0026-895X/89/060751-09$02.00/0

MOLECULAR PHARMACOLOGY, 35:751-759

Solubilization and Characterization of the Pyrilamine-Binding Protein from Cultured Smooth Muscle Cells

MASATO MITSUHASHI and DONALD G. PAYAN

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

Received November 1, 1988; Accepted March 6, 1989

SUMMARY

The cultured smooth muscle cell line DDT$_1$MF-2 expresses a large number (9.7×10^6 receptors/cell) of functional histamine H_1-type receptors [*J. Cell. Physiol.* **134**:367–375 1988]. Two different binding assays, gel filtration and polyethylene glycol precipitation, indicated that the [^{3}H]pyrilamine binding activity was solubilized by 1% digitonin with binding characteristics similar to those of intact cells. The solubilized proteins were then purified by sequential gel filtration, chromatofocusing, and reverse phase high pressure liquid chromatography. The calculated molecular weight of this purified pyrilamine-binding protein was 38–40 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. [^{3}H]Pyrilamine binding to these 38–40-kDa proteins indicated a single class of binding site with a K_d of 288 nM, which is equivalent to that of intact cells and digitonin-solubilized proteins. The computer analysis Scatfit also indicated that one molecule of [^{3}H]pyrilamine bound to one molecule of purified protein. Furthermore, a polyclonal antibody raised against the purified protein recognized the 38–40-kDa band by Western blotting techniques, specifically bound to the cell surface of DDT$_1$MF-2 cells, and inhibited [^{3}H]pyrilamine binding to these cells in a dose-dependent manner. These data strongly suggest that the purified 38–40-kDa protein is part of an antagonist binding domain on the histamine H_1 receptor on DDT$_1$MF-2 cells.

Histamine stimulates a wide variety of activities in peripheral tissues and also appears to serve as a neurotransmitter in the brain (1, 2). The action of histamine is believed to be mediated by specific cell surface receptors, which have been pharmacologically classified into three distinct groups using highly specific chemical competitive agonists and antagonists (3, 4). Although histamine receptors in the brain and peripheral tissues have been extensively studied by many laboratories, the molecular mechanisms of histamine receptor-mediated reactions are not fully understood, mainly because none of these histamine receptors has been fully characterized from the biochemical point of view.

The analysis of histamine receptors in whole tissues is difficult to interpret because of the heterologous population of cell types and the possible heterogeneity of histamine receptors (5). The advantage of using an established cell line is that a homogeneous cell population can be studied directly. In our previous studies (6, 7), the histamine H_1-specific receptor has been identified on a cloned smooth muscle cell line, DDT$_1$MF-2 (8), using a radiolabeled ligand binding assay with the H_1-specific antagonist [^{3}H]pyrilamine. Furthermore, histamine binding results in distinct functional responses, such as increased cytosolic Ca^{2+} followed by actin polymerization (6). Although [^{3}H]pyrilamine binding indicated that the affinity of pyrilamine (K_d = 219 nM) on DDT$_1$-MF-2 cells is relatively lower than that of other tissues/cells (5), DDT$_1$MF-2 cells express a large number of binding sites (9.7×10^6 binding sites/cell). Therefore, the present study was designed to purify the H_1 receptor protein(s) from DDT$_1$MF-2 cells.

Experimental Procedures

Materials

DDT$_1$MF-2 cells (American Type Culture Collection, Rockville, MD), cell culture media (Cell Culture Facility, University of California, San Francisco), [^{3}H]pyrilamine (26 Ci/mmol) and ^{125}I-protein A (35 mCi/mg) (Amersham, Arlington Heights, IL), the materials for chromatofocusing and gel filtration (Pharmacia, Sweden), reagents for polyacrylamide gel electrophoresis (Bio-Rad, Richmond, CA), and HPLC grade solutions (EM Science, Cherry Hill, NJ) were obtained from the designated suppliers. The stereoisomers of chlorpheniramine (*d*- and *l*-type) were kindly provided by Dr. W. Kreutner (Schering Corp., Bloomfield, NJ). The H_3-specific antagonist thioperamide was a kind gift from Dr. Arrang (INSERM, Paris). Other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

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

ABBREVIATIONS: HPLC, high pressure liquid chromatography; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; HBSS, Hank's balanced salt solution; NC, nitrocellulose; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; PEG, polyethylene glycol; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid.

Cell Culture

DDT$_1$MF-2 cells were grown in Dulbecco's modified Eagle's medium with 1.0 g of glucose/liter that contained 100 units/ml penicillin, 100 μg/ml streptomycin, and 5% fetal calf serum, at 37° in 5% CO_2/95% air, as previously described (6, 9). Cells were removed from flasks by vigorous shaking and were subcultured twice weekly at a ratio of 1:20. Cell viability was always more than 90%, as assessed by the exclusion of trypan blue.

Solubilization

DDT$_1$MF-2 cells were suspended, at a concentration of 2.0–3.8 × 10^8/ml, in HBSS and were mixed with 0.1–2.0% digitonin, Triton X-100, CHAPS, and *n*-octyl-β-D-glucopyranoside in the presence of 1 mM PMSF. Solubilization was carried out by gentle stirring of the detergent-protein mixture at 4° for 60 min and by vigorous mixing with a syringe for 5 min at 4°, following by centrifugation at 100,000 × *g* for 60 min at 4° (L8-m; Beckman, Palo Alto, CA) as previously described (10). The supernatants were then used immediately to assess [^{3}H] pyrilamine binding. Protein concentration was determined by a fluorimetric method (11).

C_4 Mini-column Procedure for Protein Concentration

WP′ Butyl (C_4) mini-columns (J. T. Baker, Phillipsburg, NJ) were washed with 10 ml of 100% methanol and equilibrated with 20 ml of 0.1% TFA. Supernatants from 100,000 × *g* centrifugation of digitonin cell lysates, the fractions from chromatofocusing, and the eluted proteins from polyacrylamide gels were diluted 10-fold with 0.1% TFA and applied to the columns. Unbound materials were washed out with 0.1% TFA. Bound materials were eluted with 1.0–1.5 ml of 90% acetonitrile that contained 0.1% TFA and were concentrated under vacuum rotation (Speed Vac; Savant, Hicksville, NY).

[^{3}H]Pyrilamine Binding Assays

Gel filtration. The pyrilamine-binding protein was first detected by gel filtration using a procedure similar to that employed for the study of soluble insulin (12) and substance P-receptor complexes (10). One-half milligram of 100,000 × *g* supernatants of digitonin cell lysates was incubated with 2 μM [^{3}H]pyrilamine in the presence or absence of 10^{-4} M unlabeled pyrilamine in a final volume of 0.5 ml, at 4° for 60 min, then the mixture was applied to a column (1.1 × 60 cm) of Sephadex G-75 (Pharmacia), equilibrated, and run at 4° with PBS. The flow rate was 40 ml/hr, 60 drops in each tube were collected, 100 μl of each tube content were mixed with 3.5 ml of scintillation fluid (Ecoscint; National Diagnostics, Somerville, NJ), and the β-radioactivity was determined in a liquid scintillation counter (LS 5801; Beckman, Irvine, CA).

PEG precipitation. In order to analyze the binding characteristics of [^{3}H]pyrilamine, the soluble proteins were precipitated by PEG in the presence of γ-globulin, according to the method previously described for the soluble insulin receptor (12). In each experiment, materials were suspended in HBSS that contained 5 mM histidine and 1 mg of human γ-globulin and were incubated with 3 to 1000 nM [^{3}H] pyrilamine in the presence or absence of 10^{-4} M unlabeled pyrilamine, in a final volume of 0.2 ml, at 4° for a specified length of time. An equal volume of 20% PEG was added to each tube and mixed for 7 min at 4°. The amount of radioactivity present in the PEG precipitates was determined by filtering the content of each tube under reduced pressure through Whatman GF/F glass fiber filters. To decrease the nonspecific [^{3}H]pyrilamine binding component, the filters were pretreated with 5% aqueous polyethylenimine for more than 2 hr at room temperature, as previously described (6, 10). Each filter was washed six times with 2.0 ml of cold 10% PEG, and then the β-radioactivity was determined. 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.

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 and co-workers (13, 14). This method is based on the law of mass action 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, and a two-site model was accepted only when the fit of the data were significantly ($p < 0.05$) improved, compared with the fit with a one-site model. Testing for statistical differences 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}, affinity binding constant, and concentration for each class of receptor.

Purification of the Pyrilamine-Binding Protein

Gel filtration. Supernatants (90–100 mg) from the 100,000 × *g* centrifugation of digitonin-solubilized cell lysates were applied to a column (26 × 100 cm) of Bio-Gel A-5m (Bio Rad, Richmond, CA), which had been previously equilibrated and was eluted with PBS at 4°. The column was eluted at 20 ml/hr and 2.5-ml fractions were collected. This procedure was repeated six times using different batches of cell lysates (total, 588 mg of protein). One hundred microliters of each fraction were incubated with 200 nM [^{3}H]pyrilamine in the presence or absence of 10^{-4} M unlabeled pyrilamine, and the specific binding of each fraction was determined using PEG precipitation in duplicate, as described above. The protein concentration of each fraction was also measured by a fluorimetric method (11).

Chromatofocusing. The active fractions from the Bio-Gel A-5m (tubes 75–115; see Results) were pooled, concentrated by stirred ultrafiltration (Amicon, Danvers, MA) to 80 ml in volume, and dialyzed against 0.025 M 2-aminoethanol/acetic acid, pH 9.4 (starting buffer), and then 20 ml of the fraction were applied to a chromatofocusing column (1.1 × 60 cm, PBE 94; Pharmacia), as previously described by Sluyterman and co-workers (15, 16). Unbound materials were removed with 800 ml of starting buffer. The column was then eluted with a 1:10 dilution of Polybuffer 96/acetic acid, pH 5.5, at 20 ml/hr, and 2.5-ml fractions were collected. The column was regenerated with starting buffer and experiments were repeated four times. After the pH and absorbance at 280 nM of each fraction were determined, 1.0 ml of each fraction was concentrated using C_4 mini-columns as described above. The contents of each tube were resuspended in HBSS and were incubated with 200 nM [^{3}H]pyrilamine, and the specific binding was determined using PEG precipitation in duplicate, as described above. Protein concentration was also determined by a fluorimetric method (11).

Reverse phase HPLC. The pH 6.8–7.2 fractions, which demonstrated the highest amount of the [^{3}H]pyrilamine binding activity (see Results), were pooled, diluted 10-fold with 0.1% TFA/5% acetonitrile (starting buffer), and then injected onto an HPLC column (4.6 × 250 mm) of Vydac Protein C_4, which was previously equilibrated with more than 20 column volumes of starting buffer. Chromatography was performed using a Beckman HPLC system (Model 450 data system/controller, Model 114M solvent delivery module, Model 165 variable wavelength detector; Beckman, Berkeley, CA). After UV absorbance at both 220 and 280 nm had returned to base line, the column was eluted with a 5–85% acetonitrile gradient, containing 0.1% TFA, over 80 min at a rate of 1 ml/min and fractions of 1 ml/tube were collected. The column was regenerated with more than 20 column volumes of starting buffer and experiments were repeated three times. In order to identify the peak that contained the specific [^{3}H]pyrilamine binding activity, every 10 tubes in the first experiment were pooled and concentrated under vacuum rotation. Each tube was then resuspended in HBSS and mixed with 200 nM [^{3}H]pyrilamine and the specific binding was determined using PEG precipitation in triplicate, as described above. The positive fractions (55–65% acetonitrile; see Results) were then further purified with an analytical narrow bore C_8 HPLC column (2.1 × 100 mm) of Brownlee Aquapore RP-300, which was previously equilibrated with 0.1% TFA/40% acetonitrile. The gradient was 40–75% acetonitrile

over 100 min in 0.1% TFA, at a rate of 0.2 ml/min. The eluted fractions were concentrated under vacuum rotation, resuspended in HBSS, and incubated with 200 nM [^{3}H]pyrilamine to detemine the degree of the specific binding in triplicate, as described above.

Analysis of the Purified Pyrilamine-Binding Protein

SDS-PAGE. The single active peak on C_8 HPLC (see Results) was dried using a Speed Vac concentrator and was suspended in 0.625 M Tris·HCl, pH 6.8, that contained 2% SDS, 15% glycerol, 0.0025% bromophenol blue, and 2.5% 2-mercaptoethanol (sample buffer). Electrophoresis was performed according to the method of Laemmli (17). The stacking gel was 5% acrylamide, 0.125 M Tris·HCl, pH 6.8, and 0.1% SDS. The separation gel was 10% acrylamide, 0.375 M Tris·HCl, pH 8.8, and 0.1% SDS. The electrophoresis was carried out at 100 mA constant current until the tracking dye was 0.5 cm from the bottom of the gel. Gels were stained with a silver stain reagent (Bio-Rad) and analyzed by a laser scanning densitometer (Biomed Instruments, Inc., Fullerton, CA). Molecular weight standards (Bio-Rad) were also electrophoresed on each gel for comparison purpose.

Elution of the pyrilamine-binding protein from polyacrylamide gels. After SDS-PAGE was completed, the 38–40 kDa band was cut out 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 gels, as previously described for prostaglandin E_1/prostacyclin receptors (18) and the H_1 receptor protein on BC3H1 cells (19). Homogenates were centrifuged at 3000 × *g* at 4° for 10 min, and supernatants were applied to the mini-columns of WP'Butyl (C_4), as described above. The eluted proteins were then assessed for saturation [^{3}H]pyrilamine binding, using PEG precipitation. Part of the eluted protein was also analyzed on SDS-PAGE and stained with silver-staining reagents to determine the protein concentration.

Polyclonal Antibody Against the Pyrilamine-Binding Protein

Preparation of polyclonal antibody against the pyrilamine-binding protein. The dried 38–40-kDa band from the final silver-stained gel (approximately 100 ng) was cut from the gel and implanted subcutaneously in a rabbit in the presence of Freund's adjuvant. This same procedure was repeated every 2 weeks in order to boost the antibody titers, and blood was withdrawn 10 days after each boost.

Inhibition of [^{3}H]pyrilamine binding. DDT_1MF-2 cells (1–2 × 10^5) were suspended in HBSS and incubated with 100 nM [^{3}H]pyrilamine in the presence or absence of various concentrations of control and immune sera at 4° for 1 hr. Cells were then harvested on Whatman GF/C glass fiber filters and washed with 2 ml of PBS six times under reduced pressure, and the bound radioactivity was measured as previously described (6, 7).

Recognition of cell surface molecules by the polyclonal antibody. DDT_1MF-2 cells (1 × 10^6) were suspended in HBSS and incubated with various concentrations of control and the pyrilamine-binding protein antisera at 4° for 1 hr. Cells were washed with PBS to remove unbound serum components and then incubated with fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobulin G (1:100 dilution) (Cappel, Cochranville, PA) at 4° for 1 hr. Cells were washed with PBS, suspended in fresh HBSS, and analyzed in a fluorescence-activated cell sorter (Becton Dickinson, Sunnyvale, CA) at 488 nm excitation and 522 nm emission, as previously described (20).

Detection of proteins by Western blot. DDT_1MF-2 cells (1 × 10^8) were suspended in sample buffer for SDS-PAGE analysis, in the presence of 2-mercaptoethanol, and were separated on a 5% polyacrylamide stacking gel and 10% polyacrylamide separation gel, as described above. After electrophoresis was completed, the gel was soaked in 20 mM Tris containing 150 mM glycine and 20% methanol (blotting buffer) and was transblotted onto NC paper, as previously described (20). The NC paper was then incubated with various concentations of control and specific antisera at room temperature for 2 hr, in a solution of 5% nonfat milk, 0.2% Nonidet P-40, and 2 mM $CaCl_2$ (reaction buffer), to decrease the level of the nonspecific binding. The NC paper was then washed 10 times to remove unbound antisera and incubated with 5,000,000 cpm of ^{125}I-protein A in reaction buffer at room temperature for 30 min. The NC paper was then washed 10 times with reaction buffer and dried into filter paper. Autoradiography was conducted at −70° with Kodak XAR-5 film and a DuPont Cronex intensifying screen overnight.

RESULTS

Solubilization of the Pyrilamine-Binding Protein

Because the optimal detergents to solubilize membrane-bound proteins are different in each experimental system, we first tested the efficiency of four different detergents to solubilize the pyrilamine-binding protein from DDT_1MF-2 cells. Sixty to 90% of cellular proteins were solubilized by a 1% solution of CHAPS, digitonin, and Triton X-100, and 40–60% of cellular proteins were solubilized by 1% *n*-octyl-β-D-glucopyranoside, as assessed by a fluorimetric method (11) (data not shown). However, the [^{3}H]pyrilamine binding activity was not detected in CHAPS-, Triton X-100-, and glucopyranoside-solubilized proteins (Fig. 1). The [^{3}H]pyrilamine binding activity was only solubilized by 0.75–2.0% digitonin.

[^{3}H]Pyrilamine Binding Assays

Gel filtration. Pyrilamine binding to soluble proteins was first assessed by gel filtration (Fig. 2). As a result, two peaks of radioactivity were detected, the high molecular weight fraction in the void volume (tubes 14–20) and free [^{3}H]pyrilamine (tubes 42–70). [^{3}H]Pyrilamine alone had only one peak around tube 50 (data not shown). The [^{3}H]pyrilamine peak that appeared in the void volume was significantly reduced if digitonin-solubilized cell lysates were preincubated with 10^{-4} M unlabeled pyrilamine for 1 hr at 4° before the addition of [^{3}H]pyrilamine (Fig. 2).

PEG precipitation. In order to compare the binding characteristics of 100,000 × *g* supernatants of digitonin-solubilized cell lysates and the eluted proteins from C_4 mini-columns with

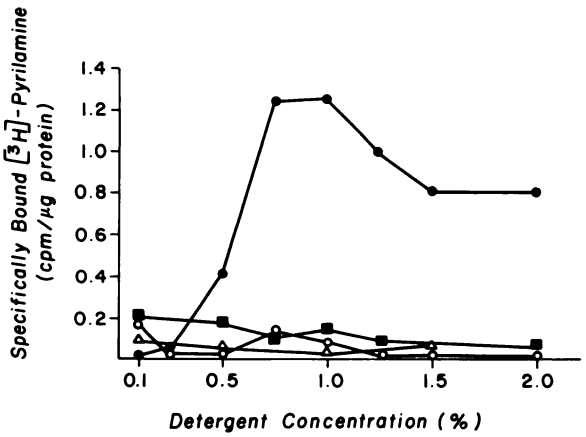

Fig. 1. Solubilization of the [^{3}H]pyrilamine binding activity from DDT_1MF-2 cells. DDT_1MF-2 cells were suspended in HBSS and incubated with various concentrations of digitonin (●), Triton X-100 (△), CHAPS (○), or *n*-octyl-β-D-glucopyranoside (■), in the presence of 1 mM PMSF at 4° for 1 hr. After solubilization, 100,000 × *g* supernatants of cell lysates were then assessed for [^{3}H]pyrilamine binding using PEG precipitation and for protein concentration, as described under Experimental Procedures. Each data point is the mean of duplicate determinations from a single experiment.

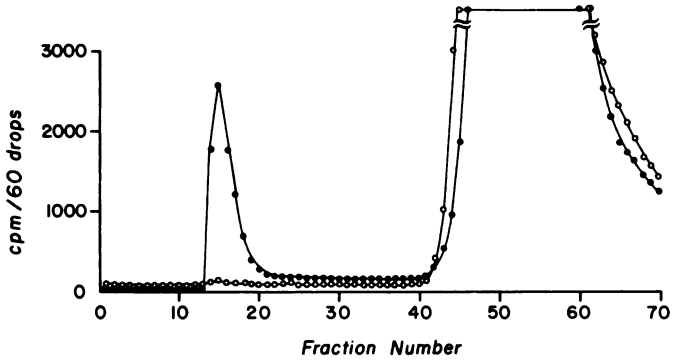

Fig. 2. Gel filtration patterns demonstrating the specific binding between [^{3}H]pyrilamine and digitonin-solubilized cell lysates. Supernatants (100,000 × *g*) of digitonin-solubilized cell lysates were incubated with 2 μM [^{3}H]pyrilamine in the presence (○) or absence (●) of 10^{-4} M unlabeled pyrilamine at 4° for 1 hr. The mixtures were then applied to a column (1.1 × 60 cm) of Sephadex G-75, equilibrated, and run at 4° with PBS. The flow rate was 40 ml/hr; 60 drops (1.0 ml) in each tube were collected, and the β-radioactivity of 100 μl of each tube content was determined.

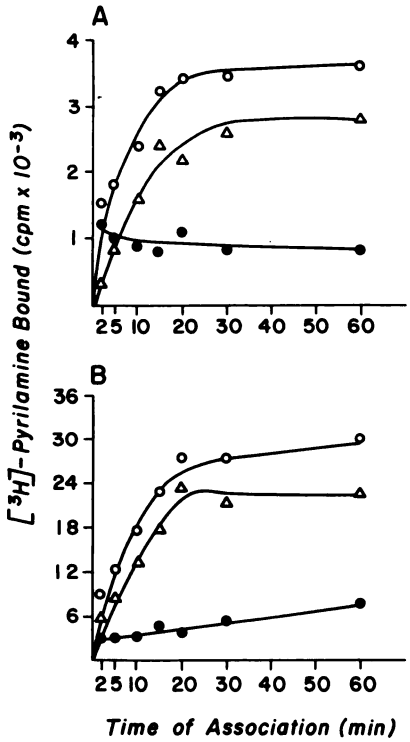

Fig. 3. Time course of [^{3}H]pyrilamine binding. Protein (2.8 mg) from digitonin-solubilized cell lysates (A) and 0.1 mg of protein from C_4 columns (B) were suspended in HBSS that contained 5 mM histidine and 1 mg of γ-globulin and were incubated with 100 nM [^{3}H]pyrilamine, in the presence (●) or absence (○) of 10^{-4} M unlabeled pyrilamine, at 4° for 2–60 min. The binding of [^{3}H]pyrilamine was assessed by PEG precipitation, as described in Experimental Procedures. Each data point was the mean of duplicate determinations from two separate experiments. △, Specific binding.

those of intact cells, the soluble materials were co-precipitated by PEG in the presence of γ-globulin. Without γ-globulin, the specific [^{3}H]pyrilamine binding was not detected. The specific binding of [^{3}H]pyrilamine to both cell lysates (Fig. 3A) and the eluted protein from C_4 mini-columns (Fig. 3B) increased rapidly and reached a steady state plateau after 15–20 min. Furthermore, the specific binding of [^{3}H]pyrilamine was reversibly dissociated by addition of unlabeled pyrilamine (Fig. 4). The specific binding of [^{3}H]pyrilamine also increased in proportion with the concentration of soluble proteins (data not shown).

[^{3}H]Pyrilamine binding at equilibrium, under saturation conditions, to both cell lysates and the eluted proteins from C_4 mini-columns occurred at concentrations of 300 nM [^{3}H]pyrilamine or higher (Fig. 5). The computer program Scatfit indicates a single class of binding sites for both cell lysates and the eluted proteins from C_4 mini-columns and gives a mean (±SE) K_d and density of binding sites of 172 ± 81 nM and 0.3 ± 0.03 pmol/mg of protein for cell lysates and 173 ± 47 nM and 29.9 ± 1.7 pmol/mg of protein for C_4 mini-columns, respectively (Table 1).

To evaluate the structural specificity of [^{3}H]pyrilamine binding, various ligands were assessed for their capacity to inhibit the specific binding of [^{3}H]pyrilamine to intact cells, cell lysates, or the eluted proteins from C_4 mini-columns (Table 2). The specific [^{3}H]pyrilamine binding was inhibited in a concentration-dependent manner by various H_1 receptor antagonists, whereas the H_2 and H_3 antagonists and ligands for other receptors were poorly effective. The stereoisomer *d*-chlorpheniramine was more potent than the *l*-form.

Purification of the Pyrilamine-Binding Protein

Gel filtration. An initial attempt to separate the pyrilamine-binding protein from other proteins in digitonin-solubilized cell lysates and to determine an approximate size of the protein was carried out by subjecting the 100,000 × *g* superna-

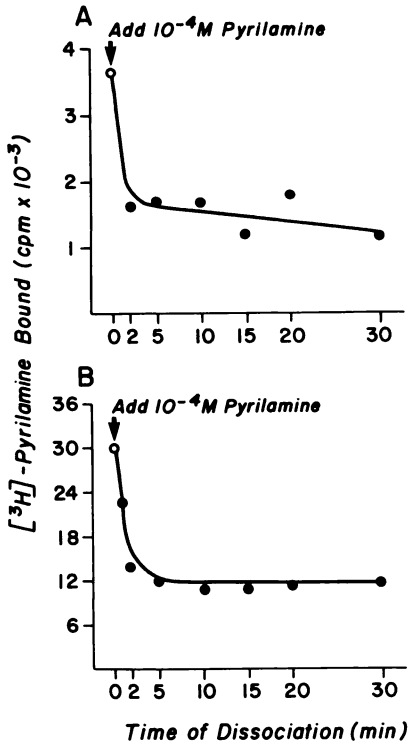

Fig. 4. Dissociation of [^{3}H]pyrilamine binding. Cell lysate (A) and the eluted proteins from C_4 mini-columns (B) were previously incubated with 100 nM [^{3}H]pyrilamine in the absence of unlabeled pyrilamine at 4° for 60 min (Fig. 3). At the end of incubation, 10^{-4} M unlabeled pyrilamine was added to each tube and incubation was continued at 4° for 1–30 min. The binding of [^{3}H]pyrilamine was assessed by PEG precipitation, as described in Experimental Procedures. Each data point was the mean of duplicate determinations from two separate experiments.

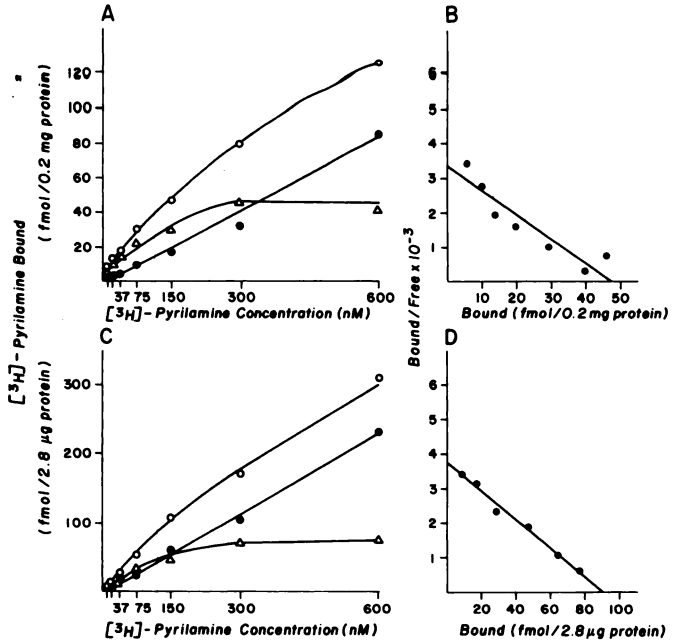

Fig. 5. Saturation [^{3}H]pyrilamine binding. Protein (0.2 mg) of digitonin-solubilized cell lysates (A and B) and 2.8 µg of the eluted protein from C_4 mini-columns (C and D) were incubated with 3–600 nM [^{3}H]pyrilamine in the presence (●) or absence (○) of 10^{-4} M unlabeled pyrilamine at 4° for 1 hr. The binding of [^{3}H]pyrilamine was assessed by PEG precipitation as described in Experimental Procedures. △, Specific binding. A and C, saturation curves; B and D, Scatchard plots of the same data. Each data point was the mean of duplicate determinations from a single representative experiment. Saturation [^{3}H]pyrilamine binding was repeated 3–5 times and the values of the K_d and the number of binding sites were summarized in Table 1.

TABLE 1

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

The data of [^{3}H]pyrilamine binding to intact cells have been reported previously (6). Methods of saturation binding to sonicated membranes were the same as those for intact cells. Saturation binding of digitonin-solubilized cell lysates and the eluted proteins from C_4 mini-columns was carried out using PEG precipitation, as described in Experimental Procedures. The K_d and the number of binding sites were calculated using the computer program Scatfit (13) (14). Each data point is the mean ± standard error of duplicate determinations from one to nine separate experiments.

	No. of experiments	K_d	Binding sites
	nM	pmol/mg of protein	
Intact cells	9	219 ± 40	16.2 ± 3.2[a]
Sonicated membranes	3	161 ± 50	1.7 ± 0.3
Digitonin-solubilized cell lysates	3	172 ± 81	0.3 ± 0.03
Eluted proteins from C_4 mini-columns	5	173 ± 47	29.9 ± 1.7
Purified 38–40-KDa protein	1	288	31,300

[a] pmol/10^6 cells.

TABLE 2

Specificity of [^{3}H]pyrilamine binding

[^{3}H]Pyrilamine (200–300 nM) was incubated with 1–2 × 10^6 cells, 0.8 mg of digitonin-solubilized cell lysates, or 10 µg of eluted proteins from C_4 mini-columns, in the presence of various concentrations of ligands, to evaluate the structural specificity. [^{3}H]Pyrilamine binding was performed using PEG precipitation as described in Experimental Procedures. The IC_{50} was calculated using the computer program Scatfit. Each data point is the mean of duplicate determinations from two separate experiments.

Ligands	IC_{50}		
	Cells	Cell lysates	C_4 columns
		µM	
Histamine	316	170	269
H_1 antagonists			
Pyrilamine	2.0	1.0	0.9
Diphenhydramine	3.6	1.9	2.5
Triplolidine	3.8		
Doxepine	0.9		
d-Chlorpheniramine	1.4		0.4
l-Chlorpheniramine	10.5		5.4
H_2 antagonist, cimetidine	>1000	>1000	>1000
H_3 antagonist thioperamide	90		>100
Others			
Acetylcholine	>1000	>1000	>1000
Epinephrine	>1000	>1000	>1000
Isoproterenol	>1000	>1000	>1000
Serotonin	>1000	>1000	>1000
γ-Aminobutyric acid	>1000	>1000	>1000
Histidine	>1000	>1000	>1000

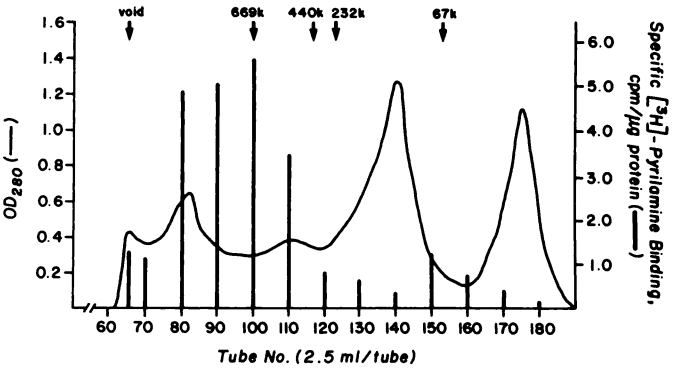

Fig. 6. Gel filtration of the solubilized [^{3}H]pyrilamine binding activity. Supernatants (100,000 × *g* of digitonin-solubilized cell lysates (approximately 100 mg of protein) were applied to a column (26 × 100 cm) of Bio-Gel A-5m, which had been previously equilibrated and was eluted with PBS at 4°. The flow rate was 20 ml/hr and 2.5-ml fractions were collected. Gel filtration was repeated six times using separate preparations of cell lysates. The absorbance at 280 nm (——) is a representative recording, and the *bars* indicate the mean of the specific [^{3}H]pyrilamine binding (cpm/µg of protein) from three separate experiments, as described in Experimental Procedures. Molecular mass standards were blue dextran 2000 (void volume), thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), and bovine serum albumin (67 kDa).

tants of digitonin-solubilized cell lysates to gel filtration, as previously described for the brain membrane histamine H_1 receptor (21). The peak representing the specific [^{3}H]pyrilamine binding activity eluted from the column over a broad range with an apparent molecular weight of 670,000 (Fig. 6). Three separate experiments indicated that the specific binding of tubes 80–110 (3.6–5.7 cpm/µg of protein) was significantly higher than that of other tubes.

Chromatofocusing. The active fractions from the gel filtration (tubes 75–115; Fig. 6) from six separate experiments were pooled, concentrated, dialyzed, and applied to a chromatofocusing column, as described in Experimental Procedures. The [^{3}H]pyrilamine binding activity was eluted at pH 7.0 ± 0.07 (mean ± SE; four experiments) (Fig. 7). The specific binding of tube 70 (480 cpm/µg of protein) was significantly higher than that of other tubes.

Reverse phase HPLC. The active fractions identified by chromatofocusing (tubes 66–78; Fig. 7) from four separate experiments were pooled and applied to a Vydac protein C_4 HPLC column, as described in Experimental Procedures. [^{3}H]

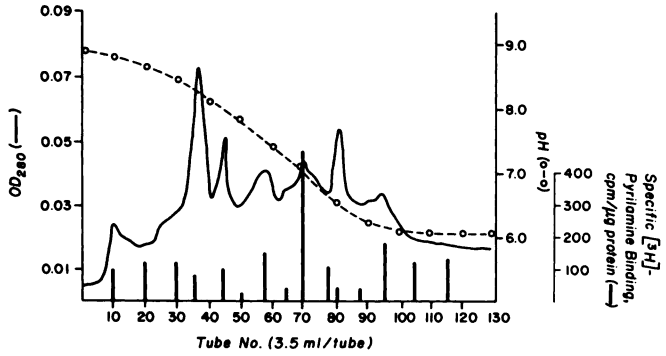

Fig. 7. Chromatofocusing of the pyrilamine binding activity. The active fractions of Bio-Gel A-5m (tubes 75–115; Fig. 6) were pooled, concentrated, dialyzed against 0.025 M 2-aminoethanol/acetic acid, pH 9.4, and then applied to a chromatofocusing column (1.1 × 60 cm). The column was eluted with a 1:10 dilution of Polybuffer 96/acetic acid, pH 5.5, at 20 ml/hr, and 2.5-ml fractions were collected. The absorbance at 280 nm (——) and pH (O) are representative recordings, and the *bars* indicate the mean of the specific [^{3}H]pyrilamine binding (cpm/μg of protein) from four different experiments, as described in Experimental Procedures.

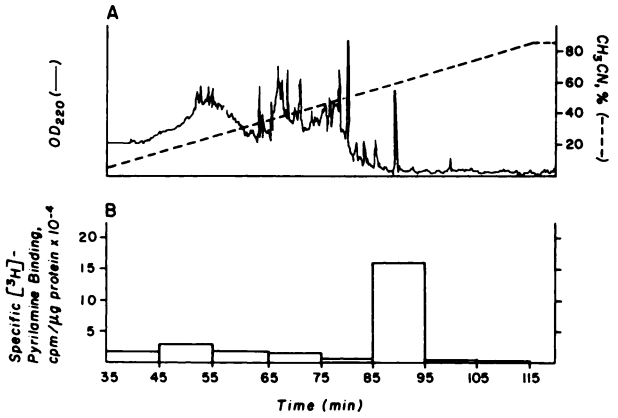

Fig. 8. C_4 reverse phase HPLC purification of the pyrilamine-binding protein. The pH 6.8–7.2 fractions from chromatofocusing (Fig. 7) were pooled, diluted 10-fold with 0.1% TFA, and then injected onto a HPLC column (4.6 × 250 mm) of Vydac protein C_4. A, The column was eluted with a 5–85% acetonitrile gradient, containing 0.1% TFA, over 80 min at a rate of 1 ml/min. The absorbance at 220 nm (——) and acetonitrile gradient (– – –) are represenative recordings from one of three separate experiments. B, For the identification of the peak of interest, every 10 tubes were mixed and assessed for [^{3}H]pyrilamine binding in triplicate, using PEG precipitation, as described in Experimental Procedures.

Pyrilamine binding of the collected fractions indicated that the binding activity was eluted with 55–65% acetonitrile (Fig. 8). This fraction was then further purified in an analytical narrow bore C_8 HPLC column of Brownlee Aquapore RP-300, as described in Experimental Procedures. As a result, the binding activity eluted at 55–60% acetonitrile in a single peak on C_8 reverse phase HPLC (Fig. 9).

Analyses of the Purified Pyrilamine-Binding Protein

SDS-PAGE. The single active peak on C_8 HPLC (Fig. 9) was then analyzed by SDS-PAGE and stained with silver stain reagents (Fig. 10). Densitometry indicated that the purity of this peak was not 100%, but the molecular weight of the most distinct band, which represented the greatest proportion of the total protein, was approximately 38–40 kDa.

Saturation binding of [^{3}H]pyrilamine to the eluted 38–40-kDa protein. In order to analyze whether the 38–40-kDa protein exhibited pyrilamine binding activity with characteristics similar to those of intact cells, the protein was eluted from polyacrylamide gels as described in Experimental Procedures and was assessed for [^{3}H]pyrilamine binding. The protein concentration in this band was estimated by comparing the densitometric tracings with a protein standard (bovine serum albumin) on a second SDS-PAGE. The computer program Scatfit indicated a single class of binding sites with a K_d of 288 nM, which is a value similar to that of intact cells (Table 1). The calculated number of binding sites was 31.3 pmol/μg of protein, which indicates an approximate 100,000-fold purification from the original cell lysate (Table 1). Moreover, the calculated receptor density suggests that one molecule of [^{3}H] pyrilamine binds to one molecule of the 38–40-kDa protein (Table 1).

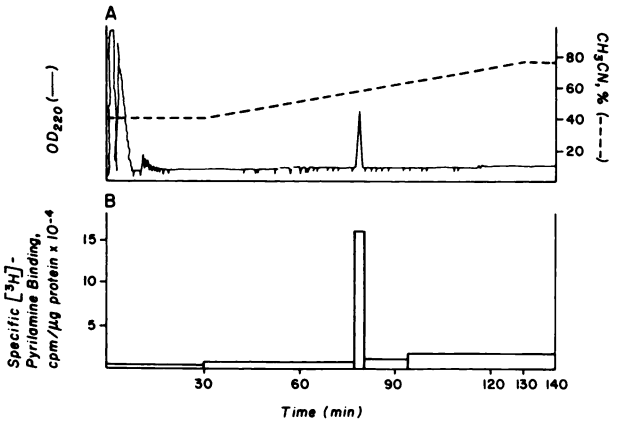

Fig. 9. C_8 reverse phase HPLC purification of the pyrilamine-binding protein. The 85–95-min fraction from C_4 reverse phase HPLC (Fig. 8) was then further purified in an analytical narrow bore C_8 HPLC column (2.1 × 100 mm) of Brownlee Aquapore RP-300. A, The gradient was 40–75% acetonitrile, over 100 min, in 0.1% TFA, at a flow rate of 0.2 ml/min (– – –). The absorbance at 220 nm was also monitored (——) and 0.2 ml/tube was collected. B, The eluted fractions were also assessed for [^{3}H]pyrilamine binding using PEG precipitation in triplicate, as described in Experimental Procedures. Protein concentration was estimated by comparing with a standard protein on SDS-PAGE.

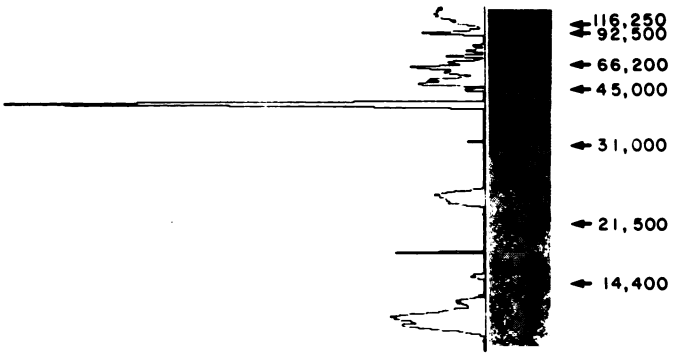

Fig. 10. SDS-PAGE assessment of the purified pyrilamine-binding protein. The single peak on C_8 HPLC (Fig. 9) was dried using a Speed Vac concentrator, suspended in sample buffer, and separated on SDS-PAGE as described, in Experimental Procedures. The gel was stained with silver stain reagents and analyzed with a densitometer (*left*). Molecular weight standards were also electrophoresed for comparison (*right*).

Immunological analysis of the pyrilamine-binding protein. In order to further identify whether the 38–40-kDa protein band was the pyrilamine-binding protein, a rabbit was immunized with this protein and the resulting polyclonal anti-

sera were tested. The immune serum 10 days after the second boost (second bleed) significantly inhibited [^{3}H]pyrilamine binding to DDT$_1$MF-2 cells in a dose-dependent manner, whereas pre-bleed antiserum and first-bleed antiserum failed to inhibit [^{3}H]pyrilamine binding (Fig. 11).

We have also tested whether these antisera recognize the cell surface of intact DDT$_1$MF-2 cells, using a fluorescence-activated cell sorter as described in Experimental Procedures. As shown in Fig. 12, the fluorescent intensity of labeled DDT$_1$MF-2 cells with antiserum from the second bleed is significantly increased, compared with pre-bleed labeled cells. Labeling with antiserum from the second bleed was dose dependent (data not shown).

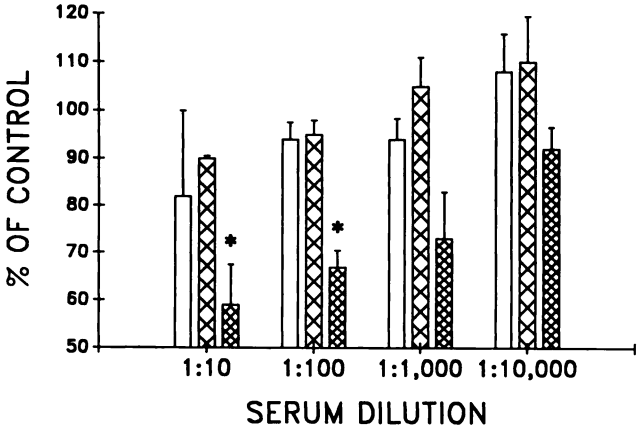

Fig. 11. Polyclonal antibody inhibition of [^{3}H]pyrilamine binding. DDT$_1$MF-2 cells (1–2 × 10^5) were suspended in HBSS and incubated with 100 nM [^{3}H]pyrilamine, in the presence of various concentrations of immune sera, at 4° for 1 h. Cells were then harvested on Whatman GF/C glass fiber filters and washed with 2 ml of PBS six times under reduced pressure, and then the β-radioactivity of each filter was determined as described in Experimental Procedures. □, ⊠, and ■ indicate the data for the pre-bleed, first bleed, and second bleed antiserum, respectively.

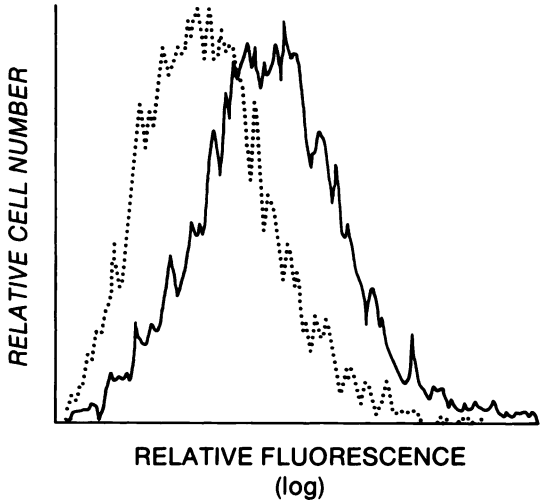

Fig. 12. Polyclonal antibody recognition of cell surface molecules. DDT$_1$MF-2 cells (1 × 10^6) were suspended in HBSS and incubated with a 1:100 dilution of the pre-bleed (· · · ·) or the second bleed (——) antiserum at 4° for 1 hr. Cells were washed with PBS to remove unbound serum components and then incubated with fluoroscein isothiocyanate-labeled goat anti-rabbit immunoglobulin G (1:100 dilution) at 4° for 1 hr. Cells were washed with PBS, suspended in fresh HBSS, and analyzed in a fluorescence-activated cell sorter at 488 nm excitation and 522 nm emission, as described in Experimental Procedures.

In order to analyze further whether the polyclonal antibody against the purified pyrilamine-binding protein recognizes the 38–40-kDa protein on intact DDT$_1$MF-2 cells, total cellular proteins were separated on SDS-PAGE, and a Western blot was performed, as described in Experimental Procedures. As a result, antiserum from the second bleed specifically recognized a 57-kDa and the 38–40-kDa protein (Fig. 13).

Discussion

In the present study, the pyrilamine-binding protein from the smooth muscle cell line DDT$_1$MF-2 was solubilized with digitonin, with the resultant preservation of [^{3}H]pyrilamine binding characteristics, and was purified with gel filtration and chromatofocusing, followed by reverse phase HPLC.

DDT$_1$MF-2 cell membranes were first prepared by three

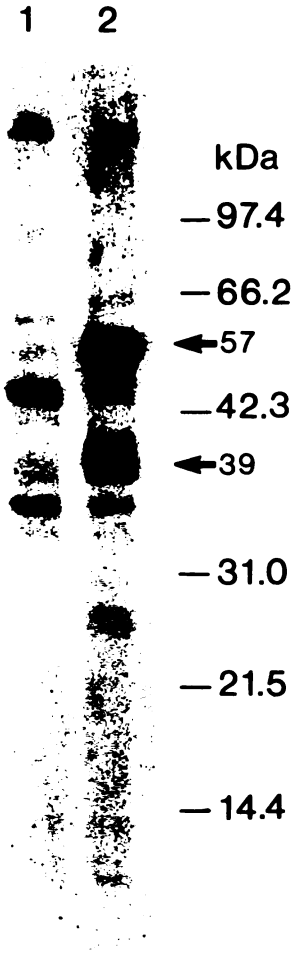

Fig. 13. Western blot analysis of polyclonal antibody. DDT$_1$MF-2 cells (1 × 10^8) were suspended in sample buffer for SDS-PAGE, in the presence of 2-mercaptoethanol, and were separated on a 5% polyacrylamide stacking gel and 10% polyacrylamide separation gel. After electrophoresis was completed, the gel was transblotted onto NC paper. The NC paper was incubated with a 1:100 dilution of control serum (pre-bleed; *lane 1*) or specific antiserum (second bleed; *lane 2*) at room temperature for 2 hr, washed extensively to remove unbound antisera, and then incubated with 5,000,000 cpm of ^{125}I-protein A at room temperature for 30 min. The NC paper was then washed extensively and dried onto filter paper. Autoradiography was conducted at −70° with Kodak XAR-5 film and a DuPont Cronex intensifying screen overnight. Molecular weight standards were also electrophoresed for comparison. The *small arrows* indicate 57- and 39-kDa proteins specifically recognized by the antiserum directed against the purified pyrilamine-binding protein.

different methods, sonication (Sonifier, Branson, Danbury, CT), cell disruption (Polytron, Kinematics, Switzerland), or nitrogen cavitation, as previously described for the substance P receptor (10). The [^{3}H]pyrilamine binding activity was present only in sonicated membranes, with a similar K_d to that described for intact cells (Table 1). However, the recovery of the [^{3}H]pyrilamine binding activity in membranes was approximately 1% of that seen in cells (data not shown). Therefore, to avoid the loss of the pyrilamine binding activity during membrane preparation, cells were directly solubilized. These results demonstrate that the [^{3}H]pyrilamine binding activity was solubilized only by digitonin, but not by CHAPS, Triton X-100, or glucopyranoside (Fig. 1). However, digitonin could solubilize only approximately 20% of the total [^{3}H]pyrilamine binding activity from intact cells (data not shown). This low recovery of the pyrilamine binding activity has also been reported for the H_1receptor in rat brain membranes (21). The computer analysis of [^{3}H]pyrilamine binding indicated the presence of a single binding site for pyrilamine on both solubilized proteins and intact cells, with similar binding characteristics (Figs. 2–5; Table 1). Furthermore, both intact cells and solubilized cells demonstrated similar H_1 receptor specificity (Table 3). Therefore, digitonin-solubilized cell lysates contain at least part of the pyrilamine-binding protein, although the solubilization procedure is not efficient.

Before receptor purification, various methods of protein concentration without loss of the binding activity were evaluated. The relative recovery of the binding activity was C_4 mini-column > freeze-dry > ultrafiltration (Amicon) ≫ methanol precipitation (data not shown). C_4 mini-columns are theoretically the same as reverse phase HPLC, but more rapid, and, therefore, it is possible to analyze many samples simultaneously using disposable mini-columns. Furthermore, because TFA and acetonitrile are easily removed by Speed Vac concentration, the [^{3}H]pyrilamine binding assay could be performed by resuspending the concentrated samples in HBSS. Moreover, the pyrilamine binding activity was preserved after elution from C_4 mini-columns with binding characteristics similar to those of digitonin-solubilized cell lysates (Figs. 3B, 4B, and 5C, and 5D; Tables 1 and 2) and to those of intact cells (6).

In the present study, the protein with the [^{3}H]pyrilamine binding activity was purified by gel filtration and chromatofocusing, followed by reverse phase HPLC. The final material was a single peak on reverse phase HPLC and the molecular weight was 38–40 kDa on SDS-PAGE. In order to identify whether the 38–40-kDa protein has the pyrilamine binding activity, the protein was eluted from the gel and [^{3}H]pyrilamine binding was assessed to estimate the K_d and the number of binding sites. The computer analysis Scatfit indicated a single class of binding sites, with a K_d of 288 nM which is equivalent to that of intact cells and digitonin-solubilized cell lysates (Table 1). Furthermore, the calculated number of binding sites indicated that one molecule of [^{3}H]pyrilamine bound to one molecule of the purified protein (Table 1). A detailed pharmacological binding analysis of the purified protein was not performed because of the difficulty in purifying a large quantity of this protein and the need to commit this material to sequencing efforts. However, we have successfully raised a rabbit polyclonal antibody against the 38–40-kDa protein by subcutaneous implantation of silver-stained SDS gel pieces containing the band of interest. Western blot analysis indicated that this polyclonal antibody recognized the protein from whole cell lysates (Fig. 13). Although this polyclonal antibody was prepared by immunization of the denatured proteins, antiserum specifically bound to the cell surface of DDT_1MF-2 cells (Fig. 12) and inhibited the specific pyrilamine binding (Fig. 11). These data strongly suggest that the 38–40-kDa protein is a minimum component of the antagonist binding domain of the histamine H_1 receptor.

The reported molecular size of membrane-bound H_1 receptors in both bovine and human cerebral cortex by target size analysis has demonstrated an approximate size of 160 kDa (22). However, in the present study, the molecular weight of the soluble pyrilamine-binding protein from DDT_1MF-2 cells was shown to be approximately 670 kDa by gel filtration. The difference in molecular weight is most likely due to the presence of a large quantity of digitonin within solubilized proteins forming micelles. Recently, Ruat *et al.* (23) have used irreversible photoaffinity labeling to specifically label H_1-binding proteins in guinea pig brain membranes. SDS-PAGE and autoradiography indicated that the molecular weight of the H_1 receptors was 350–400 kDa in the absence of 2-mercaptoethanol, and the molecular weight decreased to 56 and 47 kDa in the presence of 2-mercaptoethanol. These data suggest that the H_1 receptor protein may have multiple subunits, with one or more disulfide bridges, resulting in a higher molecular weight complex. Furthermore, in the presence of protease inhibitors, labeling of the 56-kDa peptide increased at the expense of the 47-kDa peptide. This also suggests that one of the subunits of the H_1 receptor proteins that includes an antagonist binding domain may be the 56-kDa peptide, with the 47-kDa peptide being a proteolytic product.

The molecular weight of the pyrilamine-binding protein from DDT_1MF-2 cells is 38–40 kDa, smaller than that of the 56-kDa H_1 receptors from guinea pig brain. However, this value is similar to that of the reported low affinity H_1 receptor on BC3H1 smooth muscle cells (19). Moreover, we have recently reported that the change in the molecular weight between the low and high affinity H_1 receptors on BC3H1 cells is mediated by receptor glycosylation (19) and, therefore, part of the difference in the reported molecular weights might be due to receptor glycosylation. In addition, according to our Western blot analysis (Fig. 13), the antiserum against the 38–40-kDa protein also recognizes a 57-kDa protein from whole DDT_1MF-2 cell lysates, a molecular weight similar to that of the brain histamine H_1 receptor (23). In order to further analyze the biochemical and pharmacological characteristics of the histamine H_1 receptor, it will be important to determine the amino acid sequence of this purified pyrilamine-binding protein, followed by the cloning of the gene encoding this protein.

Acknowledgments

The authors would like to thank Dr. J. P. McGillis for his helpful comments and suggestions and Dr. W. Kreutner (Schering Corp., Bloomfield, NJ) and Dr. Arrang (Centre Paul Broca de l'INSERM, Paris) for sending us the stereoisomers of chlorpheniramine and the H_3 antagonist.

References

1. Beaven, M. A. Histamine. *N. Engl. J. Med.* **294**:30–36 (1976).
2. Beaven, M. A. Histamine. *N. Engl. J. Med.* **294**:320–325 (1976).
3. Black, J. W., W. A. M. Duncan, C. J. Durant, C. R. Ganellin, and E. M. Parsons. Definition and antagonism of histamine H_2 receptors. *Nature (Lond.)* **236**:385–390 (1972).
4. Arrang, J. M., M. Garbarg, L. C. Lancelot, J. M. Lecomte, H. Pollard, M. Robba, W. Schunack, and J. C. Schwartz. Highly potent and selective ligands for histamine H_3-receptor. *Nature (Lond.)* **327**:117–123 (1987).
5. Mitsuhashi, M., and D. G. Payan. Molecular and cellular analysis of hista-

mine H_1 receptors on cultured smooth muscle cells. *J. Cell. Biochem.* **40**:1–10
6. Mitsuhashi, M., and D. G. Payan. Characterization of functional histamine H_1 receptors on a cultured smooth muscle cell line. *J. Cell. Physiol.* **134**:367–375 (1988).
7. Mitsuhashi, M., and D. G. Payan. Phorbol ester-mediated desensitization of histamine H_1 receptors on a cultured smooth muscle cell line. *Life Sci.* **43**:1433–1440 (1988).
8. Norris, J. S., J. Gorski, and P. O. Kohler. Androgen receptors in a syrian hamster ductus deferens tumor cell line. *Nature (Lond.)* **248**:422–424 (1974).
9. Mitsuhashi, M., and D. G. Payan. The mitogenic effects of vasoactive neuropeptides on a cultured smooth muscle cell lines. *Life Sci.* **40**:853–861 (1987).
10. Payan, D. G., J. P. McGillis, and M. L. Organist. Binding characteristics and affinity labeling of protein constituents of the human IM-9 lymphoblast receptor for substance P. *J. Biol. Chem.* **261**:14321–14329 (1986).
11. Udenfriend, S., S. Stein, P. Bohlen, W. Dairman, W. Leimgruber, and M. Weigele. Fluorescamine: a reagent for assay of amino acids, peptides, proteins, and primary amines in the picomole range. *Science (Wash. D. C.)* **178**:871–872 (1972).
12. Cuatrecasas, P., and G. P. E. Tell. Insulin-like activity of concanavalin A and wheat germ agglutinin: direct interactions with insulin receptors. *Proc. Natl. Acad. Sci. USA* **70**:485–489 (1973).
13. Burgisser, E., A. A. Hancock, R. J. Lefkowitz, and A. DeLean. Anomalous equilibrium binding properties of high-affinity racemic radioligands. *Mol. Pharmacol.* **19**:205–216 (1981).
14. DeLean, A., A. A. Hancock, and R. J. Lefkowitz. Validation and statistical analysis of a computer modeling method for quantitative analysis of radioligand binding data for mixtures of pharmacological receptor subtypes. *Mol. Pharmacol.* **21**:5–16 (1982).
15. Sluyterman, L. A. E., and O. Elgersma. Chromatofocusing: isoelectric focusing on ion exchange columns. I. General principles. *J. Chromatogr.* **150**:17–30 (1978).
16. Sluyterman, L. A. E., and J. Wijdenes. Chromatofocusing: isoelectric focusing on ion exchange columns. II. Experimental verification. *J. Chromatogr.* **150**:31–44 (1978).
17. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* **227**:680–685 (1970).
18. Dutta-Roy, A. K., and A. K. Sinha. Purification and properties of prostaglandin E_1/prostacyclin receptor of human blood platelets. *J. Biol. Chem.* **262**:12685–12691 (1987).
19. Mitsuhashi, M., and D. G. Payan. Receptor-glycosylation regulates the affinity of histamine H_1 receptors during smooth muscle cell differentiation. *Mol. Pharmacol.* **35**:311–318 (1989).
20. Organist, M. L., J. Harvey, J. P. McGillis, M. Mitsuhashi, P. Melera, and D. G. Payan. Characterization of a monoclonal antibody against the lymphoblast substance P receptors. *J. Immunol.* **139**:3050–3054 (1987).
21. Toll, L., and S. H. Snyder. Solubilization and characterization of histamine H_1 receptors in brain. *J. Biol. Chem.* **257**:13593–13601 (1982).
22. Kuno, T., N. Kubo, and C. Tanaka. Molecular size of histamine H-1 receptor determined by target size analysis. *Biochem. Biophys. Res. Commun.* **92**:639–644 (1985).
23. Ruat, M., M. Koerner, M. Garberg, C. Gros, J. C. Schwartz, W. Tertiuk, and C. R. Ganellin. Characterization of histamine H_1-receptor binding peptides in guinea pig brain using [^{125}I]iodoazidophenpyramine, an irreversible specific photoaffinity probe. *Proc. Natl. Acad. Sci. USA* **85**:2743–2747 (1988).

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