α -methyl D-mannopyranoside and 32% with 300 mM- α -methyl D-mannopyranoside. These results indicate the existence of two β_2 -adrenergic receptors populations in pre-confluent cells, one bearing complex chains and the other polymannosidic chains.

In the solubilized postconfluent cells, 90% of the binding activity was eluted with 300 mM- α -methyl D-mannopyranoside and little activity with 10 mM. Thus, β_2 -adrenergic receptors of A431 postconfluent cells contain almost exclusively polymannosidic chains (Fig. 4).

Fig. 5. Immunoblotting of solubilized A431 cells and of eluates of Con-A gel (a), photoaffinity labelling of β_2 -adrenergic receptor of A431 membranes (b), and electrophoresis of solubilized prelabeled A431 membranes and of eluates from Con-A gel (c)

(a) The solubilized pre-confluent (lane a), postconfluent (lane d) cells, and eluates from Con-A gel with 10 mM-mannose (lane b) and 300 mM-mannose (lane c) were treated as described under Materials and methods. (b) Membranes from A431 pre-confluent cells were incubated with [125 I]ICYP-diazirine and photolysed as described under Materials and methods. Lane a, labelling with [125 I]ICYP-diazirine; lane b, same as a in the presence of an excess of (±)-propranolol. (c) Membranes from A431 pre-confluent cells were photolabelled, solubilized by SDS and subjected to Con-A gel chromatography as described under Materials and methods. Lane a, membranes before Con-A gel treatment; lane b, 10 mM-mannose eluate; lane c, 300 mM-mannose eluate.

Table 1. Effect of glycosylation inhibitors on the incorporation of radiolabelled methionine or sugars in A431 cells

The pre- and post-confluent cells were treated and labelled as described under Materials and methods. In the control the incorporations of [³⁵S]methionine, [³H]mannose and *N*-acetyl[³H]glucosamine were respectively 400 000, 32 000 and 11 000 c.p.m./10⁶ cells. Results are the means of three independent experiments.

Addition	Incorporation (% of control)			
	Preconfluent cells		Postconfluent cells	
	Monensin	Tunicamycin	Monensin	Tunicamycin
[³ H]Mannose	118 ± 9	6 ± 0.4	100 ± 15	30 ± 1
<i>N</i> -Acetyl[³ H]glucosamine	61 ± 4	21 ± 1	50 ± 7	50 ± 7
[³⁵ S]Methionine	86 ± 10	80 ± 7	93 ± 10	80 ± 9

Solubilized pre- and post-confluent cells and the different eluates from Con-A gel were extensively dialysed, lyophilized and subjected to SDS/polyacrylamide gel electrophoresis under reducing conditions. Then they were transferred on to nitrocellulose and probed with specific anti-(β -adrenergic receptor) monoclonal antibodies (Kaveri *et al.*, 1987). The results obtained are illustrated in Fig. 5(a). In preconfluent cells (lane a) two polypeptides of 65–63 and 55–52 kDa were revealed. They had the same molecular mass as the polypeptides identified by photolabelling of membranes from preconfluent A431 cells (Fig. 5b). In postconfluent cells (lane d), only the larger polypeptide was observed. Smaller peptides of 60 and 47 kDa in preconfluent cells and of 60 kDa in postconfluent cells were also detected, resulting probably from proteolytic degradation in the course of the experiments. In the eluate of pre-confluent cells from Con A gel with 10 mM- α -methyl D-mannopyranoside (lane b), only the 55–52 kDa peptide and its proteolytic degraded peptides were revealed. In the eluate with 300 mM (lane c), the 65–63 kDa and 60 kDa bands were detected. When membranes from preconfluent cells were labelled with the β -adrenergic-specific ligand [¹²⁵I]ICYP-diazirine before being solubilized and subjected to Con-A gel chromatography, a band of 55–52 kDa was revealed in the 10 mM- α -methyl D-mannopyranoside eluate and a band of 65–63 kDa in the 300 mM- α -methyl D-mannopyranoside eluate (Fig. 5c). In this case, no minor bands were observed; it is likely that the solubilized membrane preparations were less contaminated by proteases than were the cell lysates.

These results suggest that receptors bearing complex carbohydrate chains correspond to the 55–52 kDa polypeptide, whereas those associated with polymannosidic chains correspond to the 65–63 kDa protein.

Inhibition of glycosylation of the β_2 -adrenergic receptor by tunicamycin and monensin

The effect of two inhibitors of glycosylation, tunicamycin and monensin, was studied on pre- and post-confluent cells. Tunicamycin inhibits the transfer of *N*-acetylglucosamine-1-phosphate from UDP-*N*-acetylglucosamine to dolichol monophosphate and thereby blocks the formation of glycoproteins containing *N*-linked carbohydrate (Hickman *et al.*, 1977; Damsky *et al.*, 1979); monensin is an ionophore, whose major effect on cells is to disrupt the Golgi complex (Griffiths *et al.*, 1983).

Initial experiments were performed to ensure that the inhibitors modified glycosylation without affecting protein synthesis. As judged by the incorporation of [³⁵S]methionine into trichloroacetic acid precipitable material (Table 1), none of the drugs altered protein synthesis during 24 h of treatment. However, they affected the incorporation of labelled sugars: tunicamycin dramatically reduced the incorporation of [³H]mannose and *N*-acetyl[³H]glucosamine into total cellular *N*-linked glycoprotein (Table 1). Similar results were obtained by Doss *et al.* (1985) with astrocytoma cells. As for monensin, it inhibited up to 50 % of the incorporation of *N*-acetyl[³H]glucosamine without altering that of [³H]mannose (Table 1).

Effects of drugs on antagonist binding to intact A431 cells

[³H]CGP 12,177 binding. After treatment with tunicamycin, binding of [³H]CGP 12,177 to pre- and post-confluent cells was approx. 30 % and 50 % of the control values, respectively: 17 fmol instead of 49 fmol/10⁶ preconfluent cells (Fig. 6a) and 12 fmol instead of 23.5 fmol/10⁶ postconfluent cells (Fig. 6b and Table 2). The apparent dissociation constants of the ligand (K_d 0.29 nM) were similar in every case. After monensin treatment, the number of sites for [³H]CGP 12,177 on preconfluent cells shifted from 49 fmol to 27 fmol/10⁶ cells, but the apparent dissociation constant of the ligand for the receptor remained the same (Fig. 6a). On postconfluent cells, monensin had no effect on the number of [³H]CGP 12,177 binding sites nor on the apparent dissociation constant (K_d 0.29 nM) (Fig. 6b and Table 2).

[¹²⁵I]ICYP binding. In preconfluent cells, the total number of sites per 10⁶ cells increased from 72 to 105 fmol after tunicamycin treatment, and to 90 fmol after monensin treatment (Fig. 6c and Table 2).

In postconfluent cells, monensin had nearly no effect but tunicamycin enhanced the number of sites from 31 fmol/10⁶ cells (Fig. 6d and Table 2). No change occurred in the apparent dissociation constant in any conditions (K_d 0.09 nM).

Effect of drugs on competition of (–)-isoproterenol for [³H]CGP 12,177 binding sites on intact A431 cells

Treatment of pre- and post-confluent cells with monensin or tunicamycin did not modify significantly competition curves of (–)-isoproterenol for [³H]CGP

Fig. 6. Effect of inhibitors of glycosylation on antagonists binding to intact A431 cells

A431 cells were incubated in the absence (●) or in the presence of monensin (○) or tunicamycin (▲). Specific [3 H]CGP 12,177 binding to preconfluent (a) and postconfluent (b) cells, and [125 I]ICYP binding to preconfluent (c) and postconfluent (d) cells was determined as described under Materials and methods. B is the number of fmol of radioligand specifically bound/ 10^6 cells, and F is the varying concentration of antagonist ligand. The data represent the mean of duplicate determinations of one typical experiment.

Table 2. Effect of glycosylation inhibitors on antagonist binding to intact A431 cells

Cells were treated for 24 h as described under Materials and methods; 100% of binding for 10^6 cells is about 50 fmol of [3 H]CGP 12,177 and 70 fmol of [125 I]ICYP, in preconfluent cells and 23 fmol of [3 H]CGP 12,177 and 30 fmol of [125 I]ICYP in postconfluent cells. The values represent the means of at least three experiments in duplicate.

Inhibitor	Binding (% of control)			
	Preconfluent cells		Postconfluent cells	
	[3 H]CGP 12,177	[125 I]ICYP	[3 H]CGP 12,177	[125 I]ICYP
Monensin	40–60	130	80–100	80–100
Tunicamycin	30–40	150	40–50	130–140

12,177 binding sites: the affinity for (–)-isoproterenol of the receptor was not changed significantly. The relative percentage of highest and lowest sites were 52% and 48% respectively as compared with 55% and 45% in the control cells (Figs. 7a–7c).

Effect of drugs on intracellular cyclic AMP accumulation

As can be seen in Figs. 8(a) and 8(b), tunicamycin decreased the intracellular cyclic AMP accumulation by about 50% in both pre- and post-confluent cells. Monensin diminished it by 35% in preconfluent cells but had no effect on postconfluent cells. In treated pre- or post-confluent cells, the IC_{50} for (–)-isoproterenol was of the same magnitude as that of untreated cells, 10 nM.

DISCUSSION

Using chromatography on Con-A gel (Fig. 4) and on other lectin gels (results not shown), we have established

Fig. 7. Effect of inhibitors of glycosylation on competition of (–)-isoproterenol for [^3H]CGP 12,177 binding sites on intact A431 cells

Preconfluent cells were treated with monensin (a) or tunicamycin (b) and incubated with [^3H]CGP 12,177 and various concentrations of (–)-isoproterenol for 1 min. Postconfluent cells (c) were treated in the same conditions (○, ▲, without or with monensin). Drug treatments and ligand binding determinations are described under 'Materials and methods'. Results are the means of three independent experiments done in duplicate.

that β_2 -adrenergic receptors from human carcinoma epidermoid A431 cells were glycoproteins containing N-linked carbohydrate. The presence of O-linked carbohydrate was not excluded. The main objective of our studies was to determine whether the carbohydrate moiety is involved in expression and function of these β_2 -adrenergic receptors. Since it has been shown in a variety of cells that the structure of glycoproteins (Hakimi & Atkinson, 1981; Codogno *et al.*, 1985) and the expression and function of β_2 -adrenergic receptors (Doss

Fig. 8. Effect of inhibitors of glycosylation on cyclic AMP accumulation in intact A431 cells

Preconfluent (a) and postconfluent (b) cells were treated without (●) or with monensin (○) or with tunicamycin (▲) as described under Materials and methods. Intracellular cyclic AMP accumulation was measured in response to the indicated concentrations of isoproterenol in 30 s assays. The Figure is representative of a typical experiment done in quadruplicate.

et al., 1985; Harden *et al.*, 1979) depend on cell density, experiments were performed on A431 cells at two states of growth, pre- and post-confluency.

In preconfluent A431 cells, the differential elution from Con-A gel of the β -adrenergic receptor labelled with [^{125}I]ICYP or photolabelled with [^{125}I]ICYP-diazirine, revealed the existence of two distinct populations of β_2 -adrenergic receptors, one associated with complex carbohydrate chain(s), the other with polymannosidic carbohydrate chain(s). Treatment with α -mannosidase and neuraminidase allowed Stiles *et al.* (1984) to reach the same conclusion, namely that β_2 -adrenergic receptors from hamster lung and rat erythrocytes each contain two discrete populations with a different type of carbohydrate moiety. In contrast, on turkey erythrocyte β_1 -adrenergic receptors we and others found only complex carbohydrate chains (Cervantes-Olivier *et al.*, 1985; Jürss *et al.*, 1985).

β -Adrenergic receptors from several sources, photoaffinity labelled or iodinated at different steps of purification, display two polypeptides with molecular masses of 65 kDa (major) or 55 kDa (minor) upon SDS/

polyacrylamide gel electrophoresis under reducing conditions. Certain authors have proposed that the polypeptide of lower molecular mass as a result of the proteolytic degradation of the 65 kDa polypeptide (Benovic *et al.*, 1983). Malbon and his colleagues have shown that, in fat (Moxham & Malbon, 1985) and S49 lymphoma cells (George & Malbon, 1984). The 55 kDa polypeptide represents the non-reduced receptor in the native state whereas the 65 kDa is the reduced form. As for A431 cells, both polypeptides were revealed by photoaffinity labelling. Our findings establish that the 65–65 kDa polypeptide is associated with polymannosidic chains and the 55–52 kDa one with complex carbohydrate chain. Thus the two polypeptides of 55–52 and 65–63 kDa, revealed by photolabelling of β_2 -adrenergic receptor of the membranes from preconfluent A431 cells, correspond to the two populations of receptors mentioned above and differ at least by their carbohydrate moiety.

In postconfluent cells, a single population of receptors with a major polypeptide of 65–63 kDa was found to possess polymannosidic chain(s). Thus the amount of complex carbohydrate chain(s) decreases with the age of the cells. These results are different from those of Ceccarini *et al.* (1975) and Codogno *et al.* (1985) who observed an increase of glycoprotein associated with complex chain(s). However, these authors worked with a mixture of glycoproteins whereas we focused on a specific protein, the β_2 -adrenergic receptor: its processing may yield a different pattern of oligosaccharide structure.

We have also compared antagonist and agonist binding on the β_2 -adrenergic receptor of the pre- and post-confluent cells. We found that the affinities of the antagonists binding, [3 H]CGP 12,177 and [125 I]ICYP, were the same for both types of cells, whereas the affinity of the receptor for the agonist (–)-isoproterenol changed with the age of cells. In the preconfluent cells, the curve reflecting competition of binding between [3 H]CGP 12,177 and increasing concentrations of (–)-isoproterenol is biphasic: about 55% of the receptors have a high affinity for (–)-isoproterenol and about 45% a lower affinity. The treatment of these cell lysates with Con-A gel allowed to separate two populations of β_2 receptors; one, of 55–52 kDa, is associated with complex carbohydrate chains, the other, of 65–63 kDa, is associated with polymannosidic chains. Although it is not established whether the same percentage of each type of receptor is solubilized, the correlation between the proportion of molecular form and the proportions of receptors with differing affinities for agonist is striking. Moreover in postconfluent cells, which only possess the receptors of lower affinity for (–)-isoproterenol, one only finds the population of larger molecular mass, which bears polymannosidic chains. Taken together, these results suggest that in A431 cells, the population associated with the complex chain(s) and corresponding to the polypeptide of 55–52 kDa has a high affinity for the agonist, while that associated with polymannosidic carbohydrate chain and corresponding to the polypeptide of 65–63 kDa has a lower affinity for the agonist. Thus the two populations may have a well-defined role in the function of the receptor.

In order to evaluate the contribution of the carbohydrate moiety to the biological activity of the β_2 -adrenergic receptor, we used two inhibitors of protein glycosylation, tunicamycin and monensin. Tunicamycin

was shown to block the accumulation of β_2 -adrenergic receptor in astrocytoma cells (Doss *et al.*, 1985). Monensin has a complex effect that impairs processing beyond the high-mannose stage and it interrupts the process of receptor-mediated endocytosis and exocytosis and inhibits recycling of the receptors (Ledger & Tanzer, 1984). We examined whether the changes in rate of glycosylation affected ligand binding and adenylyl cyclase activity in pre- and post-confluent cells.

In preconfluent cells, treatment with monensin or tunicamycin resulted in reduced numbers (50–70%) of cell surface β -adrenergic receptors, as assessed by binding of [3 H]CGP 12,177. Tunicamycin had a similar effect on postconfluent cells. This diminution was not due to the lack of activity of the newly synthesized receptors partially or completely devoid of carbohydrate chain(s). Indeed the carbohydrate moiety does not seem to be involved in the binding activity of the β_2 -adrenergic receptor since we observed that [125 I]ICYP-diazirine-receptor complex (from untreated cells) is retained on Con-A gel. Moreover, Stiles (1985) demonstrated that enzymically deglycosylated β_2 -adrenergic receptor of rat reticulocytes recognized the agonists and antagonists with the same potency order and affinity as the glycosylated form. The decrease of cell surface receptors is more likely due to the fact that aglycosylated or partially glycosylated β_2 -adrenergic receptors were not incorporated into plasma membrane; this could explain the accumulation of the receptors inside the drug-sensitive cells, revealed by [125 I]ICYP binding (Figs. 6c and 6d). The properties of the remaining cell surface receptors were not affected by the drug treatment: neither their affinities for agonist and antagonist nor their relative percentages were modified. In postconfluent cells, which only possess polymannosidic chains, monensin affected only slightly the number of sites determined by binding of [3 H]CGP 12,177 and [125 I]ICYP. Another molecular bearing polymannosidic oligosaccharides, fibronectin, has been also shown to be unaffected by monensin (Ledger *et al.*, 1983). In preconfluent cells, the situation was different since monensin does act on receptor associated with polymannosidic chain(s). A possible explanation of these results is that the activity or amount of the mannosidases are growth-dependent, leading to the modification of the polymannosidic glycoprotein processing.

As for adenylyl cyclase activity, a good correlation was found between the decrease of the accumulation of intracellular cyclic AMP and the diminution of the number of the sites at the cell surface after drug treatment. This correlation was strengthened by the fact that in postconfluent cells monensin, which did not affect the number of sites, had no effect on the pool of cyclic AMP. However, one cannot exclude a direct action of the drug on the adenylyl cyclase, as this enzyme has been shown recently to be a glycoprotein (Pfeuffer *et al.*, 1985). Indeed, preliminary experiments performed with preconfluent cells treated with tunicamycin on monensin show partial decrease of cyclic AMP accumulation, determined in presence of forskolin.

In conclusion, our results demonstrate that A431 cells contain two populations of β_2 -adrenergic receptors with two different affinities for isoproterenol and with molecular masses (65–63 and 55–52 kDa) related to the structure of the carbohydrate chains. Further studies using peptide maps and sequencing will be necessary to investigate

whether these differences are also associated with distinction in locations of carbohydrates, especially considering that four potential glycosylation sites have been found by analysis of β_2 receptor cDNA of hamster lung (Dixon *et al.*, 1986). Our results, added to those of Stiles (1985), suggest that *N*-linked glycosylation is required for incorporation of β_2 -adrenergic receptors into plasma membrane, but once receptors have been incorporated, deglycosylation does not modify the binding activity.

It will now be interesting to probe the role of the carbohydrate chains, particularly in the desensitization and recycling processes of the receptor.

During the preparation of this manuscript, a paper (George *et al.*, 1986) appeared on a closely related subject. Our results differ from their findings since those authors found no effect of tunicamycin on the number of β -adrenergic receptors and on the activity of isoproterenol-stimulated adenylate cyclase. The difference could be accounted for by several facts: the material studied, the state of growth of the cells and the nature of the β -adrenergic ligand. Indeed, George *et al.* (1986) worked on S49 lymphoma cells, at confluency, and they used, as β -adrenergic ligands, [125 I]ICYP which does not allow the distinction between internal and membrane-bound receptors.

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