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Use of 1-Deoxymannojirimycin to Show That Complex Oligosaccharides Regulate Cellular Distribution of the α_1-Adrenergic Receptor Glycoprotein in BC_3H_1 Muscle Cells

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SUMMARY

We have previously shown that α_1-adrenergic receptors in BC_3H_1 muscle cells are glycoproteins containing complex but not high mannose oligosaccharides. In the present study we investigated the role of the complex sugars in functional aspects of the receptor by treating BC_3H_1 cells with 1-deoxymannojirimycin (dMM), which blocks conversion of high mannose oligosaccharides to complex chains. Receptors were photoaffinity labeled in intact cells with ^{125}I-azido prazosin; drug treatment with dMM resulted in conversion of the 87-kDa receptor to 62 kDa. The 62-kDa protein was sensitive to mannosidase, indicating loss of complex sugars. Radioligand ([^{3}H]prazosin) binding analysis carried out at 37° to intact cells indicated that dMM treatment increased the affinity of the α_1-receptors for [^{3}H]prazosin 2-fold and decreased the number of total cellular receptors by 15%. In order to distinguish between surface and sequestered receptors, we assessed [^{3}H]prazosin binding to intact cells at 4° using competition by the hydrophilic agonist epinephrine to define surface receptors and by nonradioactive antagonists (prazosin and phentolamine) to define total receptors. In control cells, epinephrine competed for 90% of the total receptors, whereas for dMM-treated cells this value was only 60%. In addition, dMM treatment caused a 40% reduction in epinephrine-stimulated phosphatidylinositol turnover when compared with untreated cells. The results indicate that dMM treatment reduces the number of functional α_1-adrenergic receptors on the cell surface while increasing the number of sequestered receptors. We conclude that complex oligosaccharides are important for cellular localization and function of α_1-adrenergic receptors in BC_3H_1 cells.

The catecholamines epinephrine and norepinephrine regulate target cell function by initially binding to α- and β-adrenergic receptors on the cell surface and then activating second messenger systems via G proteins. Regulation of adrenergic receptor number by agonists, antagonists, or other classes of hormones or in various disease states provides an important mechanism whereby target cells can modulate their responsiveness to catecholamines (1–5). Much evidence suggests that such settings can alter the receptor "life cycle," in particular the balance between receptors that are present on the cell surface and those that may be located in intracellular locations (4, 5).

Although certain adrenergic receptors and other G-linked receptors have been cloned and sequenced (6–8), little information is available regarding structural aspects of the receptor glycoproteins that are involved in regulating receptor localization and turnover. Receptors must possess precise domains that are involved in regulating their trafficking between cellular organelles and in their localization within the plasma membrane. Although oligosaccharides can be involved in regulating intracellular location of certain glycoproteins (9–11), there is as yet little evidence that carbohydrate moieties are important in contributing to localization of adrenergic receptors or other G-linked receptors. Some recent data even suggest that carbohydrates play no role in cell surface localization of β-adrenergic receptors (12).

In order to investigate this question further, we initiated studies to examine the role of the oligosaccharide component in the compartmentation of α_1-adrenergic receptors between cell surface and intracellular sites in a model system, BC_3H_1 muscle cells. This cell line was chosen because the detailed physiological and pharmacological properties of BC_3H_1 cell α_1-adrenergic receptors have been well described (e.g., Refs. 3 and 13–15). In addition, in recent studies we found that these receptors are linked to a G protein (16) and that the BC_3H_1 α_1-adrenergic receptor glycoprotein contains complex but not high mannose *N*-linked oligosaccharides.[2] Similar results have been

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ABBREVIATIONS: The abbreviations used are: G protein, guanine nucleotide-binding protein; dMM, 1-deoxymannojirimycin; PI, phosphoinositide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate.

observed for α_1-receptors in liver and DDT_1-MF_2 muscle cells (17). The strategy in the present studies was to treat BC_3H_1 cells with dMM, a recently available compound that inhibits α-mannosidase IA and IB activity and thus prevents the conversion of high mannose oligosaccharides to complex chains (18, 19). The results show that this conversion is important both for cellular localization and function of α_1-adrenergic receptors.[3]

Experimental Procedures

Materials. dMM was purchased from Calbiochem Biochemicals (La Jolla, CA). [^{3}H]Prazosin and ^{125}I-azido prazosin were from New England Nuclear (Boston, MA). Gel electrophoresis reagents were from Bio-Rad (Richmond, CA). α-Mannosidase (M-7257) was from Sigma Chemical Co. (St. Louis, MO). Phentolamine and prazosin were received as gifts from GEIGY Pharmaceuticals (Ardsley, NY) and Pfizer, Inc. (New York, NY), respectively. All other reagents were from standard sources.

Culture of BC_3H_1 cells. Cells were routinely grown in Falcon T-flasks in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and maintained at 37° in a humidified atmosphere of 10% CO_2/90% air. The cells were subcultured at 3–4-day intervals using cold ATV-solution (Irving Scientific) to dissociate them from the plastic dish. For experimental purposes, cells were seeded into 35-mm or 100-mm diameter culture dishes so that they reached confluence in 3 days, at which time they were used for the experiments.

Photoaffinity labeling, mannosidase treatment, and SDS-PAGE autoradiography. Confluent cells grown on 100-mm dishes were treated with or without dMM, scraped from the dishes, pelleted by centrifuging at 1,000 × *g* for 5 min, and resuspended in 1.0 ml of Dulbecco's modified Eagle's medium. The cells were placed in 24-mm plastic dishes and ^{125}I-azido prazosin, 150 pM, was added. The samples were incubated for 1 hr at 37° in the dark; photolysis was done by placing the samples into a UV light chamber (88 watt, C.B.S. Scientific, Del Mar, CA) for 15 min. The cells were washed twice with 1.0 ml of 300 mM sucrose, 20 mM Tris·HCl, pH 7.4, 2 mM EDTA. The cells were scraped from the dishes in sucrose, EDTA, Tris·HCl buffer and microfuged for 5 min at maximum speed. The resulting pellets were either prepared for SDS-PAGE or solubilized by resuspending in 100 mM sodium citrate, pH 5.0, 50 mM EDTA, 200 mM NaCl, 1.0% digitonin, 1.0% Nonidet P-40, 0.1% SDS, 10 μg/ml leupeptin, and 5 μg/ml pepstatin A. The samples were then sonicated for 15 sec and incubated at room temperature for 1 hr. The samples were centrifuged in a microfuge for 10 min at maximal speed and the supernatant was collected. β-Mercaptoethanol, 0.2%, was added and the samples were boiled for 3 min. Aliquots of 100 μl were treated with mannosidase for 12 hr at 37° as described in the legend to Fig. 3. At this time 1 ml of ice-cold 10% trichloroacetic acid was added and 10 min later the samples were centrifuged. The resulting pellet was washed two times with acetone and finally suspended in gel sample buffer containing 50 mM Tris·HCl, pH 6.9, 1% SDS, 10% glycerol, 0.01% bromophenol blue. The samples were sonicated in order to disperse the pellets, boiled for 3 min, and loaded on a 7% SDS-PAGE gel, prepared and run according to Laemmli (20). Molecular weight markers (Bio Rad No. 161-0304) were applied to the gel as well. The gels were stained with 50% methanol, 3.75% acetic acid, and 0.2% Coomassie blue, destained, dried, and subjected to autoradiography by exposing Kodak XAR5 film for 7 days at −70°.

[^{3}H]Prazosin binding. Whole cell binding assays were carried out by adding [^{3}H]prazosin and various drugs directly to the media of cells grown on 35-mm dishes. The cells were incubated either for 1 hour at 37° or for 24 hr at 4°. For binding isotherm experiments, the concentration of label ranged from 30 to 500 pM; in all other experiments the concentration was 500 pM. The reactions were terminated by aspirating the media from the plates and the cells were washed three times with 1.5 ml of ice-cold 20 mM Tris·HCl, pH 7.4, 300 mM sucrose, 5 mM $MgCl_2$, to remove unbound label. One ml of 0.1 N NaOH plus 0.5% SDS was added to each plate and the cells were pipetted into scintillation vials. The plates were washed first with 1.0 ml of NaOH plus SDS and then with 2.0 ml of 0.2 N HCl, each time the wash was added to the liquid scintillation vials. Liquid scintillation cocktail, 10 ml, was added and the samples were counted at 40% efficiency. Superoxide dismutase and catalase were added to prevent oxidation of epinephrine (21).

Measurement of PI turnover. The growth media of BC_3H_1 cells (which had been grown to confluence on 35-mm dishes) were aspirated and replaced with 0.75 ml of 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1.6 mM $MgSO_4$, 1.0 mM Na_2HPO_4, 5.5 mM glucose, 25 mM HEPES, pH 7.4, 0.06% (w/v) bovine serum albumin, 4 μCi/ml of *myo*-[^{3}H]inositol, with or without 10 μM (−)-epinephrine. After a 30-min incubation at 37°, the medium was aspirated and the cells were washed with three 1-ml aliquots of buffer that contained no radioligand or hormone. The cells were then covered with 1.0 ml of ice-cold methanol and removed from the dishes with the aid of a cell scraper. The samples were mixed with 1 ml of chloroform and 0.9 ml of 2 M KCl and centrifuged at 2000 × *g* to separate the two phases. One ml of the upper phase was reserved for determination of its content of tritium and the remainder of the upper phase was aspirated. The samples were washed three times with 1.0 ml of chloroform/methanol/2 M KCl (3:48:47) before being transferred to scintillation vials and evaporated to dryness.

Data analysis. Most data shown represent results similar to those obtained in at least two independent experiments. Error bars represent the standard error of the mean of triplicate samples. Where error bars are not shown the data points represent the average of duplicate samples; the values of the duplicate samples did not vary more than 10% for any given data point.

Results

In initial studies we used photoaffinity labeling techniques combined with mannosidase treatment in order to determine appropriate conditions for treating cells with dMM. Fig. 1 shows the effect of a 36-hr incubation of BC_3H_1 cells with 200 μM dMM on the photoaffinity labeling pattern of the receptor. As reported previously,[2] ^{125}I-azidoprazosin specifically labels a 87-kDa protein in control cells (Fig. 1, *lane 2*); dMM treatment resulted in loss of the 87-kDa protein and labeling of a 62-kDa protein (Fig. 1, *lane 1*). Interestingly, the intensity of the 62-kDa protein seen after dMM treatment was less than the intensity of the 87-kDa protein in control cells (Fig. 1); a possible explanation for this is discussed later. As with the photoaffinity-labeled 87-kDa protein in control cells[2] (17), photoaffinity labeling of the 62-kDa protein in dMM-treated cells is blocked by 10 μM phentolamine and 100 μM epinephrine (Fig. 2). Proteins other than 87-kDa (control samples) or 62-kDa (dMM-treated cells) are photoaffinity labeled under the conditions used; photoaffinity labeling of these proteins is not blocked by phentolamine[2] (Ref. 17 and Fig. 2) or epinephrine[2] (Fig. 2).

As reported previously,[2] the 87-kDa photoaffinity-labeled receptor in control cells is insensitive to mannosidase treatment (compare Fig. 3 *lanes 1* and *2*). The 62-kDa protein from dMM-treated cells is sensitive to glycosidase (compare Fig. 3, *lanes 3*, *4*, and *5*) consistent with the absence of complex sugars. The intensity of the specifically labeled protein in dMM-treated cells appears to decrease; this was observed in three experiments. For the following reasons we do not believe that this effect is due to proteolysis. First, α-mannosidase had no effect

[3] Portions of this work have been presented in preliminary form: *Clin. Res.* 35:378A (1987).

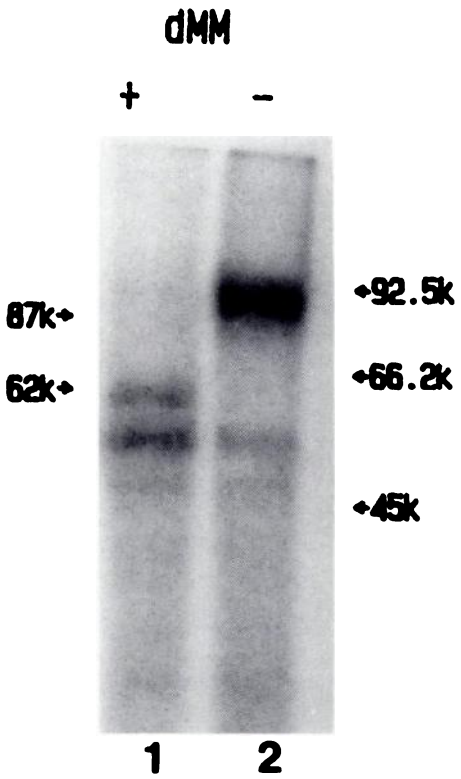

Fig. 1. Photoaffinity labeling of BC_3H_1 α_1-receptors after treatment with dMM. BC_3H_1 cells, grown to confluence on two 35-mm cell culture dishes, were incubated with or without 200 μM dMM for 36 hr. The cells were incubated with 150 pM ^{125}I-azido prazosin for 1 hr at 37° in the dark and then photolyzed by placing the dishes into a UV light chamber (C.B.S. Scientific) for 15 min. The cells were washed with ice-cold 300 mM sucrose, 20 mM Tris·HCl, pH 7.4, 5 mM $MgCl_2$, to remove unincorporated label. The cells were scraped from the dishes in 1.4 ml of 20 mM Tris·HCl, pH 7.4, 1 mM EDTA, and centrifuged, and the pellets were resuspended in gel sample buffer. SDS-PAGE autoradiography was done as described in the Experimental Procedures. The sample shown in *lane 1* was prepared from dMM treated cells and the sample in *lane 2* was from control cells.

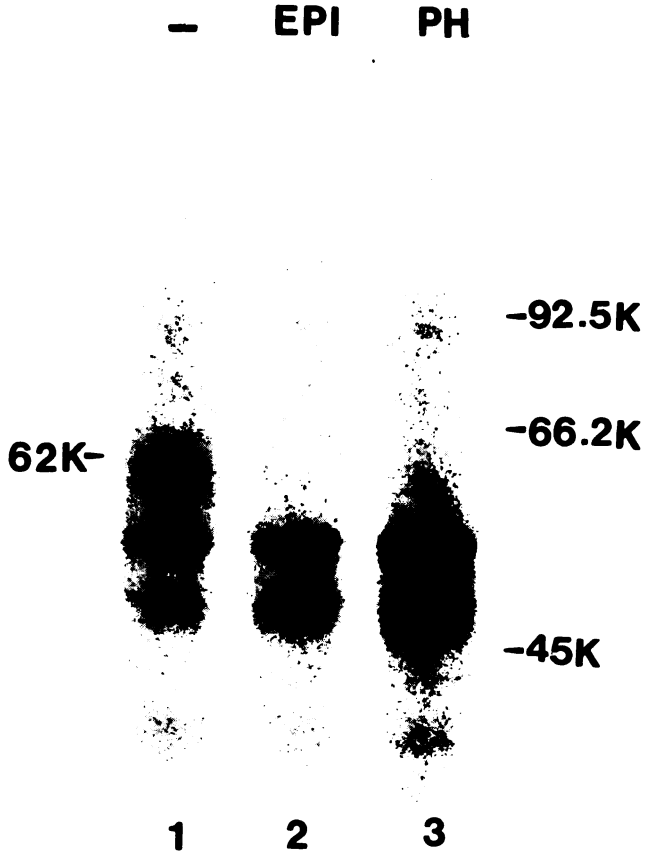

Fig. 2. Photoaffinity labeling of α_1-receptors in dMM-treated BC_3H_1 cells. BC_3H_1 cells, grown to confluence on three 35-mm cell culture dishes were incubated for 48 hr with 200 μM dMM. The cells were chilled to 4° and ^{125}I-azido prazosin (150 pM) was added to all dishes. Epinephrine (100 μM) (*EPI*) and phentolamine (10 μM) (*PH*) were added to samples as indicated in the figure. The cells were incubated in the dark at 4° for 24 hr at which time they were photolyzed and prepared for SDS-PAGE autoradiography as described in the Experimental Procedures.

on the 87-kDa protein in control cells and this protein should possess an amino acid sequence that would subsume that of the 62-kDa protein observed after dMM treatment. Secondly, a number of protease inhibitors (see Experimental Procedures) were added during the experiment and the samples were boiled before mannosidase treatment. Thirdly, when four proteins that lack oligosaccharides (phosphorylase B, bovine serum albumin, ovalbumin, and α-chymotrypsinogen) were incubated under identical conditions used for the experiment shown in Fig. 3, no effect was seen on the molecular weights of the proteins, indicating lack of protease activity. It is possible that the conditions used for the experiment shown in Fig. 3 were insufficient for complete glycosidase activity, although increasing the concentration of α-mannosidase to 10 U/ml had no further effect (data not shown).

In this particular experiment (Fig. 3) the intensity of nonspecifically labeled proteins was greater in dMM-treated than control samples; this was observed in other similar experiments (Fig. 1) but not in all experiments. We do not believe that the increased intensity of label in these proteins was derived from the 87-kDa protein observed in control cells, because photoaffinity labeling of the nonspecific proteins was insensitive to α-mannosidase (Fig. 3). The increased intensity of the nonspecific proteins may have been due to increased protein in the dMM-treated cells.

We tested for the effect of dMM on the growth rate and viability of the BC_3H_1 cells. When 200 μM dMM was added to confluent cells (conditions used for other experiments) no effect could be detected on the total amount of cellular protein [as determined by the method of Bradford (22)]. Addition of drug (200 μM) to freshly seeded cells had no effect on the time required to reach confluence (data not shown).

Fig. 4 shows the effect of dMM treatment on [^{3}H]prazosin binding to BC_3H_1 cells. For this experiment, cells were incubated with the radioligand for 1 hr at 37°. Scatchard (23) analysis of radioligand binding revealed a single class of binding sites after drug treatment; the affinity of the receptor for antagonist increased about 2-fold in dMM-treated cells whereas the number of receptors decreased 15%. The effect on dMM treatment on the affinity of the receptor for antagonist was consistent in all (four) experiments in which it was tested. The effect of treatment on the number of cellular receptors was more variable, ranging from no effect (Fig. 5) to 30% (Fig. 6). The cause of this variable effect is unclear.

Both the [^{3}H]prazosin and phentolamine (which was used to define nonspecific binding) are hydrophobic and thus the specific binding observed in Fig. 4 is likely to represent total (both surface and intracellular) receptors in the cells. To test the

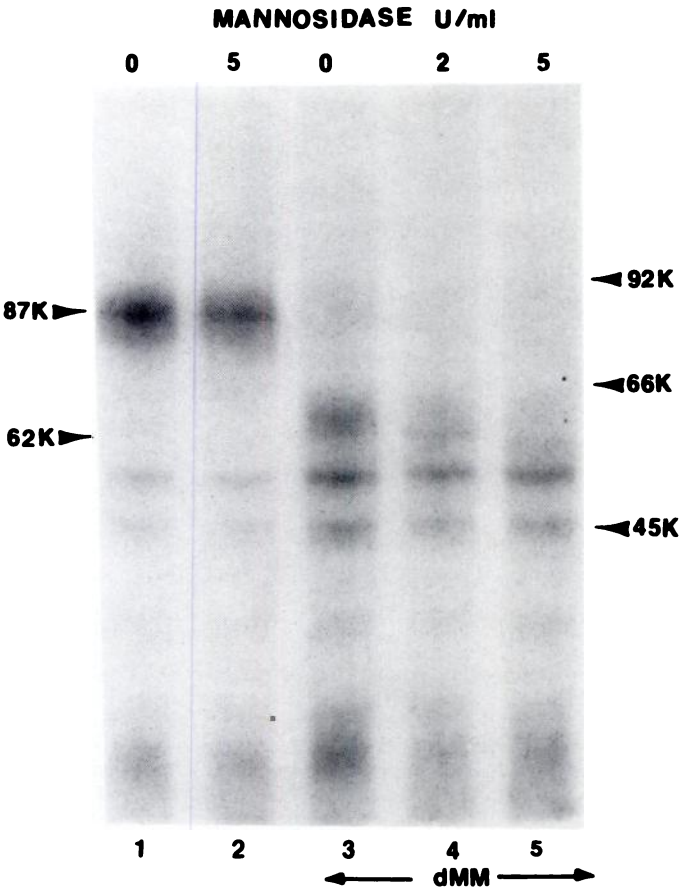

Fig. 3. Mannosidase treatment of photoaffinity-labeled α_1-receptors from control and dMM-treated BC_3H_1 cells. BC_3H_1 cells, grown to confluence on two 100-mm cell culture dishes, were incubated with or without 200 μM dMM for 48 hr. Photoaffinity labeling of the cells with ^{125}I-azido prazosin, solubilization, and denaturation of the labeled receptor, as well as glycosidase treatment and SDS-PAGE autoradiography, were done as described in the Experimental Procedures. The concentrations of α-mannosidase in the samples were as follows: *lane 1*, control cells without mannosidase; *lane 2*, control cells with 5 units/ml mannosidase; *lane 3*, dMM-treated cells without glycosidase; *lane 4*, dMM-treated cells with 2 units/ml mannosidase; *lane 5*, dMM-treated cells with 5 units/ml mannosidase.

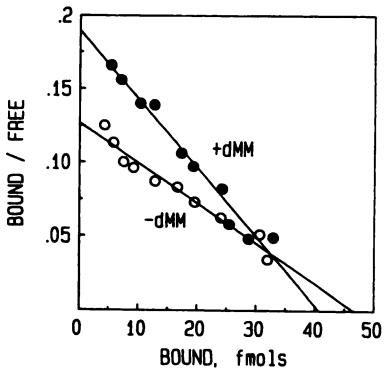

Fig. 4. [^{3}H]Prazosin binding to control and dMM-treated BC_3H_1 cells. BC_3H_1 cells, grown to confluence on 35-mm cell culture plates, were incubated with or without 200 μM for 48 hr. [^{3}H]Prazosin, 20–500 pM, was added to the dishes; 10 μM phentolamine was added to appropriate dishes as well. After a 1-hr incubation at 37°, the assay was terminated and the amount of radioactivity bound to the cells was determined as described in the Experimental Procedures. Scatchard analysis of the data was done as described (22).

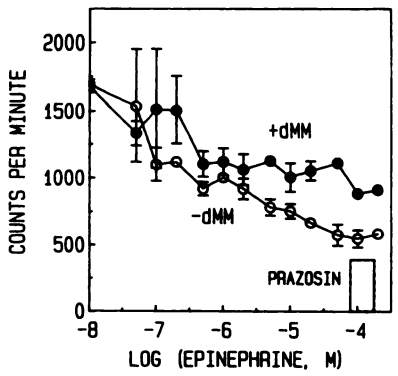

Fig. 5. Epinephrine competition for [^{3}H]prazosin binding in control and dMM-treated BC_3H_1 cells. BC_3H_1 cells, grown to confluence on 35-mm cell culture plates, were incubated for 36 hr with or without 200 μM dMM. The cells were chilled to 4° and [^{3}H]prazosin (0.5 nM), epinephrine, and prazosin (5 μM) were added to appropriate dishes. Radioligand binding was done by incubating the cells at 4° for 24 hr as described in the Experimental Procedures. The *bar* at the *lower right* of the figure represents the amount of radioactivity that was bound in the presence of unlabeled prazosin in both control and dMM treated cells.

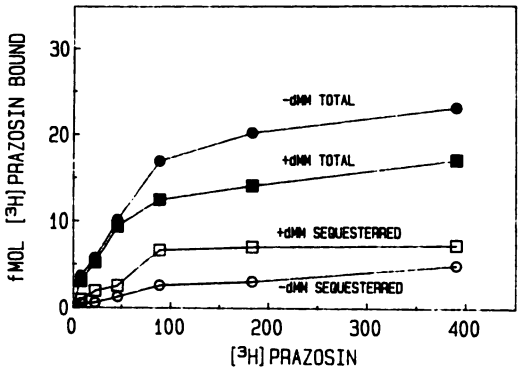

Fig. 6. The effect of dMM on surface and intracellular α_1-receptors in BC_3H_1 cells. BC_3H_1 cells, grown to confluence on 35-mm cell culture plates, were incubated for 48 hr with or without 200 μM dMM. The cells were chilled to 4° and [^{3}H]prazosin (30–400 pM), phentolamine (10 μM), and epinephrine (100 μM) were added to appropriate dishes. After a 24-hr incubation, radioligand binding was done as in Fig. 3. The data points represent the average of duplicate values obtained by subtracting the amount of binding seen in the presence of phentolamine.

effect of dMM treatment on cellular distribution of α_1-adrenergic receptors, we distinguished surface from intracellular receptors by adapting an approach used in previous studies for α_1- (24, 25), α_2- (26, 27), and β-adrenergic receptors (24, 28, 29). Whole cell radioligand binding was done at 4° in the presence and absence of hydrophilic or hydrophobic compounds. Prazosin and phentolamine are hydrophobic and will compete for [^{3}H]prazosin binding at both surface and intracellular sites; the difference in radioactivity seen with and without these antagonists therefore represents total cellular receptors. Epinephrine, on the other hand, is hydrophilic and will preferentially compete for [^{3}H]prazosin binding at the cell surface; the difference between radioactivity detected with and without epinephrine is thus a measure of surface sites. The difference between total sites and sites detected by competition with epinephrine is thus considered to represent intracellular receptors.

Fig. 5 shows the result of epinephrine competition for [^{3}H]prazosin binding in a 24-hr incubation at 4°. Unlabeled prazosin competed for a similar number of [^{3}H]prazosin sites in control and dMM-treated cells. In control cells, epinephrine competed

for 90% of the sites detected by unlabeled prazosin, a result compatible with the conclusion that 90% of the receptors were at the cell surface and 10% were intracellular. In dMM-treated cells, however, epinephrine competed for only 60% of the sites detected by unlabeled prazosin, a result suggesting that 40% of the receptors were intracellular. Fig. 6 shows the effect of dMM treatment on radioligand binding isotherm done at 4° for 24-hr in the presence of 10 μM phentolamine to define total cellular receptors and 100 μM epinephrine to define surface receptors. It can be seen that whereas the total number of cellular receptors decreased some 30% after dMM treatment, the number of intracellular receptors (as defined by the difference in [^{3}H] prazosin sites in the presence of epinephrine and phentolamine) was substantially increased by dMM treatment.

It is interesting that although epinephrine competes with [^{3}H]prazosin for 60% of the receptors after dMM treatment (Fig. 5), the agonist appears to completely block photoaffinity labeling of the receptor (Fig. 2). These two experiments were done in an identical manner except that in one case the cells were incubated with [^{3}H]prazosin for 24 hr at 4° (Fig. 5) and in the other case cells were incubated with ^{125}I-azido prazosin (Fig. 2). One explanation for this is that only surface receptors are photoaffinity labeled. We would expect that [^{125}I-azido prazosin is hydrophobic and should bind to both surface and intracellular receptors (it is not soluble in H_2O at a concentration of 10 mM and must be suspended in an organic solution such as dimethyl formamide at this concentration); perhaps the cell surface quenches the photolysis of intracellular sites and only surface receptors can covalently incorporate the probe. Consistent with this explanation is the finding that the intensity of labeling of the 62-kDa protein in dMM-treated cells is substantially less than that of the 87-kDa protein in control cells (Fig. 1) whereas the number of [^{3}H]prazosin sites is only slightly different (Fig. 4).

Fig. 7 compares the effect of dMM treatment on [^{3}H]prazosin binding to cellular receptors and their functional ability to stimulate PI turnover. In these cells there is a close correlation between the number of cellular receptors and agonist-stimulated PI hydrolysis (phospholipase C activity) (15). The rationale for the experiment was to test the hypothesis that only surface α_1-receptors would couple to phospholipase C and that hormone-stimulated PI turnover should fall in proportion to the depletion of surface receptors produced by dMM treatment. The data in Fig. 7A (showing a depletion of surface receptors by dMM treatment) and Fig. 7B (showing a decrease in epinephrine-stimulated PI turnover) confirm this prediction.

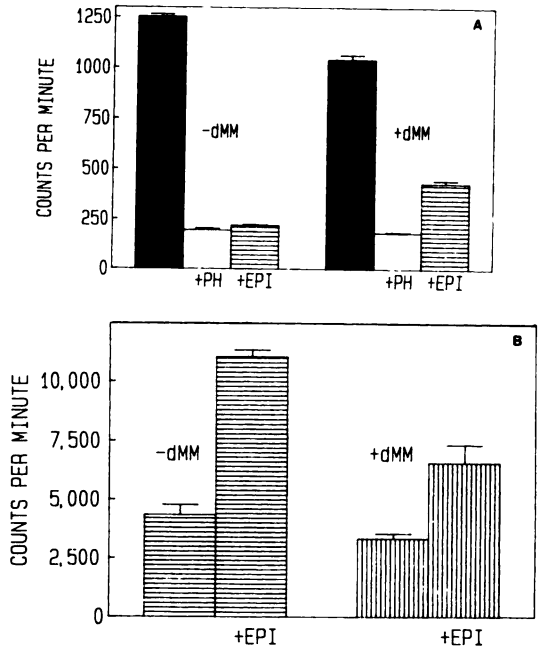

Fig. 7. The effect of dMM treatment of the number of cell surface receptors in BC_3H_1 cells. A, The effect of dMM treatment on [^{3}H]prazosin binding. The experiment was done exactly as described in the legend to Fig. 3 except for the following modifications. 1) The cells were incubated for 48 hr with dMM. 2) Only a single concentration of epinephrine (100 μM) (*EPI*) was used. 3) Phentolamine (100 μM) (*PH*) was used to define total cellular receptors. B, The effect of dMM treatment on epinephrine-stimulated PI turnover. The cells were cultured and treated with dMM as described in A. Epinephrine-stimulated PI turnover was done as described in the Experimental Procedures. The amount of [^{3}H]inositol incorporated into the cells was the same in both control and dMM-treated samples. The experiment shown here was done on the same day with the same population of cells as the experiment shown in A.

Discussion

In this manuscript we asked whether proper glycosylation of α_1-adrenergic receptors is important for their cellular distribution and function. The strategy taken to monitor receptor distribution was based on differences in hydrophobicity between different adrenergic agents. McKernan *et al.* (27) have experimentally determined the lipophilicity index (the ratio of ligand that partitions into octanol versus a buffer similar to that used in our studies) and found that for phentolamine this index is 650-fold greater than for epinephrine. The approach of using ligands of differing hydrophobicity derives from the observation that incubation of cells at 37° with hydrophilic agonists results in receptors with multiple agonist affinity states and that these affinity states differ from those observed in experiments done with intact cells at 4° or with isolated membranes at 37° (e.g., Refs. 24–29). Further characterization of this effect has revealed that in many cases the multiple agonist affinity states seen with cells at 37° may be explained by agonist-induced sequestration of surface receptors. Previous studies on β- (24, 28, 29), α_2- (26, 27), and α_1- (24, 30) receptors have used this approach to provide evidence for compartmentation and redistribution of adrenergic receptors. In the studies reported here, we found that treatment of BC_3H_1 cells with dMM decreased the number of surface α_1-receptors, increased the number of intracellular receptors, and blunted α_1-receptor-mediated functional response. We conclude that proper glycosylation plays a role in the distribution and function of α_1-receptors in BC_3H_1 cells.

We cannot unequivocally rule out interpretations of our data other than as a dMM-mediated change in cellular distribution of α_1-adrenergic receptors. Because changes in agonist binding and function were the primary techniques used to determine effects of dMM treatment, it is possible that the dMM-induced conversion of the receptor from 87 kDa to 62 kDa alters the ability of the receptor to bind and respond to agonist, although the receptor remains on the cell surface. There is evidence in the literature (24, 25, 31) that α_1-adrenergic receptors can exist in two affinity states for agonist. Towes (24) has reported that incubation of DDT_1-MF_2 cells with agonist at 37° decreases the affinity of the receptor for agonist but the distribution of receptors between sequestered versus surface membranes (as

determined by sucrose density centrifugation) does not change. These results emphasize the need to characterize more precisely both the structural nature of high and low agonist affinity receptors and the locations within the cell where sequestered receptors reside. In preliminary experiments we observe that crude membranes (prepared by centrifuging cellular homogenates at 45,000 × g) from dMM-treated cells contain 30–40% fewer receptors (as determined by [^{3}H]prazosin binding) than did control cells (data not shown). These results are consistent with the notion that the loss in epinephrine binding sites (Fig. 5) and loss in agonist-induced functional properties (Fig. 7) represent loss in receptors from surface membranes.

Cells treated with dMM showed preferential expression of a 62-kDa protein labeled by a photoaffinity probe but these cells could still bind agonist and antagonist and could still express epinephrine-stimulated PI turnover. The blunting in PI turnover was similar to the loss in surface receptor number that was determined by whole cell radioligand binding. These results are consistent with the evidence that BC_3H_1 cells do not contain spare α-receptors (15) and with the conclusion that the loss in epinephrine-stimulated phospholipase C activity is due to a decrease in receptor number on the cell surface. It is possible, but we believe less likely, that other cellular effects due to dMM treatment may have blunted the PI response, such as alterations in the number or function of G proteins (16) or membrane-associated phospholipases. Although the α_1-receptor-associated phospholipase C has not been extensively characterized, based on studies with other phospholipases (32) it may be a soluble, not glycosylated, enzyme.

The results presented in this manuscript suggest that conversion of high mannose to complex asparagine-linked oligosaccharides is required for maintaining the proper number of surface receptors. There are several possible cellular mechanisms that may explain these results. It is possible that sialic acid residues or one of the other complex sugars on newly synthesized receptors interact with other cellular components to regulate either the rate at which receptors move to the cell surface or the trafficking of receptors within the cell. A second potential mechanism is that complex sugars regulate the turnover rate of the receptor at the cell surface; that is, without proper glycosylation the rate of receptor disappearance increases, resulting in a decreased number of surface sites. It is also possible that conversion of high mannose oligosaccharides to complex chains of some other cellular protein is required for regulating the number of surface α_1-receptors. Future experiments will need to test those possibilities.

Our finding that inhibition of glycoprotein processing alters the number of cell surface α_1-receptors is consistent with previous findings for certain other receptors. For example, Reed *et al.* (10) have shown that tunicamycin prevents movement of insulin receptors to the cell surface; similar findings have been reported for acetylcholine receptors (11). Information about G-linked receptors is very scanty; a recent report has shown that neither tunicamycin nor swainsonine alters the number of surface β-receptors in S49 mouse lymphoma cells (12). Other work also suggests that the role of carbohydrate moieties may be different for β-adrenergic receptors relative to what we have found for α_1-adrenergic receptors (33, 34).

dMM has been used to study the role of complex sugars in the cellular distribution of another surface membrane protein, the transferrin receptor (35). Swainsonine has been used in the past (e.g., Refs. 12, 17, and 33) to alter the conversion of high mannose to complex oligosaccharide chains but glycoproteins still retain a partial complex nature after swainsonine treatment. For this reason we would suggest that dMM may prove advantageous because it blocks completely the addition of complex sugars to newly synthesized glycoproteins (19). It has been suggested that complex sugars, more specifically sialic acid residues, play significant roles in the function of glycoproteins. For example, addition of sialic residues to newly synthesized glycoproteins has been suggested to regulate the turnover rate of insulin receptors at the cell surface (36) and treatment of synaptosomes with neuraminidase, which removes sialic acid, inhibits sodium dependent uptake of γ-aminobutyric acid (37). Based on previous data and the findings described here, we believe that dMM should prove useful in further studies addressing the role of complex sugars in the function of receptor glycoproteins.

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