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ARTICLES

Total Cellular Activity and Distribution of a Subpopulation of Galactosyl Receptors in Isolated Rat Hepatocytes Are Differentially Affected by Microtubule Drugs, Monensin, Low Temperature, and Chloroquine

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Abstract We studied the effects of low temperature (20–37°C), monensin, chloroquine, and microtubule drugs on the cellular distribution and activity of galactosyl (Gal) receptors in isolated rat hepatocytes. After equilibration at 37°C, hepatocytes were incubated at 37°C, 31°C, 25°C, or 20°C or treated with or without inhibitors at 37°C in the absence of ligand. The cells were then assayed at 4°C for ^{125}I-asialo-orosomucoid binding, to measure receptor activity, or ^{125}I-anti-Gal receptor IgG binding, to measure receptor protein. Surface or total (surface and intracellular) Gal receptor activity and protein were measured on intact or digitonin-permeabilized cells, respectively. These inhibitors fell into two categories. Type I inhibitors (sub-37°C temperatures or colchicine) induced receptor redistribution but not inactivation. Treated cells lost up to 40% of surface Gal receptor activity and protein. Lost surface receptors were recovered intracellularly with no loss of receptor activity. Type II inhibitors (monensin or chloroquine) induced receptor inactivation but not redistribution. Treated cells lost 50–65% of their surface Gal receptor activity but only $\leq$15% of their surface receptor protein. These cells lost up to 60% of total cellular Gal receptor activity with no loss of total receptor protein. Of the total inactive Gal receptors, up to 50% and 75%, respectively, were present intracellularly in monensin- and chloroquine-treated cells. Loss of ligand binding to permeable treated cells was not due to changes in receptor affinity. A third category, Type III inhibitors (metabolic energy poisons that deplete ATP) induce both Gal receptor redistribution and inactivation (Biochemistry 27:2061, 1988). We conclude that only one of the two previously characterized subpopulations of Gal receptors on hepatocytes, termed State 2 receptors (J Biol Chem 265:629, 1990), recycles constitutively. The activity and distribution of State 2 but not State 1 Gal receptors are differentially affected by these specific drugs or treatments.

Key words: Gal receptors, colchicine, digitonin, asialoglycoprotein, receptor inactivation

Cells regulate the number of endocytic receptors on their surfaces by mechanisms that are incompletely understood. Many cells alter their surface receptor activity in response to extracellular stimuli. For instance, isolated rat hepatocytes and adipocytes incubated continuously with excess insulin reversibly lose a fraction of surface insulin receptor activity [1,2]. This ligand-dependent down-regulation, characteristic of class I receptors [3], is a physiological response to high, sustained levels of hormone. Class II receptors, exemplified by the Gal receptor of mammalian hepatocytes, can alter surface receptor activity in a ligand-independent manner. Different cell types treated in the absence of added ligand with metabolic energy poisons, ionophores, weak bases, phorbol esters, or microtubule disrupters lose 40–70% of their active surface low-density lipoprotein receptors [4], mannose-6-phosphate receptors [5], mannose receptors [6], α_2-macroglobulin receptors [7], transferrin receptors [8], or Gal receptors [9–15]. Most investigators have concluded that this lost surface receptor activity reflects the intracellu-

Abbreviations used: Gal, galactosyl; ASOR, asialo-orosomucoid; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; GalNAc, N-acetyl-*D*-galactosamine; HBSS, Hanks' buffered salt solution; IgG^R, affinity purified anti-Gal receptor goat IgG.

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lar entrapment of constitutively recycling receptors. In most instances, however, this interpretation is based on the assumption that cells treated with these perturbants do not alter the ligand-binding activity of the particular receptor under study.

Hepatocytes accumulate inactive Gal receptors after treatment with certain agents that interrupt receptor endocytosis and recycling during endocytosis. Azide and monensin, two commonly used inhibitors, induce hepatocytes to inactivate the ligand-binding activity of a subset of Gal receptors. Fiete et al. [15] demonstrated that surface Gal receptor activity on cultured rat hepatocytes treated with 100 μM monensin without ligand decreased by ≃50% with very little loss of surface receptor protein. Hepatocytes depleted of ATP by ≥90% with NaN_3 reversibly inactivate up to half of all cellular Gal receptors; most inactive receptors are located intracellularly [10,16]. Therefore, the loss of surface receptor activity induced by a given perturbant does not necessarily indicate a concomitant loss of surface receptor protein but rather may reflect receptor inactivation. Moreover, these results suggest that reversible inactivation of Gal receptors may represent an important regulatory mechanism for this receptor system.

Hepatocytes express two distinct subpopulations of Gal receptors, State 1 and State 2 receptors [17]. These two receptor populations mediate ligand uptake and processing and receptor recycling by two different pathways [18]. In a recent study [9], we showed that hepatocytes treated in the absence of ligand with either microtubule depolymerizing drugs, monensin, chloroquine, or metabolic energy poisons reduce the surface activity of only a subset of Gal receptors. Only State 2 Gal receptors are sensitive to these perturbants. Regardless of the inhibitor treatment, hepatocytes maintain active surface State 1 Gal receptors. In the present study we find that these agents elicited one of two effects on State 2 Gal receptor distribution and activity: 1) translocation of surface Gal receptors to the cell interior with no loss in receptor activity (reduced temperature, microtubule drugs) or 2) inactivation of ≃50% of all cellular Gal receptors with little or no alteration in receptor distribution (monensin, chloroquine).

MATERIALS AND METHODS

Materials

Human orosomucoid (α_1-acid glycoprotein), bovine serum albumin (BSA; fraction V), collagenase (type I), neuraminidase (type X), Percoll, chloroquine, monensin, and colchicine were obtained from Sigma Chemical Co. BSA (clinical reagent grade) was also obtained from Armour Pharmaceutical Co. Collagenase (type IV) was also obtained from Serva. Digitonin was obtained from Eastman Kodak. N-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonic acid was from Research Organics, Inc. Bisbenzimide (Hoechst dye 33258) was from Behring Diagnostics. 1,3,4,6-Tetrachloro-3α,6α-diphenylglycouril was from Pierce Chemical Co. $Na^{125}I$ (10–20 mCi/μg of iodine) was from Amersham Corp. All other chemicals were reagent grade. Medium 1 contains modified Eagle's medium (Grand Island Biological Co., catalogue 420–1,400) supplemented with 2.4 g/liter N-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonic acid, pH 7.4, and 0.22 g/liter $NaHCO_3$. Medium 1/BSA is medium 1 containing 0.1% (w/v) BSA. Hank's balanced salt solution (HBSS) was prepared according to the Grand Island Biological Co. catalogue formulation. Orosomucoid was desialylated and iodinated as described previously [19].

Hepatocytes

Male Sprague-Dawley rats (150–200 g; Harlan Breeding Labs, Houston, TX) were maintained with standard laboratory chow and water ad libitum. Hepatocytes were prepared by a modification of a collagenase perfusion procedure [20] as described previously [21]. Cells were kept at ~30°C during the filtration and differential centrifugation steps. Final cell pellets suspended in ice-cold medium 1 were ≥85% viable and single cells. Experiments were performed in medium 1/BSA in the absence of serum. Prior to experiments, cell suspensions (2×10^6 cells/ml in medium 1/BSA, 10% of the flask volume) were equilibrated at 37°C for 60 min to increase and stabilize the number of surface receptors per cell [22]. Cell viability was determined by trypan blue exclusion.

Anti-Gal Receptor Antibody

Affinity-purified goat antirat Gal receptor IgG (IgG^R), isolated and characterized previously [10], was iodinated [19] to specific activities of

100–650 dpm/fmol. ^{125}I-IgG^{R} recognizes all three Gal receptor subunits, binds to both intact and digitonin-permeabilized hepatocytes with moderately high affinity ($K_d \simeq 8 \times 10^{-8}$ M) and with high specificity and recognizes equally well both active and inactive Gal receptors [10].

Hepatocyte Treatments

Following equilibration at 37°C, hepatocytes in medium 1/BSA (2×10^6 cells/ml) were incubated either below 37°C or at 37°C with monensin, chloroquine, or colchicine at the concentrations and durations designated in the figure legends and table footnotes. Untreated and treated hepatocyte suspensions were rapidly chilled by transfer to 3–5 volumes of ice-cold HBSS on ice. The cells were collected by centrifugation, washed, and assayed at 4°C for receptor activity and protein.

Binding Assays

The assays to detect differences in Gal receptor activity or Gal receptor protein have been described [10,16]. Briefly, control or treated hepatocytes ($0.5–1 \times 10^6$ cells/sample) were incubated in HBSS containing either 1.5 μg/ml ^{125}I-asialo-orosomucoid (ASOR) or 13 μg/ml ^{125}I-IgG^{R} at 4°C for 60 min, with occasional mixing. Total (surface and intracellular) binding was measured in the presence of 0.055% (w/v) digitonin (23) added 10 min prior to the addition of ^{125}I-ASOR or ^{125}I-IgG^{R}. Surface binding only was measured in the absence of digitonin. Digitonin at 0.055% permeabilizes cells without solubilizing Gal receptors, releases cytosolic proteins of ≥200 kDa and makes intracellular receptors accessible to added ligand. Binding of either ligand is essentially complete by 60 min in intact or permeable cells. The cells, kept on ice, were then washed twice by centrifugation with HBSS, resuspended in 0.5 ml of 100 mM NaCl, 50 mM sodium phosphate, pH 7.4, and 5 mM EDTA, sonicated for 60–120 sec at 80 W in a water bath sonicator (Laboratory Supplies Co.) and assayed for DNA and radioactivity. Nonspecific binding of ^{125}I-ASOR or ^{125}I-IgG^{R}, determined in the presence of a 50-fold excess of unlabeled probe, was routinely <15% of the total binding. Binding of ^{125}I-ASOR or ^{125}I-IgG^{R} to intact or permeable cells was linear with increasing Gal receptor number (i.e., cell number) under the conditions used. All determinations were done in triplicate and the means ± standard deviations are presented.

General Procedures

Cellular DNA was determined by the Hoechst dye method [24] using calf thymus DNA as standard. Hepatocytes contain 18.7 μg of DNA/10^6 cells. Protein was determined by the Bicinchoninic acid protein assay procedure [25] (Pierce Chemical Co.) using BSA as the standard. Centrifugation of cell suspensions was at 800 rpm for 2 min at 4°C in a Beckman refrigerated TJ-6 tabletop centrifuge. ^{125}I-radioactivity was determined using a Packard Multiprias 2 gamma spectrometer.

RESULTS

Effect of Reduced Temperatures on Modulation of Gal Receptor Activity and Protein

Isolated rat hepatocytes reversibly reduce their surface Gal receptor activity by up to 65% when incubated in the absence of ligand between 20°C and 37°C [22]. Receptor down-modulation is proportional to the temperature decrease below 37°C but does not occur below ~20°C. This down-modulation could reflect either the translocation of active surface Gal receptors to the cell interior or the inactivation of a subset of Gal receptors at the surface. Therefore, we determined whether loss of surface Gal receptor activity from cells incubated at sub-37°C temperatures was due to the loss of surface Gal receptor protein. To test this, hepatocytes were incubated without ligand at 37°C, 31°C, 25°C, or 20°C for 90 min, then rapidly chilled to 4°C and assayed for either ^{125}I-ASOR binding or ^{125}I-IgG^{R} binding to measure Gal receptor activity or protein, respectively. Surface Gal receptor activity and protein were measured on intact cells, whereas total cellular (surface and intracellular) Gal receptor activity and protein were measured on permeable cells (Table I). Surface binding of both ^{125}I-ASOR and ^{125}I-IgG^{R} decreased progressively as the equilibration temperature decreased. Cells incubated at 20°C lost 30–35% of their surface Gal receptor activity and protein. These lost ^{125}I-ASOR and ^{125}I-IgG^{R} binding sites were recovered in permeable cells, indicating that the lost active surface Gal receptors (equivalent to ≃40,000 ^{125}I-ASOR binding sites/cell) were translocated to the cell interior. In no instance did we observe the complete loss of all

TABLE I. Effect of Temperature on Surface or Total Gal Receptor Activity and Protein*

Second incubation temperature (°C)	^{125}I-ASOR bound (fmol/μg DNA)		^{125}I-IgG^{R} bound (fmol/μg DNA)	
	Surface	Total	Surface	Total
37	10.4 ± 0.1 (100)	30.1 ± 2.2 (100)	25.2 ± 2.5 (100)	37.3 ± 2.8 (100)
31	8.5 ± 0.1 (82)	30.7 ± 1.5 (102)	24.7 ± 1.5 (98)	40.6 ± 2.5 (109)
25	7.0 ± 0.7 (67)	33.5 ± 1.0 (111)	17.2 ± 1.0 (68)	37.2 ± 4.0 (100)
20	6.8 ± 0.1 (65)	35.7 ± 1.7 (119)	18.4 ± 2.3 (73)	37.1 ± 3.0 (99)

*After equilibration at 37°C, hepatocytes were incubated for 90 min at the designated second temperatures, rapidly chilled to 4°C, and then assessed for ^{125}I-ASOR and ^{125}I-IgG^{R} binding as described in Materials and Methods. Numbers in parentheses are the percentages relative to the control, 37°C-equilibrated cells.

TABLE II. Effect of Colchicine on Surface or Total Gal Receptor Activity and Protein*

Exp.	Colchicine (M)	^{125}I-ASOR bound (fmol/μg DNA)		^{125}I-IgG^{R} bound (fmol/μg DNA)	
		Surface	Total	Surface	Total
1	0	16.0 ± 0.3 (100)	43.2 ± 2.0 (100)	88.1 ± 1.8 (100)	158.3 ± 6.4 (100)
	1×10^{-7}	11.0 ± 1.8 (69)	47.3 ± 3.3 (109)	64.6 ± 3.5 (73)	162.7 ± 5.1 (103)
2	0	12.5 ± 0.4 (100)	26.2 ± 2.7 (100)	31.3 ± 2.3 (100)	43.5 ± 0.9 (100)
	1×10^{-4}	9.1 ± 0.2 (73)	29.6 ± 1.9 (113)	21.1 ± 3.6 (67)	44.5 ± 6.4 (102)

*After equilibration at 37°C for 1 hr, cells were incubated without or with colchicine for 2 hr, rapidly chilled, and then assessed for ^{125}I-ASOR and ^{125}I-IgG^{R} binding as described in Materials and Methods. Numbers in parentheses are the percentages relative to the control, untreated cells.

surface Gal receptor activity or protein, indicating that only a subset of receptors was sensitive to temperature-induced alteration [22]. Thus hepatocytes incubated at 20–37°C concomitantly reduce surface Gal receptor activity and protein. Regardless of incubation temperature, hepatocytes retained their total Gal receptor activity and protein.

Effect of Colchicine on Modulation of Gal Receptor Activity and Protein

Microtubule-disrupting agents diminish surface Gal receptor activity on isolated rat hepatocytes in the absence of ligand [9,26] and hamper recycling of Gal receptors during endocytosis [9,27]. To determine if colchicine-treated hepatocytes redistributed and/or inactivated Gal receptors, cells were incubated without ligand at 37°C with different amounts of colchicine, then assayed for surface and total Gal receptor activity and protein at 4°C (Table II). Hepatocytes treated with either 0.1 or 100 μM colchicine lost ≃30% of their surface ^{125}I-ASOR and ^{125}I-IgG^{R} binding sites. Permeable treated cells, however, retained full ASOR and antibody binding, indicating that treated hepatocytes had shifted active Gal receptors to the cell interior. Lumicolchicine (2 μM) had no affect on cellular receptor distribution or activity. The loss of ^{125}I-ASOR surface binding sites on colchicine-treated cells represented the net internalization of 40,000–50,000 surface receptors/cell. Hepatocytes treated with vinblastine sulfate (2 μM) also elicited this pattern of Gal receptor redistribution without receptor inactivation (not shown). Thus hepatocytes subjected to microtubule disruption redistributed Gal receptors from the cell surface to the cell interior but did not alter Gal receptor activity. The response of colchicine-treated hepatocytes and cells incubated at reduced temperatures were essentially identical.

Effect of Monensin on Modulation of Gal Receptor Activity and Protein

Monensin is a carboxylic acid ionophore that intercalates into biomembranes and dissipates Na^{+} and H^{+} gradients [28]. Monensin-treated rat hepatocytes in the absence of added ligand reduce their surface Gal receptor activity by 50–70% [9,11,15]. The sensitive receptors consti-

tute a subpopulation of receptors we term State 2 Gal receptors [9]. The monensin concentration (100 μM) used to inactivate Gal receptors in rat hepatocytes by Fiete et al. [15], however, was tenfold greater than that eliciting the loss of surface receptor activity [9]. Therefore, we determined if hepatocytes accumulate inactive surface Gal receptors or merely redistribute surface Gal receptors after treatment with monensin at lower concentrations sufficient to reduce surface receptor activity. Hepatocytes treated with 1 μM monensin lost neither ^{125}I-ASOR nor ^{125}I-IgG^{R} binding at the cell surface (Table III). Hepatocytes treated with higher monensin concentrations progressively reduced their surface ^{125}I-ASOR binding in a concentration-dependent manner, with a maximal loss of 60% at 50 μM monensin. This represented a reduction of ≃91,000 surface ^{125}I-ASOR binding sites/cell. These same cells, however, retained 96% of their surface ^{125}I-IgG^{R} binding.

Equilibrium binding of ^{125}I-ASOR to permeable untreated and monensin-treated hepatocytes at 4°C revealed that the loss of total ^{125}I-ASOR binding capacity reflects a decreased number of ASOR-binding sites rather than a significant change in receptor-ligand affinity (Fig. 1). Surprisingly, however, the total cellular Gal receptor activity loss exceeded the loss at the cell surface alone, indicating that cells also accumulated inactive Gal receptors intracellularly (Table III). Cells treated with 3 μM monensin lost ≃42,000 surface ASOR binding sites/cell and ≃6,000 intracellular ASOR binding sites/cell. Hepatocytes treated with 50 μM monensin lost 80,000–90,000 ^{125}I-ASOR binding sites, both at the surface and internally. Hence the fraction of total cellular inactive Gal receptors at the surface decreased as the monensin concentration increased. In addition, only a subset of all cellular Gal receptors were monensin-sensitive and inactivated.

Effect of Chloroquine on Modulation of Gal Receptor Activity and Protein

Disruption of intracellular H^{+} gradients with the weak base chloroquine results in a 50–70% loss of Gal receptor activity from the surface of isolated rat hepatocytes [9,12]. The kinetics and final extent of receptor activity loss in chloroquine-treated cells were similar to those observed for monensin-treated cells [9]. Therefore, we determined whether chloroquine-treated hepatocytes altered their Gal receptor activity as did monensin-treated cells. In the absence of added ligand, isolated rat hepatocytes were treated with different chloroquine concentrations at 37°C, then assessed for surface and total binding of ^{125}I-ASOR and ^{125}I-IgG^{R} at 4°C (Table IV). Hepatocytes reduced their surface ^{125}I-ASOR binding in proportion to the chloroquine concentration. Cells lost ≃50% of their surface Gal receptor activity after a 90 min treatment with 250 μM chloroquine. This loss corresponded to a reduction of ≃90,000 surface ^{125}I-ASOR binding sites/cell. These same cells, however, retained 85% of their initial surface ^{125}I-IgG^{R} binding, indicating that the large loss of surface Gal receptor activity did not correlate with the small loss of receptor protein. Treatment of permeable dead cells at 37°C with 0.3 mM chloroquine for 60 min did not cause a decrease in Gal receptor activity.

When chloroquine-treated cells were assessed for total cellular Gal receptor activity, we found that ^{125}I-ASOR binding was reduced by 60–66%

TABLE III. Effect of Monensin on Surface or Total Gal Receptor Activity and Protein*

Exp.	Monensin (μM)	^{125}I-ASOR bound (fmol/μg DNA)		^{125}I-IgG^{R} bound (fmol/μg DNA)	
		Surface	Total	Surface	Total
1	0	18.2 ± 0.9 (100)	40.7 ± 1.3 (100)	38.8 ± 4.3 (100)	91.6 ± 3.5 (100)
	1	16.2 ± 0.6 (89)	41.7 ± 0.6 (102)	41.7 ± 5.5 (107)	91.8 ± 5.7 (100)
	3	14.3 ± 0.8 (79)	36.3 ± 1.8 (89)	33.4 ± 2.8 (86)	87.4 ± 3.4 (95)
	10	12.5 ± 0.7 (69)	31.6 ± 2.3 (78)	32.8 ± 1.3 (85)	85.8 ± 6.1 (94)
2	0	14.2 ± 1.4 (100)	34.1 ± 2.0 (100)	16.9 ± 2.5 (100)	32.6 ± 2.3 (100)
	50	5.8 ± 1.1 (41)	18.3 ± 2.6 (54)	16.2 ± 3.3 (96)	32.5 ± 7.3 (100)

*After equilibration at 37°C, hepatocytes were incubated with monensin for 2 hr, rapidly chilled, then assessed for ^{125}I-ASOR and ^{125}I-IgG^{R} binding as described in Materials and Methods. Numbers in parentheses are the percentages relative to untreated control cells.

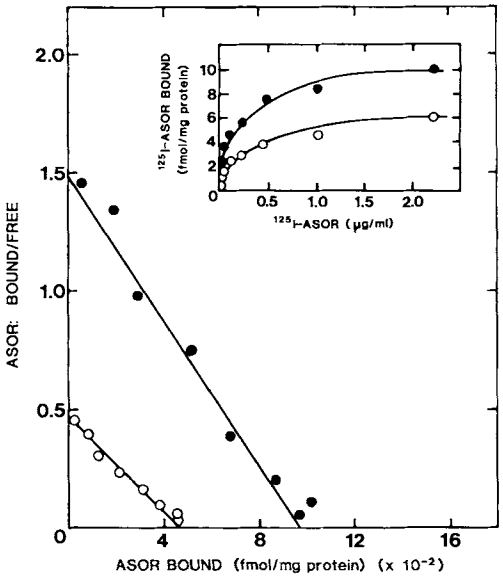

Fig. 1. Effect of monensin treatment on the equilibrium binding of ^{125}I-ASOR to permeable hepatocytes. After equilibration at 37°C, cells were incubated in medium 1/BSA with (○) or without (●) 25 μM monensin in the absence of ligand at 37°C for 90 min. After rapid chilling and permeabilization with digitonin, cells were incubated at 4°C with different concentrations of ^{125}I-ASOR for 60 min. The cells were centrifuged, and the amounts of unbound ^{125}I-ASOR in the supernatants were determined. The cells were then washed twice, and bound radioactivity and protein were determined. Specific binding of ^{125}I-ASOR to both cells samples was ≥ 95%. The **inset** shows the ^{125}I-ASOR binding isotherms. The specific binding data were analyzed and plotted according to Scatchard [47]. The data, analyzed using the LIGAND program of Munson and Rodbard [48], were best fit by a one-site model ($P < .05$) in each case. Control cells exhibited $2.86 \pm 0.15 \times 10^5$ ^{125}I-ASOR binding sites/cell with a dissociation constant (K_d) = $0.61 \pm 0.10 \times 10^{-9}$ M. Monensin treated cells exhibited $1.35 \pm 0.08 \times 10^5$ ^{125}I-ASOR binding sites/cell with a $K_d = 0.98 \pm 0.14 \times 10^{-9}$ M.

TABLE IV. Effect of Chloroquine on Surface or Total Gal Receptor Activity and Protein*

Time (min)	Chloroquine (μM)	^{125}I-ASOR bound (fmol/μg DNA)		^{125}I-IgG^R bound (fmol/μg DNA)	
		Surface	Total	Surface	Total
Experiment 1					
90	0	16.5 ± 0.9 (100)	50.9 ± 3.3 (100)	39.3 ± 2.5 (100)	75.8 ± 0.9 (100)
90	50	12.5 ± 1.1 (76)	36.8 ± 2.0 (72)	40.8 ± 2.7 (104)	84.8 ± 2.4 (112)
90	100	10.9 ± 0.9 (66)	22.4 ± 0.8 (44)	34.3 ± 0.8 (87)	84.5 ± 0.8 (111)
90	250	8.3 ± 0.7 (50)	17.3 ± 1.4 (34)	31.9 ± 2.3 (81)	73.3 ± 12.5 (97)
Experiment 2					
60	0	11.7 ± 0.2 (100)	36.8 ± 2.8 (100)	28.4 ± 2.1 (100)	75.5 ± 1.2 (100)
60	500	7.7 ± 0.9 (66)	14.6 ± 0.5 (40)	28.9 ± 2.3 (100)	75.2 ± 10.8 (100)

*Cells equilibrated at 37°C were incubated with the indicated concentration of chloroquine, rapidly chilled and assessed for ^{125}I-ASOR and ^{125}I-IgG^R binding as described in Materials and Methods. Numbers in parentheses are the percentages relative to control, untreated cells.

in a concentration-dependent manner (Table IV). The loss for hepatocytes treated with 250 μM chloroquine for 90 min was 365,000 ^{125}I-ASOR binding sites/cell. About 75% of these lost ^{125}I-ASOR binding sites were intracellular. This 1:3 ratio of surface to intracellular inactive Gal receptors was observed regardless of the chloroquine concentration tested. Total ^{125}I-IgG^R binding, however, remained essentially unchanged. In addition, total ^{125}I-ASOR binding to permeable treated cells decreased without a significant change in receptor-ligand affinity (Fig. 2). Thus

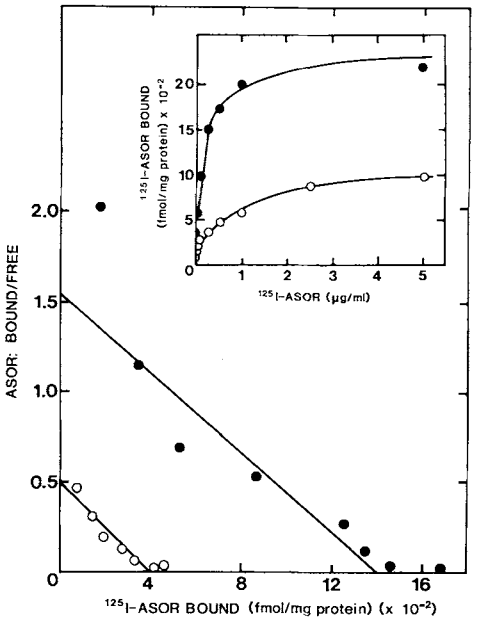

Fig. 2. Effect of chloroquine treatment on the equilibrium binding of ^{125}I-ASOR to permeable hepatocytes. After equilibration at 37°C, cells were incubated at 37°C in medium 1/BSA with (○) or without (●) 300 μM chloroquine for 70 min. After chilling and digitonin-permeabilization, the cells were assessed for bound and free ^{125}I-ASOR, and the data were analyzed as described for Figure 1. Specific ^{125}I-ASOR binding to both cell types was ≥95%. The **inset** shows the ^{125}I-ASOR binding isotherms. Analysis using LIGAND [48] showed that the best fit was to a one site model for both the control ($P < 0.03$) and the chloroquine treated ($P < 0.06$) data. Control cells exhibited $4.09 \pm 0.37 \times 10^5$ ^{125}I-ASOR binding sites/cell with a $K_d = 0.84 \pm 0.17 \times 10^{-9}$ M. Chloroquine-treated cells exhibited $1.18 \pm 0.10 \times 10^5$ ^{125}I-ASOR binding sites/cell with a $K_d = 0.80 \pm 0.15 \times 10^{-9}$ M.

hepatocytes treated with chloroquine accumulated inactive Gal receptors at the cell surface and within the cell but exhibited only a minor change in the distribution of Gal receptor protein between these two cell compartments.

DISCUSSION

To understand the cellular itinerary of migrating Class II [13] endocytic receptors, investigators have used a variety of inhibitors and treatments that selectively interrupt the function of certain cellular organelles or block pathways needed for receptor and membrane movement. Inhibitor studies have employed metabolic energy poisons, which deplete cells of adenosine triphosphate (ATP) [10,16,29–32]; ionophores and weak acids or bases, which diminish or eliminate ion and pH gradients across membranes [4,6,8,9,11,13,15,33,34]; and alkaloid poisons, which disrupt microtubule networks [26,27]. Regardless of the receptor system under study, treated cells reduce their surface receptor activity in the absence of ligand by 40–70%. In all these cases, ligand binding to the receptor is not directly affected by the inhibitor. For example, monensin [13,15,21] sodium azide [10,31], or weak bases [34] do not inhibit ^{125}I-ASOR binding to the Gal receptor at 4°C. Loss of receptor activity requires incubation of live cells at 37°C with these agents. Most investigators have assumed that receptor activity lost from the cell surface represents the intracellular entrapment of constitutively recycling receptors. There are potential problems, however, with this general interpretation. In most instances, intracellular accumulation of receptors was not verified, and receptors were assessed using only radiolabeled ligand, so that receptor inactivation induced by inhibitor treatment would have gone undetected. These concerns are reinforced by the demonstration that isolated rat hepatocytes accumulate inactive Gal receptors following either monensin treatment [15] or ATP depletion [10,16]. It is important, therefore, to examine other agents commonly used to interrupt Class II receptor dynamics for their effect on Gal receptor activity and distribution.

The findings in this present study were as follows. 1) Hepatocytes, either equilibrated at sub-37°C temperatures or treated with colchicine, shift ≃35% of their surface Gal receptors to the cell interior. Treated cells lose no immunodetectable Gal receptor protein, and Gal receptors remain active. 2) Hepatocytes treated with either chloroquine or monensin lose 50–60% of their surface and *total* Gal receptor activity with no loss of receptor protein. The accumulation of inactive surface Gal receptors on monensin-treated hepatocytes agrees with the report of Fiete et al. [15]. This result was extended by the present finding that chloroquine- and monensin-treated cells accumulate inactive Gal receptors both at their surfaces and internally. Treated cells show little or no net redistribution of surface and intracellular Gal receptor protein. As a result, these treated cells accumulate as many or more inactive Gal receptors intracellularly as on the cell surface. Some cell surface Gal receptors may normally be inactive. We consistently observe in untreated cells that the ratio of surface:total cell IgG^R binding is greater than that for ASOR binding. A greater percent of receptor protein compared with receptor activity is on the surface and therefore a larger portion of surface receptors is probably inactive. This fraction varied from experiment to experiment with different independent cell preparations (Tables I–IV).

Based on the results presented here and elsewhere [10,14], we have classified perturbants into three categories depending on their effects on Gal receptor activity and Gal receptor distribution (Table V). 1) Type I perturbants such as 20–37°C temperatures, microtubule depolymerizing drugs, and phorbol esters [14], induce redistribution of receptors from the cell surface to the cell interior with no affect on receptor activity. 2) Type II perturbants, such as monensin and chloroquine, induce receptor inactivation with only a small affect on receptor distribution between the cell surface and interior. 3) Type III perturbants, such as metabolic energy poisons and anoxia [10], induce both receptor redistribution and receptor inactivation. Active receptors are lost from the cell surface and inactive receptors accumulate intracellularly.

Regardless of the incubation time and perturbant concentration used in the present study, treated hepatocytes retained residual surface and internal Gal receptor activity. Also, ATP-depleted hepatocytes inactivate only about half of all cellular Gal receptors [10]. We demonstrated in a related study [9] that only State 2 Gal receptors are modulated on hepatocytes treated with colchicine, chloroquine, monensin, and metabolic energy poisons; surface State 1 Gal receptors are unaffected. We conclude, therefore, that State 2 Gal receptors are the receptors that cells convert to an inactive form after treatment with Type II and Type III agents.

The following summarizes our working model based on these and other results [18]. Isolated rat hepatocytes depleted of ATP in the absence of ligand convert up to 50% of all cellular Gal receptors to an inactive form, and these receptors are reactivated after restoration of cellular ATP [10]. Both inactivation and reactivation of Gal receptors occurs intracellularly, not on the cell surface [10]. Thus, under normal conditions, hepatocytes inactivate and then reactivate constitutively recycling Gal receptors. In the absence of ATP, the receptor reactivation process is blocked and inactive receptors accumulate. Since all of the perturbants studied here affect the same population of Gal receptors [9],

TABLE V. Effects of Perturbants on Gal Receptor Distribution and Activity*

Category	Treatment	Receptor redistribution	Receptor inactivation
Type I	Low temperature (20-37°C)	Yes	No
	Microtubule depolymerizing drugs	Yes	No
	Phorbol esters [14]	Yes	No
Type II	Monensin	No	Yes
	Chloroquine	No	Yes
Type III	Azide/fluoride [10, 16]	Yes	Yes
	N_2 atmosphere [10, 16]	Yes	Yes

*Different perturbants can be classified into three categories depending on their effects on State 2 Gal receptor activity or distribution, as discussed in the text.

we propose that Type II and Type III agents induce cells to accumulate inactive receptors not by causing inactivation per se but by preventing the normal receptor reactivation during constitutive recycling.

Reinsertion of recycling receptors at the cell surface is blocked by Type I and Type III but not Type II perturbants. Receptor reactivation and recycling to the cell surface are not obligatorily linked, since they are uncoupled by different categories of perturbants. In support of this model, we found that hepatocytes pretreated with hyperosmotic medium, which disrupts the formation and function of clathrin coated pits in hepatocytes and prevents receptor internalization [35–37], do not lose surface Gal receptor activity when subsequently treated with monensin or chloroquine [35]. This result indicates that chloroquine- and monensin-induced modulation of Gal receptor activity occurs intracellularly and that inactive receptors are then put back on the cell surface. The results with chloroquine and monensin suggest, therefore, that inactive State 2 Gal receptors, like active receptors, may also undergo constitutive recycling. The overall recycling time for inactive receptors may be slowed in the presence of these Type II agents as indicated by the small decrease in cell surface Gal receptor number. A complete block of an intracellular step during recycling is not likely, since this would cause a redistribution of receptors to the cell interior, which is not seen. Fiete et al. [15] concluded that monensin-treated hepatocytes accumulate inactive surface Gal receptors by a transmembrane mechanism induced by ionophore-mediated alkalinization of the cytoplasm. It is possible that cytoplasm alkalinization blocks reactivation of constitutively recycling State 2 Gal receptors.

The presence and characterization of two Gal receptor pathways in isolated hepatocytes (an in vitro model) appears to reflect the physiological situation in vivo in intact liver. Kloppel [38] recently demonstrated two intracellular transport pathways for asialoglycoproteins in perfused rat liver. Gal receptor activity is only partially lost on hepatocytes isolated from rats treated with a variety of drugs [39–44] or subjected to partial hepatectomy [40] or jejunoileal bypass [45]. Chronic phenobarbital [39] or ethanol [41] treatment of rats decreases both total immunodetectable Gal receptors and asialoglycoprotein surface binding to hepatocytes by about 50%. Notably, in the latter study, cells were unimpaired in their ability to diacytose ^{125}I-ASOR [42], a property of the State 1 but not the State 2 endocytic pathway [46], suggesting that only the State 2 Gal receptor population was lost from these cells. Hepatocytes isolated from diabetic rats lose 35% of all cellular Gal receptor activity with no loss of Gal receptor protein [44]. Lost Gal receptor activity was not caused by the occupation of receptors with endogenous ligand [43]. These results suggest that, during chronic liver dysfunction, hepatocytes can lose a functional State 2 Gal receptor pathway, either by loss of receptor protein or by the accumulation of inactive receptors.

The patterns of surface activity modulation for the Gal receptor and other Class II receptors in response to various perturbants are very similar. It is likely that the reversible inactivation/reactivation cycle observed in the Gal receptor system is common to other recycling receptors, such as the low-density lipoprotein and mannose receptors. Examination of other migratory receptors for reversible inactivation of ligand-binding activity, therefore, is warranted.

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