0026-895X/89/030311-08$02.00/0

MOLECULAR PHARMACOLOGY, 35:311-318

Receptor Glycosylation Regulates the Affinity of Histamine H1 Receptors during Smooth Muscle Cell Differentiation

MASATO MITSUHASHI and DONALD G. PAYAN

Howard Hughes Medical Institute and Department of Medicine, University of California Medical Center, San Francisco, California 94143

Received March 11, 1988; Accepted December 9, 1988

SUMMARY

During the cellular differentiation of the cultured smooth muscle cells, BC3H1, the K_d and the number of binding sites for [^{3}H]-pyrilamine were decreased transiently from 276 nM to 46.5 nM and from 13.3 × 10^6/cell to 2 × 10^6 cell, respectively. Concanavalin A (Con-A), wheat germ agglutinin, and lentil lectin inhibited [^{3}H]pyrilamine binding to differentiated BC3H1 cells but not to undifferentiated cells. The [^{3}H]pyrilamine binding activity of digitonin-solubilized membranes from differentiated cells was successively recovered from Con-A agarose affinity chromatography but not from undifferentiated cell membranes. The glycosylation inhibitors tunicamycin and swainsonine inhibited the expression of high affinity pyrilamine binding sites during BC3H1 cell differentiation. The molecular weight of high affinity [^{3}H]pyrilamine binding sites on differentiated cells were approximately 68,000 as analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, which after treatment with *N*-glycanase was shifted to 40,000, a molecular weight similar to that of low affinity [^{3}H]-pyrilamine binding sites on undifferentiated cells. These data suggest that one element contributing to H1-receptor heterogeneity is receptor-*N*-glycosylation.

Histamine receptors are currently classified into three groups, based on pharmacological experiments using competitive agonists and antagonists and the distinct functional responses of unique target tissues. H1R are mainly involved in smooth muscle contraction and the alteration in vascular permeability (1). H2 receptors mediate gastric acid secretion (2), whereas H3 receptors are present at presynaptic locations in the central nervous system and inhibit the release of histamine from depolarized slices of rat brain (3).

The contractile response of guinea pig ileum smooth muscle to histamine has led to detailed functional and structural studies of H1R, because this tissue expresses only H1-type histamine receptors (2). An early demonstration of the presence of H1R in the membrane fractions of guinea pig ileum smooth muscle was carried out by Hill *et al.* (4) using the radiolabeled H1-specific antagonist [^{3}H]pyrilamine. Furthermore, the dissociation constant (K_d) derived from [^{3}H]pyrilamine binding studies correlated with the EC_{50} and IC_{50} obtained from the contractile response of guinea pig ileum to histamine, thus suggesting that [^{3}H]pyrilamine binding sites were functional H1-specific receptors. However, recent studies also suggest that H1R in different tissues are not a homogeneous population. This is based on the wide variation in the K_d values of [^{3}H]-pyrilamine in various tissues, ranging from 0.7 to 219 nM (4–8), and on the analysis of radiolabeled ligand binding, which indicates the presence of H1R subclasses (9–13).

In order to investigate whether the variation of H1R is related to the state of cellular differentiation, we have examined [^{3}H]-pyrilamine binding to a cultured smooth muscle cell line, BC3H1 (14), at various stages of cellular maturation. These cells can be induced to differentiate by altering the concentration of FCS in culture medium and have been used extensively in the study of smooth muscle cell differentiation. After more than 5 days in tissue culture medium containing 0.1–0.05% FCS, BC3H1 cell shape can be seen to change to a more elongated spindle form (15). Under these conditions, it has been previously shown that BC3H1 cells undergo maturational changes from undifferentiated to differentiated cells during Days 4–8, because the mRNA of muscle-specific creatine phosphokinase is expressed during differentiation (16), and nicotinic acetylcholine receptors (17), voltage-dependent Ca^{2+} channels (18), and muscle-specific α-isoactin (19) also appear. Moreover, the presence of functional H1R on the undifferentiated form of BC3H1 cells has also been reported by Brown *et al.* (20) using ^{45}Ca efflux studies.

In the present study, we demonstrate that [^{3}H]pyrilamine binding characteristics are significantly changed during BC3H1

This work was supported in part by Grants AI19784 and NS21710 from the National Institutes of Health, and by the Howard Hughes Medical Institute.

ABBREVIATIONS: H1R, histamine H1 receptors; BC3H1, mouse brain derived smooth muscle like cell line; FCS, fetal calf serum; Con-A, concanavalin A; WGA, wheat germ agglutinin; TFA, trifluoroacetic acid; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; STV, saline-trypsin-versine; PEG, polyethylene glycol; HBSS, Hanks' balanced salt solution; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; TCA, trichloroacetic acid; DME, Dulbecco's modified Eagle's medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride.

cell differentiation, and these changes are based in part on H1R receptor glycosylation.

Experimental Procedures

Materials. BC3H1 cells were obtained from the American Type Tissue Culture Collection (Rockville, MD). Media for tissue culture were obtained from the Cell Culture Facility (University of California, San Francisco). [^{3}H]Pyrilamine (26 Ci/mmol), [^{3}H]mannose (16.3 Ci/mmol), [^{3}H]fucose (72 Ci/mmol), [^{3}H]leucine (171 Ci/mmol) (Amersham, Arlington Heights, IL), chymostatin, leupeptin (Peninsula Laboratories, Belmont, CA), tunicamycin (Behringer Diagnostics, La Jolla, CA), swainsonine (Boehringer Mannheim, Indianapolis, IN), WGA, agarose-bound Con-A (Vector Laboratories, Burlingame, CA), lentil lectin (Pharmacia, Sweden), *N*-glycanase (Genzyme, Boston, MA), Whatman GF/C and GF/F grade glass fiber filters (VWR Scientific, San Francisco, CA), TFA (Applied Biosystems, Foster City, CA), acetonitrile (EM Science, Cherry Hill, NJ), and reagents for SDS-PAGE (Bio-Rad, Richmond, CA) were obtained from the designated suppliers. The H3-specific antagonist thioperamide was a kind gift from Dr. Arrang (INSERM, Paris, France). Other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell culture. BC3H1 cells were grown in DME with 3.0 g of glucose/liter, containing 100 units/ml penicillin, 100 μg/ml streptomycin, and 20% FCS, at 37° in 5% CO_2/95% air, as previously described (21). Cells were removed from flasks by treatment with 0.025% trypsin-saline containing 0.02% EDTA (STV) and subcultured twice weekly at a ratio of 1:4. For the differentiation of BC3H1 cells, the culture medium was replaced with fresh DME containing 0.5% FCS every other day and the cell culture was continued for another 11 days. Cell viability was always more than 90%, as assessed by the exclusion of trypan blue.

Membrane preparation. Both undifferentiated and differentiated BC3H1 cells were removed from flasks with Ca^{2+}/Mg^{2+}-free PBS containing 0.02% EDTA and were suspended in PBS containing 40% sucrose, 50 mM HEPES, 25 mM $MgCl_2$, 1 mM PMSF, 2 μg/ml chymostatin, 4 μg/ml leupeptin, and 25 μg/ml bacitracin, at a concentration of 2.4×10^7 cells/ml. Cell suspensions were then sonicated (Sonifer cell disruptor 350; Branson, Danbury, CT) with 10 pulses at 4° and centrifuged at 450 × *g* for 10 min at 4°. Supernatants were further centrifuged at 100,000 × *g* for 60 min at 4°, and pellets were resuspended in HBSS and assessed for [^{3}H]pyrilamine binding. To solubilize [^{3}H]pyrilamine binding activity from membranes, membrane preparations were incubated with 1% digitonin in the presence of 1 mM PMSF at 4° for 60 min and then the reaction mixture was centrifuged at 100,000 × *g* for 60 min at 4°.

Binding of [^{3}H]pyrilamine. The [^{3}H]pyrilamine binding assay has been described previously (8, 22). In brief, cells and membrane preparations were suspended in HBSS containing 5 mM histidine and were incubated with 0.6 nM to 600 nM [^{3}H]pyrilamine in the presence and absence of 10^{-4} M unlabeled pyrilamine, in a final volume of 0.2 ml, at 4° for a specific length of time in order to determine the nonspecific and specific binding components, respectively. The amount of radioactivity bound to cells or membranes was determined by rapidly filtrating the contents of each tube under reduced pressure through GF/C, for cells, for GF/F, for membranes, glass fiber filters. Each filter was washed five times with 3 ml of cold PBS containing 5 mM histidine, and then the β-radioactivity was determined in a liquid scintillation counter (LS 5801; Beckman Instruments, Irvine, CA). The amount of radioactivity bound in the presence of unlabeled pyrilamine determined the level of nonspecific binding. The amount of specifically bound [^{3}H]-pyrilamine was calculated by subtracting the nonspecific binding from the total binding.

To determine soluble H1R binding, soluble proteins were precipitated by PEG in the presence of γ-globulin, according to the method previously described for the insulin receptor (23). In brief, each tube was resuspended in 0.2 ml of HBSS containing 5 mM histidine and 1 mg of γ-globulin and was incubated with [^{3}H]pyrilamine in the presence and absence of 10^{-4} M unlabeled pyrilamine at 4° for 60 min. An equal volume of 20% PEG was then added, and the amount of radioactivity present in precipitates was determined by filtering the content of each tube under reduced pressure through GF/F filters. Each filter was washed six times with 2.0 ml of cold 10% PEG, and then the β-radioactivity was determined as above.

The data from saturation and competition binding experiments were analyzed by weighted nonlinear least-squares curve fitting using the computer program Scatfit developed by DeLean *et al.* (24). This method is based on the law of mass action (25) and allows the analysis of the binding of a radioligand to multiple classes of binding sites. The data were fitted to one- and two-site models successively, and a two-site model was accepted only when the fit of the data was significantly improved, compared with the fit with a one-site model ($p < 0.05$). Testing for statistical difference between models was performed by comparing the residual variance of the fits of the data according to the "extra sum of squares" principle using an *F* ratio test. The computer analysis also yields the IC_{50}, Hill coefficient, K_i, K_d, and concentration for each class of receptor. The binding data were also analyzed by the computer program Lundon-1 and Lundon-2 (Lundon Software, Inc., Middlefield, OH) (26) to confirm the Scatfit analysis. Because, unlike Scatfit, the Lundon-1 program computes the parameter estimates directly from the data set, user-supplied initial parameter estimates are not required.

Con-A affinity chromatography. In order to analyze the glycoprotein nature of H1R, solubilized membrane proteins from both undifferentiated and differentiated BC3H1 cells were applied to Con-A-bound agarose columns. Solubilized membrane proteins (1.0 mg) were diluted 5 times with 0.02 M Tris·HCl, pH 7.4, containing 0.5 M NaCl, 1 mM $CaCl_2$, and 1 mM $MnCl_2$ (starting buffer) and were applied to the Con-A-agarose columns. Unbound proteins were completely eluted off the columns with starting buffer, then bound proteins were eluted with starting buffer containing various concentrations of methyl α-D-mannopyranoside. The eluted fractions were assessed in a fluorimetric assay to determine protein concentration (27), and the remaining materials were further applied to WP'Butyl (C_4) mini-columns (J. T. Baker, Phillipsburg, NJ) for concentration. Bound proteins were eluted with 90% acetonitrile/0.1% TFA, concentrated under vacuum rotation, resuspended in HBSS, and assessed for soluble receptor binding as described above.

[^{3}H]Mannose, [^{3}H]fucose, and [^{3}H]leucine incorporation. Confluent culture of cells in 24-well plates were incubated with 0.3 μg/ml tunicamycin or 2 μg/ml swainsonine for 8 hr, then 10 μCi of tritiated mannose, fucose, or leucine were added into each well and incubated for an additional 12 hr. Supernatants were removed, cells were detached from wells with STV, and cellular proteins were precipitated by the addition of an equal volume of 20% TCA. Precipitates were then trapped onto GF/F filters under reduced pressure and washed five times with 3 ml of TCA, and the β-radioactivity was determined.

SDS-PAGE. Electrophoresis was performed in the presence of 2-mercaptoethanol according to the methods of Laemmli (28). The stacking gel was 5% acrylamide, 0.125 M Tris·HCl, pH 6.8, and 0.1% SDS. The separation gel was 7.5–10% acrylamide, 0.375 M Tris·HCl, pH 8.8, and 0.1% SDS. After electrophoresis was completed, the gel was cut into slices (approximately 3 mm in width) and homogenized with a glass-Teflon homogenizer in 4 ml of 50 mM Tris·HCl, pH 7.4, containing 5 mM $MgCl_2$, 0.1% digitonin, and 1 mM PMSF, at 4° overnight to elute proteins from the SDS-containing gels, as described for the prostaglandin E_1/prostacyclin receptors (29). Homogenates were centrifuged at 3000 × *g* at 4° for 10 min, and supernatants were applied to WP'Butyl (C_4) mini-columns as described above. Eluted proteins were then assessed for soluble receptor binding.

Statistical analysis. The Student *t* test was performed after the test of similarity of variance of two groups.

Results

Kinetics of [^{3}H]pyrilamine binding. The specific binding of differentiated cells (Day 6) increased more rapidly than that

of undifferentiated cells and reached a steady state plateau after 15–20 min (Fig. 1A). A pseudo-first order rate plot of these results (Fig. 1A, *inset*) indicated that the two regression lines were statistically different from each other ($p < 0.01$). The observed rate constants, K_{obs}, of undifferentiated and differentiated cells were equal to 0.094 and 0.17 min^{-1}, respectively.

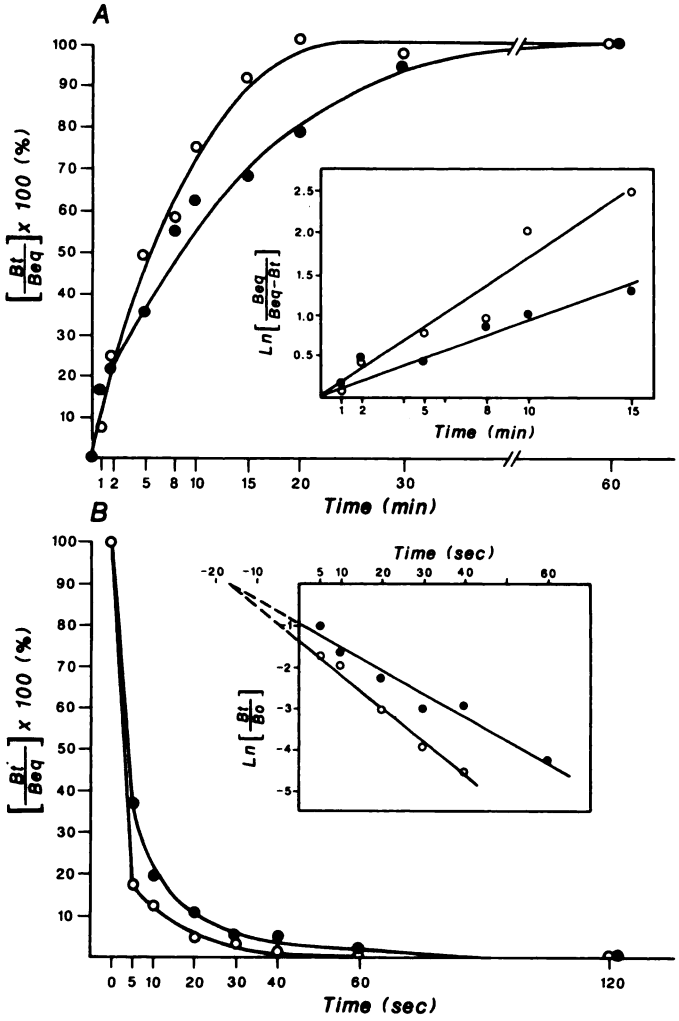

Fig. 1. Kinetics of [^{3}H]pyrilamine binding to undifferentiated and differentiated (Day 6) BC3H1 smooth muscle cells. Cells (4×10^6) suspended in 0.2 ml of HBSS containing 5 mM histidine were incubated with 200 nM [^{3}H]pyrilamine for undifferentiated cells or 50 nM [^{3}H]pyrilamine for differentiated cells, in the presence and absence of 10^{-4} M unlabeled pyrilamine, at 4° for 1 to 60 min. The difference of added [^{3}H]pyrilamine between undifferentiated and differentiated cells was based on the K_d values from saturation binding. The amount of radioactivity bound to cells was determined by rapidly filtering the content of each tube under reduced pressure as described in Experimental Procedures. The amount of specifically bound [^{3}H]pyrilamine to undifferentiated (●) and differentiated cells (○) was calculated by subtracting the nonspecific binding from the total binding. A, Association of specific [^{3}H]pyrilamine binding to BC3H1 cells. *Inset*, transformation of the data from the first 15 min of binding from the association curve into semilogarithmic form, to give pseudo-first order kinetics. B_t and B_{eq} refer to the specific binding at each time and at equilibrium, respectively. B, Dissociation of specific [^{3}H]-pyrilamine binding to BC3H1 cells after a 40-min incubation at 4°, by the addition of 10^{-4} M unlabeled pyrilamine, and continued incubation at 4° for 5 sec to 2 min. *Inset*, semilogarithmic plot from the first 40–60 sec from the dissociation curve. B_o refers to the specific binding at time 0. Each *data point* represents the mean of two separate experiments, performed in duplicate.

The dissociation of [^{3}H]pyrilamine from BC3H1 cells at different stages of differentiation is shown in Fig. 1B. The specific binding of [^{3}H]pyrilamine to differentiated cells decreased more rapidly than that to undifferentiated cells, following the addition of unlabeled pyrilamine. The first order rate constant, K_{-1}, for the dissociation of pyrilamine-receptor complexes (Fig. 1B, *inset*) was equal to 4.4 min^{-1} for undifferentiated and 7.8 min^{-1} for differentiated cells and also indicated that the two regression lines were statistically different from each other ($p < 0.01$). The x intercept of both regression lines was −17 sec. This suggests that a fraction of the specifically bound [^{3}H]pyrilamine was dissociated from the cells during the filter-washing procedure. The rate of association of pyrilamine-receptor complexes for undifferentiated and differentiated cells was calculated using the equation $K_{+1} = (K_{obs} - K_{-1})/(L)$, where (L) = ligand concentration, with the equilibrium constant $K_d = K_{-1}/K_{+1}$ being calculated to be 196 nM for undifferentiated and 49 nM for differentiated cells.

Saturation [^{3}H]pyrilamine binding. [^{3}H]Pyrilamine binding during cellular differentiation, analyzed by both Scatfit and Lundon-1, best fitted a one-site model. As shown in Fig. 2, during differentiation the K_d was transiently decreased from 276 nM to 46.5 nM, and the receptor number was also decreased, from 13.3×10^6/cell to 2×10^6/cell. Lundon-1 also calculated the K_d and receptor density from the linear analysis, based on the methods of Eadie/Hofstee (30, 31), or Scatchard/Rosenthal (32, 33). However, these values are not significantly different from those of the nonlinear analysis.

Competition [^{3}H]pyrilamine binding. To evaluate the structural specificity of [^{3}H]pyrilamine binding to both undifferentiated and differentiated cells, unlabeled histamine, chlorpheniramine, pyrilamine (H1 antagonists), cimetidine (H2 antagonist), thioperamide (H3 antagonist), and other ligands for various receptors were assessed for their capacity to inhibit the specific binding of [^{3}H]pyrilamine. The IC_{50} calculated by Scatfit demonstrated that [^{3}H]pyrilamine binding sites on both cells were highly H1 specific and the IC_{50} of pyrilamine to undifferentiated cells was significantly higher than that of differentiated cells (Table 1). The values of the Hill coefficient of pyrilamine in undifferentiated and differentiated cells were 1.02 ± 0.05 and 0.8 ± 0.06 (mean ± standard error), respectively, and the nonlinear analysis using Scatfit and Lundon-2 also indicated a single class of binding sites. The computer-calculated values for the K_i and number of binding sites for pyrilamine were 950 ± 86 nM and 23.3 ± 10.8 × 10^6/cell for undifferentiated cells and 171 ± 77 nM and 1.7 ± 1.5 × 10^6/cell for differentiated cells, respectively.

Lectin inhibition of [^{3}H]pyrilamine binding. In order to examine the role of cell surface carbohydrate molecules in receptor-ligand interaction, the influence of different lectins on [^{3}H]pyrilamine binding to membrane preparations of BC3H1 cells was examined as previously described for insulin receptors (34). Membrane preparations from both undifferentiated and differentiated cells were suspended in HBSS, containing 1 mM $MnCl_2$, at 200–400 μg/ml, and [^{3}H]pyrilamine binding was assessed, as described in Experimental Procedures, in the presence of various concentrations of Con-A, WGA, lentil lectin, and peanut lectin. As a result, Con-A inhibited [^{3}H]-pyrilamine binding in a dose-dependent manner in differentiated cell membranes but had no effect on undifferentiated cell membranes (Fig. 3). High concentrations (200 μg/ml) of

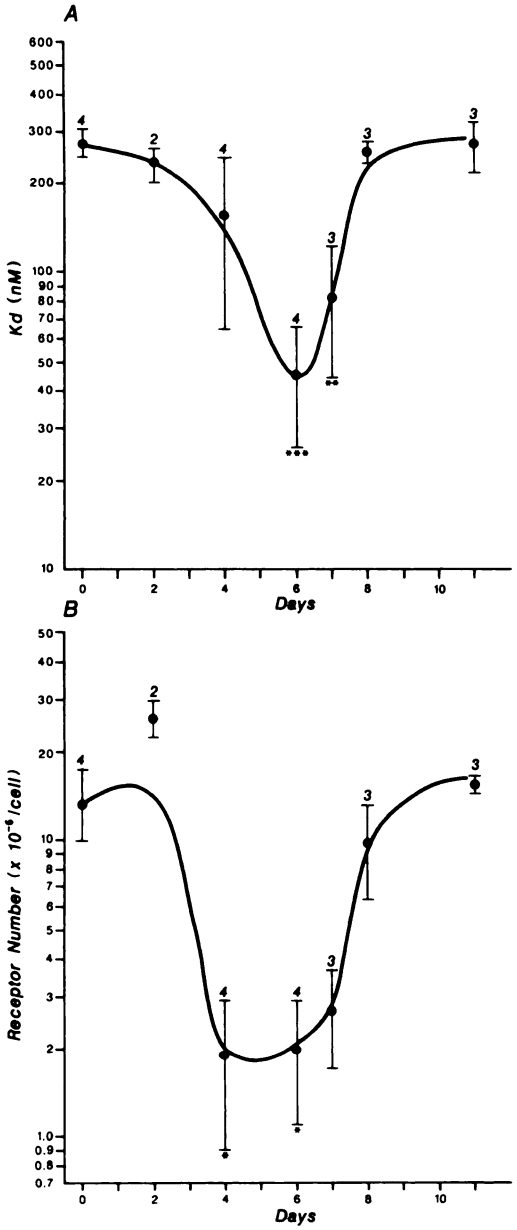

Fig. 2. Saturation binding of [^{3}H]pyrilamine to BC3H1 cells during smooth muscle cell differentiation. Cells (2–4 × 10^4) were incubated with 1–600 nM [^{3}H]pyrilamine in the presence and absence of unlabeled pyrilamine (10^{-4} M). The amount of radioactivity bound to cells was determined as described in Experimental Procedures. The data were analyzed by the computer programs Scatfit (24) and Lundon-1 (26). A, The changes in the K_d during smooth muscle cell differentiation. B, The changes in the receptor number. Each *data point* is the mean ± standard error from two to four separate determinations indicated at each *bar*, performed in duplicate. The statistical significance was determined by a Student *t* test. *, **, and *** indicate $p < 0.05$, $p < 0.02$, and $p < 0.01$, respectively.

TABLE 1

Structural determinants of [^{3}H]pyrilamine binding to undifferentiated and differentiated BC3H1 cells

Concentrations of 200 nM [^{3}H]pyrilamine for undifferentiated cells and 50 nM [^{3}H]-pyrilamine for differentiated cells were mixed with 4 × 10^4 cells in the presence of 10^{-9} to 10^{-3} M unlabeled ligands. The difference of added [^{3}H]pyrilamine between undifferentiated and differentiated cells was based on the K_d values derived from saturation binding. The amount of the radioactivity bound to cells was determined as described in Experimental Procedures and the data were analyzed by the computer programs Scatfit and Lundon-2. Each data point represents the mean ± standard error of two to four separate determinations, performed in duplicate.

Ligands	IC_{50}	
	Undifferentiated cells	Differentiated cells
	μM	
Histamine	833 ± 167	127 ± 77[a]
H1 antagonists		
Pyrilamine	1.26 ± 0.08	0.47 ± 0.14[a]
Chlorpheniramine	1.06 ± 0.32	0.37 ± 0.32
H2 antagonist		
Cimetidine	>1,000	>1,000
H3 antagonist		
Thioperamide	>100	>100
Others		
Acetylcholine	>1,000	>1,000
Epinephrine	>1,000	>1,000
Isoproterenol	>1,000	>1,000
Serotonin	>1,000	>1,000
GABA	>1,000	>1,000
Histidine	>1,000	>1,000

[a] $p < 0.05$ compared with undifferentiated cells.

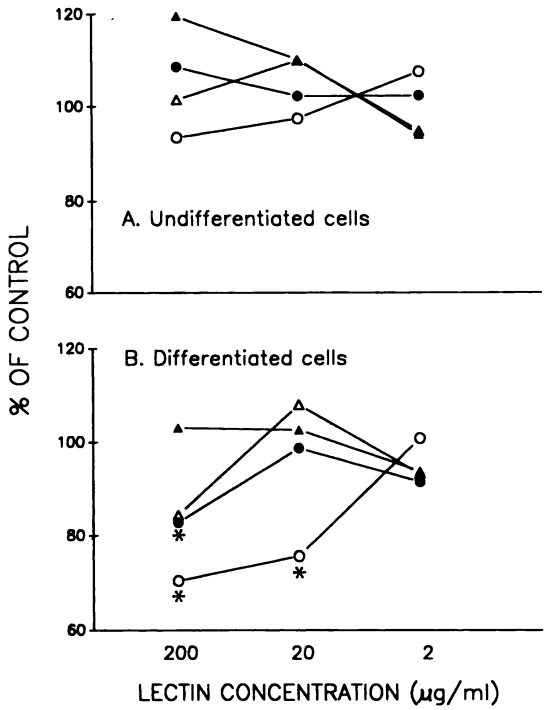

Fig. 3. Lectin inhibition of [^{3}H]pyrilamine binding to both undifferentiated and differentiated cell membranes. Membrane preparations were suspended in HBSS containing 1 mM $MnCl_2$ at 200–400 μg/ml, and [^{3}H]-pyrilamine binding was assessed in duplicate, as described in Experimental Procedures, in the presence of 2–200 μg/ml concanavalin A (○), WGA (●), lentil lectin (△), and peanut lectin (▲). Each *data point* represents the mean of two to three separate experiments. * indicates $p < 0.05$.

WGA and lentil lectin also inhibited [^{3}H]pyrilamine binding on differentiated cell membranes but not on undifferentiated cell membranes (Fig. 3).

Con-A affinity chromatography. In order to further analyze whether lectin-receptor interactions are mediated by specific carbohydrate molecules, soluble membrane proteins were applied to the Con-A-agarose columns and eluted with specific sugars. A large portion of the applied proteins were not bound to the column. However, [^{3}H]pyrilamine binding activity was specifically eluted by 0.05–0.1 M methyl α-D-mannopyranoside from differentiated cell membranes, but not from undifferentiated ones (Table 2).

Effect of glycosylation inhibitors. The role of glycosyla-

TABLE 2

Con-A affinity chromatography of solubilized [^{3}H]pyrilamine binding activity from both undifferentiated and differentiated BC3H1 cells

Solubilized membrane proteins from both undifferentiated and differentiated BC3H1 cells were applied to the Con-A-bound agarose columns, then bound proteins were specifically eluted with various concentrations of methyl α-D-mannopyranoside. The protein concentration of each fraction was measured by a fluorimetric assay (27), and the remaining materials were assessed for [^{3}H]pyrilamine binding, as described in Experimental Procedures.

Fractions	Undifferentiated		Differentiated	
	Binding	Protein	Binding	Protein
	cpm (%)	*μg (%)*	*cpm (%)*	*μg (%)*
Solubilized membranes	28,220 (100)	1,000 (100)	18,779 (100)	1,000 (100)
Unbound	25,561 (91)	740 (74)	14,355 (77)	810 (81)
Methyl mannopyranoside				
0.05 M	250 (1)	43 (4)	2,100 (11)	<10 (<1)
0.1 M	150 (1)	36 (4)	4,250 (28)	10 (1)
0.2 M	430 (2)	43 (4)	500 (3)	70 (7)
0.5 M	500 (2)	48 (5)	50 (0)	30 (3)

TABLE 3

The effect of tunicamycin and swainsonine on glycosylation and protein synthesis of undifferentiated and differentiated BC3H1 cells

Confluent cultures of both cells in 24-well plates were incubated with 0.3 μg/ml tunicamycin or 2 μg/ml swainsonine for 8 hr, then 10 μCi of [^{3}H]mannose, [^{3}H]-fucose, or [^{3}H]leucine were added into each well and incubated for another 12 hr. The radioactivity of TCA-precipitates was determined as described in Experimental Procedures. Each data point represents the mean ± standard error of three separate experiments, performed in duplicate.

Radioisotopes	Incorporation			
	Tunicamycin		Swainsonine	
	Undifferentiated	Differentiated	Undifferentiated	Differentiated
	% of control			
[^{3}H]Mannose	7.7 ± 1.3	2.0 ± 0.2	68.7 ± 19.7	80.0 ± 12.7
[^{3}H]Fucose	19.2 ± 3.5	4.4 ± 0.2	82.1 ± 14.2	89.5 ± 8.7
[^{3}H]Leucine	96.3 ± 18.5	80.1 ± 4.0	107.0 ± 17.4	106.3 ± 11.1

tion in the regulation of the affinity of H1R was evaluated by examining the effect of glycosylation inhibitors, tunicamycin (35) and swainsonine (36), on [^{3}H]pyrilamine binding to BC3H1 cells. As shown in Table 3, 0.3 μg/ml tunicamycin or 2 μg/ml swainsonine significantly inhibited [^{3}H]mannose and [^{3}H]fucose incorporation into both undifferentiated and differentiated cells but had no effect on [^{3}H]leucine incorporation. Data from saturation binding experiments indicated that the K_d values of [^{3}H]pyrilamine in differentiated cells were converted from high affinity to low affinity by the incubation of BC3H1 cells with tunicamycin or swainsonine, without a significant change in the receptor density (Table 4). Tunicamycin and swainsonine had no effect on the K_d of low affinity H1R in undifferentiated cells (Table 4).

SDS-PAGE and *N*-glycanase treatment. The molecular weight of both high and low affinity H1R on BC3H1 cells were compared in order to examine whether there was a correlation between the receptor size, ligand affinity, and degree of glycosylation. In order to examine this hypothesis, soluble membrane preparations of both undifferentiated and differentiated BC3H1 cells were separated by SDS-PAGE. Proteins were eluted from the sliced gel pieces following the method of Dutta-Roy and Sinha (29), who have reported the recovery of prostaglandin E_1/prostacyclin receptors from SDS-PAGE slices. The recovery of [^{3}H]pyrilamine binding activity from the gel pieces was approximately 80%. [^{3}H]Pyrilamine binding to the

TABLE 4

The effect of glycosylation inhibitors on saturation binding of [^{3}H]pyrilamine to undifferentiated and differentiated BC3H1 cells

Cells were incubated with 0.3 μg/ml tunicamycin or 2 μg/ml swainsonine for 20 hr, then saturation binding was assessed as described in Experimental Procedures. Each data point represents the mean ± standard error of three separate experiments, performed in duplicate.

Inhibitors	Undifferentiated		Differentiated	
	K_d	B_{max}	K_d	B_{max}
	nM	*×10^{-6}/cell*	*nM*	*×10^{-6}/cell*
Control	276 ± 32	13.3 ± 3.9	47 ± 20	2.0 ± 0.9
Tunicamycin	221 ± 22	10.3 ± 2.1	224 ± 63*	4.8 ± 1.2
Swainsonine	223 ± 41	6.2 ± 1.7	226 ± 50*	2.6 ± 0.3

*$p < 0.05$ compared with control.

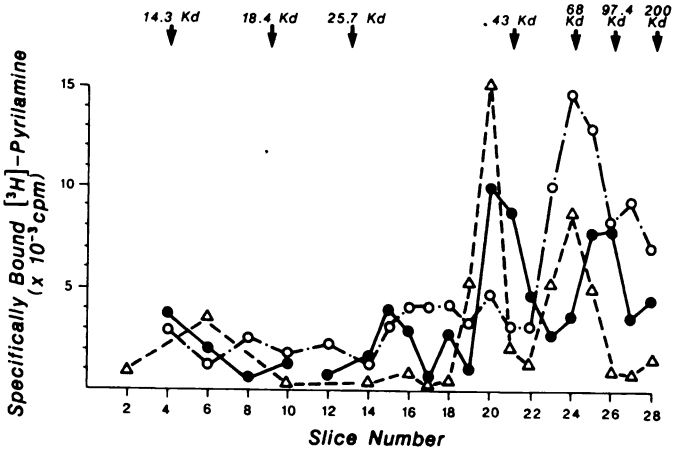

Fig. 4. Binding of [^{3}H]pyrilamine to BC3H1 cell membrane-associated proteins following elution from SDS-PAGE; 0.2 mg of protein of soluble membrane preparations of both undifferentiated and differentiated cells were analyzed on SDS-PAGE in the presence of 2-mercaptoethanol according to the method of Laemmli (28). The gels were then cut into slices (approximately 3 mm in width) and eluted proteins were assessed for [^{3}H]pyrilamine binding using PEG precipitation as described in Experimental Procedures. ● and ○, Specific binding to undifferentiated and differentiated cells, respectively. [^{3}H]Pyrilamine-binding proteins of differentiated cells eluted from SDS-PAGE slices (slice number 23–25) were further treated with 30 units/ml *N*-glycanase. After overnight incubation at 37°, the reaction mixture was separated by SDS-PAGE and assessed for [^{3}H]pyrilamine binding as described above (△). Molecular mass standard proteins were lysozyme (14.3 kDa), β-lactoglobulin (18.4 kDa), α-chymotrypsinogen (25.7 kDa), ovalbumin (43 kDa), bovine serum albumin (68 kDa), phosphorylase B (97.4 kDa), and myosin heavy chain (200 kDa).

eluted proteins from the sliced gel pieces indicated that [^{3}H]-pyrilamine binding activity of undifferentiated cells had two receptor populations with different molecular masses, approximately 40 kDa and 68–97 kDa (Fig. 4). According to the saturation binding analysis using these fractions, both the high and low molecular mass fractions on undifferentiated cells were low affinity with K_d values similar to those on intact cells (Table 4). In contract, [^{3}H]pyrilamine binding activity of differentiated cells exhibited a single population with a molecular mass of approximately 68 kDa (Fig. 4). The saturation binding analysis also indicated that this 68-kDa fraction was of high affinity, with the K_d value similar to that in intact differentiated cells (Table 4).

In order to further examine whether the difference in molecular mass of [^{3}H]pyrilamine binding sites between undifferentiated and differentiated cells was due to receptor glycosylation, the eluted 68-kDa proteins from differentiated cell membranes

(Fig. 4, slice numbers 23–25) were suspended in 0.2 M phosphate buffer, pH 8.6, containing 10 mM 1,10-phenanthroline hydrate and 0.2% SDS and were treated with 30 units/ml *N*-glycanase, which cleaves all *N*-linked sugars (37). After overnight incubation at 37°, the reaction mixture was separated by SDS-PAGE and assessed for [^{3}H]pyrilamine binding, as described above. As a result of *N*-glycanase treatment, approximately half of the binding activity was shifted from 68 to 40 kDa, a molecular mass similar to that of the low molecular mass fraction of undifferentiated cells (Fig. 4). Furthermore, the saturation binding analysis also indicated that the 40-kDa fraction of *N*-glycanase-treated differentiated cell membranes was of low affinity, with a K_d value similar to that of undifferentiated cells (Table 4).

Discussion

In the present study, [^{3}H]pyrilamine binding characteristics in BC3H1 smooth muscle cells were shifted from low affinity to high affinity during cellular differentiation (Figs. 1 and 2; Table 1); lectin inhibition of [^{3}H]pyrilamine binding (Fig. 3), Con-A affinity chromatography (Table 2), experiments using glycosylation inhibitors (Tables 3 and 4), changes in the molecular mass (Fig. 4), and the effect of *N*-glycanase (Fig. 4; Table 5) all suggest that the affinity of [^{3}H]pyrilamine binding sites is regulated by glycosylation.

In a previous study, we demonstrated the presence of functional H1R on the smooth muscle cell line DDT$_1$MF-2 (8, 21). [^{3}H]Pyrilamine binding to this cell line indicated a single class of binding sites demonstrated by three separate methods of determination, kinetics (rate of association/dissociation), saturation (binding of labeled ligand), and competition experiments (displacement of labeled ligand by unlabeled ligands) with resulting similar K_d values ranging from 104 to 953 nM (8). The K_d values of [^{3}H]pyrilamine in DDT$_1$MF-2 cells were 100–1000-fold higher than those in several H1R systems (4–6). However, the K_d values correlated well with the IC_{50} values of the H1 antagonist chlorpheniramine in both histamine-induced increased in $[Ca^{2+}]_i$ and histamine-induced actin polymerization in DDT$_1$MF-2 cells (8). More recently, we found that cell surface [^{3}H]pyrilamine binding sites in DDT$_1$MF-2 cells were down-regulated by TPA, which activates protein kinase C; the histamine-induced increase in cytosolic free Ca^{2+} was also inhibited by incubation with TPA (22). These data suggest that [^{3}H]pyrilamine binding sites on DDT$_1$MF-2 cells are functional H1R, which exhibit low affinity binding characteristics. In the present study, we also demonstrate the presence of [^{3}H]pyrilamine binding sites in the undifferentiated form of BC3H1 smooth muscle cells with the binding characteristics similar to those shown in DDT$_1$MF-2 cells.

Cuatrecasas and Tell (34) have investigated the glycoprotein nature of insulin receptors using Con-A and WGA and reported that 40–500 μg/ml Con-A inhibited ^{125}I-insulin binding to fat cells and liver membranes. In the present study, Con-A also inhibited [^{3}H]pyrilamine binding to differentiated BC3H1 cells but not to undifferentiated cells (Fig. 3). The difference in inhibitory activity among various lectins to differentiated cell membranes suggests the presence of specific carbohydrate molecules close to the [^{3}H]pyrilamine binding site. In order to further analyze whether Con-A inhibition of [^{3}H]pyrilamine binding was due to the interaction between Con-A and specific carbohydrate molecules of membrane-bound proteins, bound proteins were competitively dissociated by methyl-α-D-mannopyranoside from the Con-A-agarose affinity columns (Table 2). A large portion of the solubilized membrane proteins were not bound to the columns (Table 2). The low efficiency of Con-A affinity chromatography for H1R has been reported by Garbarg *et al.* (38) and was shown not to be due to the capacity of the columns. Digitonin may compete with the membrane proteins for binding to the lectin matrix, by virtue of the glucose residues present in digitonin (39). However, under identical conditions, part of the [^{3}H]pyrilamine binding activity was recovered from differentiated cells but not from undifferentiated cells. This suggests that Con-A may reversibly bind to specific carbohydrate molecules on membrane-bound proteins of differentiated cells and inhibit [^{3}H]pyrilamine binding to H1R (Fig. 3).

Tunicamycin has been shown to specifically inhibit the synthesis of *N*-acetylglucosaminylpyrophosphorylpolyisoprenol, leading to the inhibition of protein glycosylation (35), whereas swainsonine inhibits the biosynthesis of complex-type of oligosaccharides by inhibiting the Golgi mannosidase II, thereby causing the accumulation of high-mannose glycoproteins (36). Because there is a large cell-to-cell variation in the sensitivity to these glycosylation inhibitors, we first verified that under our experimental conditions these drugs effectively block glycosylation, but not protein synthesis. The incorporation of [^{3}H]-mannose, [^{3}H]fucose, and [^{3}H]leucine into TCA-precipitates of both undifferentiated and differentiated cells indicated that 0.3 μg/ml tunicamycin or 2 μg/ml swainsonine inhibited only glycosylation (Table 3). Under these conditions, both tunicamycin and swainsonine blocked the expression of high affinity [^{3}H]pyrilamine binding sites on differentiated BC3H1 cells. Thus, the changes in the K_d in differentiated cells from high affinity to low affinity might be due to the inhibition of glycosylation during cellular differentiation (Table 4).

The reported molecular size of membrane-bound H1R in both bovine and human cerebral cortex by target size analysis

TABLE 5

Summary of saturation [^{3}H]pyrilamine binding

A total of 2–4 × 10^4 cells or 20–50 μg of protein of membrane preparations of both undifferentiated and differentiated BC3H1 cells were suspended in 0.2 ml of HBSS and assessed for saturation [^{3}H]pyrilamine binding as described in Experimental Procedures. Digitonin-solubilized proteins (0.5–1.0 mg) were separated on SDS-PAGE in the presence of 2-mercaptoethanol and the gels were cut into 35–45-kDa and 60–90-kDa fractions according to the prestained molecular weight standards. Proteins were eluted from the sliced gels and assessed for saturation [^{3}H]pyrilamine binding as described in Experimental Procedures. The 60–90-kDa fraction of differentiated cells was further treated with 30 units/ml *N*-glycanase at 37° overnight, then the reaction mixture was separated on SDS-PAGE. The gels were cut into fractions representing 35–45-kDa and 60–90-kDa proteins, and the eluted proteins from the gels were assessed for [^{3}H]pyrilamine binding as described in Experimental Procedures. The data were analyzed by the computer programs Scatfit and Lundon-1. Each data point represents mean ± standard error from two or three separate experiments, performed in duplicate.

Fractions	K_d	
	Undifferentiated	Differentiated
	nM	
Intact cells	276 ± 32	46.5 ± 20.0
Membranes	156 ± 33	54.7 ± 12.7
Solubilized membranes		
35–45 kDa	151 ± 18	
60–90 kDa	296 ± 149	51.9 ± 20.6
N-Glycanase treatment		
35–45 kDa		281.9 ± 20.1
60–90 kDa		87.7 ± 18.3

has demonstrated an approximate size of 160 kDa (40). Recently, Ruat *et al.* (41) have used irreversible photoaffinity labeling to specifically label H1-binding proteins in guinea pig brain membranes. SDS-PAGE indicated that the molecular mass of H1R was 350–400 kDa in the absence of 2-mercaptoethanol, and the molecular mass decreased to 56 and 47 kDa in the presence of 2-mercaptoethanol. This suggests that H1R may have one or more disulfide bridges resulting in a higher molecular mass complex. Furthermore, in the presence of protease inhibitors, labeling of the 56-kDa protein increased at the expense of the 47-kDa protein. This may also suggest that the 56-kDa protein is at least the binding domain of H1R and the 47-kDa protein is a proteolytic degradation product of the 56-kDa protein.

In the present study, [^{3}H]pyrilamine binding activity was successfully recovered from the sliced SDS gels with binding characteristics similar to those in intact cells (Table 5). Because the recovery of [^{3}H]pyrilamine binding activity was more than 80% from SDS gels, the eluted proteins may be H1R. The molecular mass of H1R in undifferentiated BC3H1 cells was approximately 40 kDa and 97 kDa in the presence of 2-mercaptoethanol and both fractions exhibited low affinity binding characteristics similar to those of intact undifferentiated cells. This may suggest that the 40-kDa protein is a proteolytic product of the 97-kDa protein or, alternatively, the 97-kDa protein is glycosylated without affecting the [^{3}H]pyrilamine binding characteristics. Recently, we have purified the H1R from DDT$_1$MF-2 cells by sequential gel filtration, chromatofocusing, and reverse phase high pressure liquid chromatography (42). Interestingly, the molecular mass of this purified H1R protein was approximately 40 kDa and the eluted purified H1R protein from SDS gels also exhibited low affinity binding characteristics similar to those of the 40-kDa fraction from undifferentiated BC3H1 cells. This suggests that the 40-kDa protein contains the binding domain of the low affinity H1R. In contrast to undifferentiated cells, the H1R in differentiated BC3H1 cells was a single component with approximately 68-kDa molecular mass (Fig. 4), and this fraction showed high affinity binding characteristics similar to those of intact differentiated cells. Furthermore, after *N*-glycanase treatment, this 68-kDa protein was shifted to 40 kDa and the saturation binding analysis also demonstrated that the K_d of the 40-kDa protein was of low affinity. These data strongly suggest that the 68-kDa protein is *N*-glycosylated and this *N*-glycosylation is crucial for the expression of high affinity binding characteristics.

Receptor glycosylation is an important event in structural and functional maturation of various cell surface receptors (43–45). However, the deletion of *N*-linked carbohydrates from β-receptors (39) and the deletion of *O*-linked carbohydrates from low density lipoprotein receptors (46) have no significant effect on receptor function. The present study indicates that the affinity of H1R on the cultured smooth muscle cells BC3H1 is regulated by receptor glycosylation. However, the functional difference between high and low affinity [^{3}H]pyrilamine binding sites on BC3H1 cells is still unclear. Inasmuch as Brown *et al.* (20) have reported that histamine H1-specific stimulation increased $^{45}Ca^{2+}$ efflux and substantial transmembrane $^{45}Ca^{2+}$ influx into undifferentiated BC3H1 cells and [^{3}H]pyrilamine binding indicated a single class of binding sites on undifferentiated cells, low affinity [^{3}H]pyrilamine binding sites on undifferentiated BC3H1 cells might be functional H1R. However, because the computer analysis of [^{3}H]pyrilamine binding would not necessarily detect a small percentage of high affinity H1R, the functional responses of undifferentiated cells might be mediated by high affinity H1R. Further studies will be necessary to understand H1R heterogeneity.

Acknowledgments

The authors would like to thank Dr. C. Koo, J. Sherman, J. P. McGillis, and C. G. Davis for their helpful comments and suggestions and Dr. Arrang (Centre Paul Broca de l'INSERM, Paris) for sending us the H3 antagonist.

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Send reprint requests to: Masato Mitsuhashi, M.D., 533 Parnassus Ave. U-426, University of California Medical Center, San Francisco, CA 94143-0724.