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Reconstitution of Galactosyl Receptor Inactivation in Permeabilized Rat Hepatocytes Is ATP-dependent*

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Jheem D. Medh and Paul H. Weigel†

From the Department of Human Biological Chemistry and Genetics, The University of Texas Medical Branch, Galveston, Texas 77550

A subpopulation of galactosyl receptors (GalRs) on isolated rat hepatocytes undergo a reversible inactivation and reactivation process during constitutive recycling (McAbee, D. D., and Weigel, P. H. (1988) *Biochemistry* 27, 2061-2069). Here, we report the reconstitution of this GalR inactivation in digitonin-permeabilized rat hepatocytes. Permeabilization of freshly isolated cells at 4 °C with 0.002% (w/v) digitonin releases cytosol containing 35-40% of the total cellular protein, 10-15% of a lysosomal marker, and 5-10% of an early endosomal marker. Incubation of permeabilized cells with cytosol at 37 °C results in a time-dependent reduction of total ¹²⁵I-asialoorosomucoid binding activity, which proceeds with first order kinetics ($t_{1/2} = 11.3$ min). Only half of the total cellular GalRs are affected; maximal GalR activity loss, obtained by 30 min, is $50.5 \pm 9.5\%$ ($n = 21$) of the control (4 °C) value. Increasing the digitonin concentration up to 0.055% does not increase the extent of inactivation. Permeabilized cells with reduced GalR activity were assessed for GalR protein content by Western blot analysis and by binding of anti-GalR antibody. The results show that the reduced ¹²⁵I-asialoorosomucoid binding is due to GalR inactivation rather than receptor protein degradation. GalR inactivation does not occur in the absence of cytosol or in the presence of dialyzed cytosol. The cytosol also loses its GalR inactivating ability in the presence of an ATP-depleting system. GalR inactivation in the absence of cytosol is achieved by incubating permeabilized washed cells at 37 °C with ATP but not with ADP, AMP, or other NTPs. The rate and extent of inactivation are dependent on the ATP concentration. Half-maximal and maximal GalR inactivation are obtained at 0.3 and 3.0 mM ATP, respectively. In the presence of cytosol, permeabilized hepatocytes could replenish cytosolic ATP by oxidative phosphorylation. As a result, similar levels of GalR inactivation were obtained with 500-fold lower ATP concentrations. We conclude that ATP is the only cytosolic component necessary for GalR inactivation in permeabilized rat hepatocytes.

asialoglycoprotein receptor or GalR¹ has been extensively characterized and serves as a convenient model for dissecting the endocytic pathway. Treatment of hepatocytes with functionally diverse agents such as metabolic energy poisons (6-9), lysosomotropic amines (10-13), microtubule depolymerizing drugs (14, 15), or proton ionophores (11, 16, 17) causes an inactivation and/or redistribution of cell surface GalRs and disrupts the recycling process (18). We have previously shown that in intact rat hepatocytes, depletion of ATP in the absence of ligand causes 50% of the cell surface GalRs to be trapped intracellularly in an inactive form (6, 19). Restoration of cellular ATP to normal levels allows reactivation and reexpression of these GalRs on the cell surface, even in the absence of protein synthesis. Detailed kinetic analysis of GalR internalization, inactivation, reactivation, and externalization was performed using anti-GalR ¹²⁵I-IgG and ¹²⁵I-ASOR to follow GalR protein and GalR activity, respectively. This study revealed that both the GalR inactivation and the GalR reactivation processes occur intracellularly (19). Additional evidence to support an intracellular site of GalR inactivation was obtained when hepatocytes were treated with hyperosmolar medium to inhibit coated pit formation (20) and disrupt internalization (21, 22). In this case, GalRs failed to undergo inactivation in cells treated with monensin or chloroquine (23). Such an intracellular inactivation step during normal GalR recycling would explain why the segregation of dissociated receptor and ligand into different intracellular compartments for subsequent differential processing is so efficient.

Since the biochemical basis for GalR inactivation is not yet known, we have used digitonin-permeabilized rat hepatocytes to develop a cell-free *in vitro* system to study the requirements for GalR inactivation. At subsaturating concentrations, digitonin binds to and disrupts preferentially the cholesterol-enriched plasma membrane (24). Fiskum *et al.* (25) have shown by electron microscopy that rat hepatocytes permeabilized with 0.005% digitonin maintain an extensive cytoskeleton and intact intracellular organelles. Such selective permeabilization allows one to bypass the permeability barrier across the plasma membrane with minimum disturbance of the normal intracellular structural organization. It is thus possible to monitor directly the responses of various intracellular organelles to exogenously added agents (26). In fact, such permeable cells are metabolically active and are capable of continuing complex processes such as lipid synthesis, oxidative phosphorylation, and secretion (27, 28). Several inves-

Most class II receptors (1) are believed to undergo constitutive recycling (2-6). The biochemical events that drive the receptor recycling process and the itinerary of these receptors inside the cell are not yet clearly understood. The hepatic

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† To whom correspondence should be addressed.

¹ The abbreviations used are: GalR, galactosyl receptor; ASOR, asialoorosomucoid; BSA, bovine serum albumin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenedibis(oxyethylenetriol)]tetraacetic acid; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

tigators have used permeabilized cells to study various cellular events including mobilization of calcium ion fluxes (29, 30), modulation of enzyme activity (31, 32), receptor phosphorylation (33, 34), and the requirements for receptor-ligand dissociation (35, 36). Here, we report the time, temperature-, and ATP-dependent partial inactivation of GalRs in permeabilized rat hepatocytes.

EXPERIMENTAL PROCEDURES

Materials—Male Sprague-Dawley rats (200–300 g) were from Harlan Breeding Laboratories, Houston, TX. Human orosomucoid, Percoll, neuraminidase (type X), CNBr-activated Sepharose-4B, calf thymus DNA, Triton X-100, Nonidet P-40, 2-mercaptoethanol, SDS, ATP (catalog number A 6144), other NTPs, AMP, ADP, hexokinase (catalog number H 5500), atractyloside (catalog number A 6822), N-ethylmaleimide, phenylmethylsulfonyl fluoride, pepstatin, leupeptin, bacitracin, and diisopropylfluorophosphate were obtained from Sigma. Na¹²⁵I (10–20 mCi/ μ g of iodine) was from Amersham Corp. 1,3,4,6-Tetrachloro-3 α ,6 α -diphenylglycouril (ODO-GEN) was from Pierce Chemical Co. ¹²⁵I-ASOR was prepared by desialylation of orosomucoid with neuraminidase and subsequent iodination as described previously (37). Digitonin was from Eastman Kodak Co., collagenase (type D) was from Boehringer Mannheim, bisbenzimidazole (Hoechst dye 33258) was from Behring Diagnostics, BSA was from Armour Pharmaceutical Co. (grade CRG-7), HEPES was from Research Organics Inc., and acrylamide (twice recrystallized) was from U. S. Biochemical Corp. Nitrocellulose (0.45 μ m) was from Schleicher & Schuell. Nitro blue tetrazolium, 5-bromo-4-chloro-3-indolylphosphate, ammonium persulfate, N,N'-methylenebis(acrylamide), and SDS-PAGE molecular weight markers were from Bio-Rad. All other chemicals were reagent grade.

Antibodies—Total IgG fractions were isolated from antisera of a goat inoculated subcutaneously with 100 μ g of affinity-purified GalR as described previously (19). Total IgY was isolated from yolks of eggs collected before and after injections of chickens with affinity-purified GalR by a modification (38) of the procedure described by Polson *et al.* (39). Rabbit anti-chicken IgG and alkaline phosphatase-conjugated rabbit anti-chicken IgG were from Jackson Laboratories. Rabbit anti-chicken IgG was iodinated as described previously (19).

Buffers and Media—Medium 1 contains modified Eagle's medium (GIBCO catalog number 420-1400) supplemented with 2.4 g/liter HEPES, pH 7.4, and 0.22 g/liter NaHCO₃. Medium 1/BSA is medium 1 with 0.1% (w/v) BSA. Hanks' balanced salt solution contains 1.26 mM CaCl₂, 5.36 mM KCl, 0.3 mM KH₂PO₄, 0.5 mM MgCl₂, 0.4 mM MgSO₄, 137 mM NaCl, 0.33 mM Na₂HPO₄, 5.5 mM glucose, 0.001% (w/v) phenol red, and 4.2 mM NaHCO₃, pH 7.4. Buffer 1 contains 143 mM NaCl, 6.8 mM KCl, and 10 mM HEPES, pH 7.4. BIC 10 is buffer 1 with 10 mM CaCl₂. Extraction buffer is buffer 1 with 1% Triton X-100. Cytosolic buffer contains 10 mM NaCl, 140 mM KCl, 2 mM MgCl₂, 2 μ M CaCl₂, 10 mM HEPES, pH 7.4. Phosphate-buffered saline contains 137 mM NaCl, 8 mM sodium phosphate, 2.7 mM KCl, and 1.5 mM potassium phosphate, pH 7.4. Tris-buffered saline contains 10 mM Tris-HCl and 154 mM NaCl, pH 7.4. DNA assay buffer contains 100 mM NaCl, 50 mM Na₂HPO₄, and 5 mM EDTA, pH 7.4.

Preparation of Hepatocytes—Rat hepatocytes, isolated by a modification (40) of the collagenase perfusion procedure of Seglen (41), were suspended in medium 1/BSA (2×10^6 cells/ml) and incubated at 37 °C for 1 h in a gyratory water bath to increase and stabilize the cell surface GalR number (42). Viable cells (>95%) selected by centrifugation at 350 $\times g$ through discontinuous Percoll gradients (7) were counted, washed, and resuspended in cytosolic buffer (2×10^6 cells/ml).

Permeabilization of Hepatocytes and Preparation of Cytosol—To maintain an appropriate environment for intracellular organelles, the permeabilization of hepatocytes was done in cytosolic buffer to mimic the cytoplasm with respect to ion concentrations. Cells prepared as above were resuspended in cytosolic buffer (2×10^6 cells/ml) with or without digitonin and incubated at 4 °C for 20 min. For most experiments (unless specified otherwise), 0.006% digitonin was used to obtain mildly permeabilized hepatocytes. The permeabilized cell suspension was either used directly, or the cells were first centrifuged at 350 $\times g$ for 5 min to remove the supernatant (cytosol), washed twice more with cytosolic buffer, and resuspended in the specified incubation medium (2×10^6 cells/ml). The cytosol, which has ~800 μ g of protein/ml, contains cytosolic components at roughly 1–2% of the concentration present in the cytoplasm of intact cells.

Antibody and Ligand Binding Assays—Cell suspensions prepared

as above were incubated at either 4 or 37 °C with continuous gyration at 100 rpm. Aliquots (1 ml each) were removed at different times into 2 ml of ice-cold cytosolic buffer. The cell suspensions were then centrifuged, and the supernatant was removed by aspiration. To measure total cellular GalR activity, cell pellets were resuspended and incubated at 4 °C for 1 h in 500 μ l of medium 1/BSA with 1.5 μ g/ml ¹²⁵I-ASOR (specific activity, ~50–100 cpm/fmol) and 0.055% digitonin. The presence of 0.055% digitonin fully permeabilizes the cells and allows for the assessment of total (surface and internal) receptor number and activity (43). To measure total cellular antibody binding, cell pellets were resuspended and incubated for 1 h at 4 °C in 500 μ l of Hanks' with 100 μ g/ml immune anti-GalR chicken IgY (for total binding) or preimmune chicken IgY (for nonspecific binding) with 7.5 mM EGTA and 0.055% digitonin. After removing unbound primary antibody with three washes each of 4 ml of Hanks' containing 7.5 mM EGTA, the cells were incubated for 45 min at 4 °C with 2 μ g/ml of rabbit antichicken ¹²⁵I-IgG (specific activity ~50–100 cpm/fmol). After incubation with either ¹²⁵I-ASOR or ¹²⁵I-IgG, the cells were washed three times with 4 ml each of Hanks', and the final cell pellets were resuspended in either DNA assay buffer or 0.3 N NaOH. Aliquots were then assessed for radioactivity and assayed for DNA or protein content. Nonspecific ¹²⁵I-ASOR binding, determined by the radioactivity remaining after stripping specifically bound ligand with EGTA, was only 5–10% of the total in all cases. Nonspecific ¹²⁵I-IgG binding determined by using preimmune chicken IgY as the primary antibody was 25–35% of the total. All binding assays were done in duplicate, and the error bars indicate the sample standard deviation.

SDS-PAGE and Western Blot Analysis—The procedure was the same as that described previously (38). Permeabilized hepatocytes incubated at either 4 or 37 °C were washed at 4 °C and extracted with 1% Triton X-100. The solubilized GalR was immunoprecipitated using anti-receptor goat IgG-Sepharose, washed three times with BIC 10 buffer, resuspended in 150 μ l of the same buffer, dissociated from the Sepharose by boiling for 5 min, and separated by SDS-PAGE after reduction with 5% 2-mercaptoethanol. The proteins were then transferred to nitrocellulose and processed for Western blotting using anti-receptor chicken IgY and alkaline phosphatase conjugated to rabbit anti-chicken IgG as the primary and secondary antibodies, respectively. The receptor bands were visualized by incubation with 0.225 mg/ml 5-bromo-4-chloro-3-indolylphosphate and 0.45 mg/ml nitro blue tetrazolium as described previously (38).

General—Cell viability was determined by the ability to exclude trypan blue. ATP concentrations were quantitated by a modification (44) of the luciferin-luciferase procedure of Stanley and Williams (45). Protein content was measured by the method of Bradford (46) using BSA as a standard. N-Acetylglucosaminidase was measured by a colorimetric assay using p-nitrophenyl-N-acetylglucosaminide as the substrate and p-nitrophenol as the standard (47). Cellular DNA was determined spectrofluorometrically by the DNA binding dye Hoechst 33258 procedure of Labarca and Paigen (48) with calf thymus DNA as the standard. ¹²⁵I-Radioactivity was determined using a Packard Multiprias 2 γ -spectrometer.

RESULTS

GalR Inactivation Occurs in Permeable Cells—When hepatocytes were permeabilized at 4 °C with 0.006% digitonin and then directly incubated at 37 °C, there was a time-dependent reduction in their ability to bind ¹²⁵I-ASOR when subsequently assayed at 4 °C (Fig. 1). This reduction in GalR activity was not seen after incubation of permeabilized cells at 4 °C or in intact cells incubated at either 4 or 37 °C. The loss of ligand binding activity proceeded with first order kinetics and a $t_{1/2}$ of 11.3 min (Fig. 1). GalR activity loss was almost complete by about 30–45 min and, in a large number of experiments, resulted in $50.5 \pm 9.5\%$ ($n = 21$) reduction in ligand binding compared with controls incubated at 4 °C. Extended incubation times of up to 2 h did not show any further reduction in GalR activity. Several alternative explanations for the decreased GalR activity were investigated. For example, degradation of GalR by proteases released due to permeabilization of lysosomes could result in decreased binding. However, addition of a mixture of protease inhibitors including phenylmethylsulfonyl fluoride, N-ethylmaleimide,

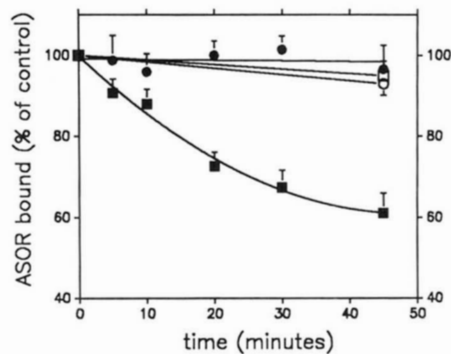


FIG. 1. Time- and temperature-dependent loss of GalR activity in permeabilized hepatocytes. Isolated cells were incubated in cytosolic buffer with (■, □) or without (●, ○) 0.005% digitonin for 20 min at 4 °C. The cells were then incubated at either 4 °C (○, □) or 37 °C (●, ■). At the indicated times, aliquots were removed and assayed for total cellular GalR binding activity as described under "Experimental Procedures."

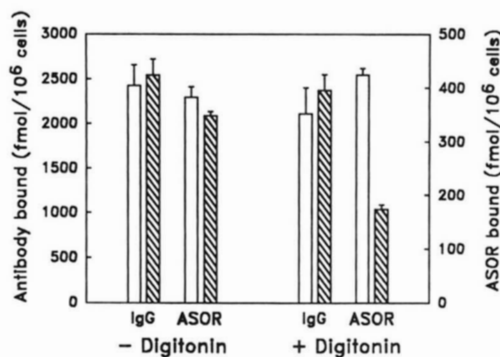


FIG. 2. GalR activity but not GalR protein is lost in permeabilized hepatocytes treated at 37 °C. Hepatocytes were incubated at 4 °C for 20 min in cytosolic buffer with or without 0.005% digitonin and then incubated for 30 min at either 4 °C (open bars) or 37 °C (hatched bars). The cells were then washed at 4 °C and allowed to bind either 125 I-ASOR or anti-receptor chicken IgY followed by rabbit anti-chicken 125 I-IgG as described under "Experimental Procedures." Cells were washed by centrifugation, and the cell-associated radioactivity was determined. Results are averages of six determinations \pm sample standard deviation.

EDTA, pepstatin, leupeptin, and bacitracin during permeabilization and the subsequent 37 °C incubation gave similar results (not shown). Degradation by proteases is therefore probably not responsible for the decreased binding. Another possibility is that partially processed glycoproteins with terminal galactosyl residues could be released from the Golgi as a result of permeabilization. Binding of these endogenous ligands to GalR would then interfere with the subsequent binding of 125 I-ASOR. However, an EGTA wash at 4 °C to remove such endogenously bound ligands also did not prevent the GalR activity loss (not shown).

To obtain more quantitative data in support of *bona fide* GalR inactivation rather than protein degradation, cells were assayed for both 125 I-ASOR and anti-GalR IgG binding as a measure of GalR activity and GalR protein, respectively (Fig. 2). Almost identical amounts of 125 I-IgG bound to both intact and permeabilized cells incubated at either 4 or 37 °C. However, permeabilized cells treated at 37 °C bound only ~40% of the 125 I-ASOR bound by intact cells or by permeabilized cells incubated at 4 °C. These results provide evidence for GalR inactivation since there is no loss of GalR protein but substantially reduced GalR activity in mildly permeabilized cells incubated at 37 °C in the presence of cytosol. The results also show that the chicken anti-GalR antibody specifically

binds to both active and inactive GalR protein.

Partial degradation of GalR protein could eliminate ligand binding without interfering with antibody binding. This possibility was examined by Western blot analysis (Fig. 3). GalR was immunoprecipitated with goat anti-receptor IgG from extracts of permeabilized cells incubated at either 4 or 37 °C. Although cells treated at 37 °C had only 42% of the 125 I-ASOR binding activity of those at 4 °C, the positions and the staining intensity of the three GalR subunits were identical in both cases, showing that there was no detectable GalR degradation. Based on these observations, we conclude that incubation of permeabilized unwashed cells at 37 °C causes inactivation of GalRs. This inactivation does not require the cytosolic buffer, since similar results were obtained when buffer 1 was used for permeabilization and the subsequent 37 °C incubation (not shown).

Only One of Two GalR Subpopulations Is Inactivated in Vitro—In the experiments described above, there was always only a 40–60% reduction in total cellular GalR activity. The average extent of GalR inactivation, calculated from 21 experiments, was $50.5 \pm 9.5\%$ of the control. To address the possibility that GalR inactivation may depend on the extent of permeabilization, hepatocytes were treated with different concentrations of digitonin. The extent of permeabilization of various intracellular compartments was assessed using different markers (Fig. 4A), and the extent of GalR inactivation induced at 37 °C was also measured at each digitonin concentration (Fig. 4B). At <0.001% digitonin, there was no significant permeabilization of hepatocytes. Between 0.001 and 0.002% digitonin, the extent of plasma membrane permeabilization increased very sharply as evidenced by the increase in the percentage of total protein released and the decrease in cell viability. Maximum loss of protein (40% of total cellular content) was seen at $\geq 0.002\%$ digitonin. Permeabilization of lysosomes started at 0.002% digitonin with a 15% loss of the lysosomal marker enzyme, *N*-acetylglucosaminidase. The percentage release steadily increased with the concentration of digitonin. Significant amounts of an early endosomal marker (internalized 125 I-ASOR) were released only at concentrations of $>0.003\%$ digitonin. At 37 °C, ~50% of the total cellular GalR activity was lost at 0.002% digitonin. This extent of GalR inactivation did not

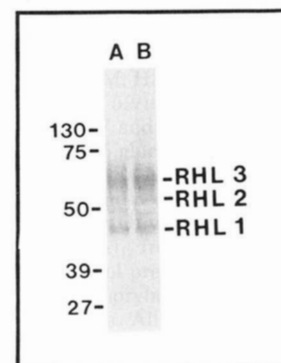


FIG. 3. Effect of GalR inactivation on Western blot analysis of receptor subunits from digitonin-permeabilized cells. Isolated hepatocytes were suspended in cytosolic buffer in the presence of 0.005% digitonin for 20 min at 4 °C. The cells were incubated at either 4 (lane A) or 37 °C (lane B) for 30 min and then chilled to 4 °C and extracted with 1% Triton X-100. Solubilized receptor was immunoprecipitated, separated by SDS-PAGE, transferred to nitrocellulose, and probed as described under "Experimental Procedures." The three Gal receptor subunits are labeled as RHL (rat hepatic lectin) 1, -2, and -3 (62). Molecular mass standards are indicated in kDa on the left.

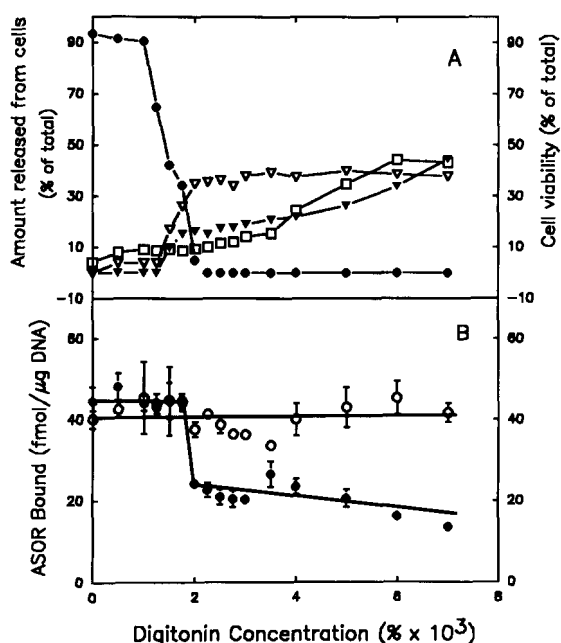


FIG. 4. Effect of digitonin concentration on the extent of GalR inactivation and cell permeabilization. Isolated hepatocytes were suspended in cytosolic buffer with the indicated concentrations of digitonin for 20 min at 4 °C. A, some of the cells were then incubated at 37 °C for 40 min, and cell viability (●) was determined. Samples were also chilled to 4 °C and centrifuged, and the amounts of protein (V) and *N*-acetylglucosaminidase (V) released into the supernatant and retained in the cell pellet were measured. To estimate the extent of permeabilization of early endosomes (□), intact cells were first allowed to internalize surface-bound ¹²⁵I-ASOR at 37 °C for 5 min (as in Ref. 7) before chilling to 4 °C and washing with medium 1 containing 7.5 mM EGTA to remove residual surface-bound ligand. Cells were then washed and resuspended in cytosolic buffer and permeabilized with the indicated concentration of digitonin at 4 °C for 20 min. After permeabilization, the supernatant and cells were separated by centrifugation. ¹²⁵I-ASOR in permeabilized vesicles but still bound to receptor was released by washing the pellet once with 7.5 mM EGTA. The first supernatant and the EGTA wash were combined, and radioactivity was determined. This value is expressed as the percentage of the total internalized radioactivity. B, aliquots of permeabilized cells were further incubated at 4 °C (○) or 37 °C (●) for 40 min and were assessed for total cellular GalR activity as described under "Experimental Procedures."

increase substantially with increasing concentrations of digitonin and did not correlate with the amount of lysosomal or endosomal markers released. Even at 0.005% digitonin, when most intracellular vesicles were permeabilized and almost 100% of both lysosomal and endosomal markers were released, the extent of inactivation was still ~50% (not shown). This latter result also suggests that GalR degradation by lysosomal proteases is not responsible for the decreased ligand binding activity of GalR.

The above results indicate that only a portion of the total cellular GalR content can be inactivated in the *in vitro* permeable cell system. This partial inactivation could reflect the behavior of two different GalR subpopulations. We have previously characterized two functionally distinct GalR populations in isolated rat hepatocytes, which we have termed State 1 and State 2 GalR (49–51). These two GalR populations process internalized ligand in two different intracellular pathways, and their activity is differentially modulated by a variety of drugs, including azide (52). The State 2 GalRs are inactivated and accumulate inside the cell in azide-treated cells (19, 52). State 1 GalRs remain active under these conditions. It is possible, therefore, that only one of these two GalR subpopulations (the State 2 GalRs) becomes inactivated in the

permeable cells. To test this, intact cells were treated at 37 °C with sodium azide to down-modulate the State 2 GalRs. When these cells were subsequently washed, permeabilized at 4 °C, and then incubated at 37 °C in the presence of cytosol, no GalR inactivation occurred (Fig. 5). Furthermore, the control (non-azide-treated) permeable cells lost an amount of GalR activity *in vitro* equal to what the azide-treated cells had lost *in vivo*. We conclude that GalR inactivation in the permeable cells represents the loss of State 2 GalR activity; State 1 GalR activity appears to be unaffected.

ATP Is the Only Cytosolic Factor Required for GalR Inactivation.—To identify the components responsible for inactivation, permeabilized cells and the released cytosol were separated from each other. GalRs in permeabilized cells that had been washed twice to remove cytosolic components and then resuspended in cytosolic buffer were not inactivated after incubation at 37 °C (Table I). Incubation of these washed cells with cytosol resulted in GalR inactivation, indicating that at least one inactivating factor(s) is cytosolic. The permeabilized washed hepatocytes thus provide a convenient system to reconstitute the GalR inactivation process. When cytosol was extensively dialyzed at 4 °C, it failed to cause GalR inactivation in permeabilized washed hepatocytes (Table I). Storage of cytosol at 4 °C for the same length of time only marginally decreased its GalR inactivating ability, suggesting that the inability of dialyzed cytosol to inactivate GalR was due to depletion rather than inactivation of a factor(s). This result indicates that a relatively small (molecular mass < 10 kDa) cytosolic component is required for GalR inactivation. Since any proteases, if present, would still be in the dialyzed cytosol, this result also corroborates the conclusion that GalR degradation by proteases is not responsible for the decreased GalR activity.

The finding that a cytosolic factor is responsible for GalR inactivation suggests that the resistance of a fraction of the total cellular GalRs to inactivation, as discussed above, may be due to the depletion or denaturation of a cytosolic inactivating factor during the incubation at 37 °C. To address this possibility, permeabilized hepatocytes were first incubated at 37 °C with cytosol. After this first incubation, only 50–60% of the total cellular GalRs were active. These cells were washed and then again incubated at 37 °C with fresh cytosol.

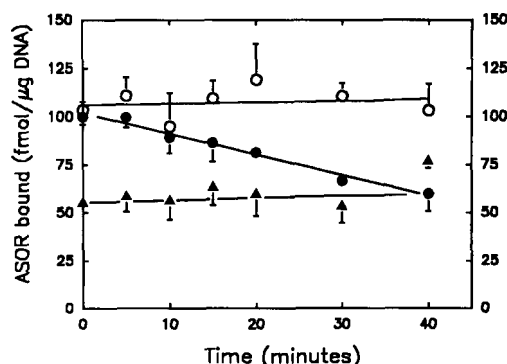


FIG. 5. Inactivation of the State 2 but not State 1 GalR population in permeabilized hepatocytes. Freshly isolated cells were incubated in either the presence (▲) or absence (○, ●), of 15 mM sodium azide for 1 h at 37 °C. They were then rapidly chilled to 4 °C and centrifuged through discontinuous Percoll gradients to select viable cells (~75% recovery). The cells were then washed and resuspended in cytosolic buffer, permeabilized at 4 °C for 20 min, and incubated at 4 °C (○) or 37 °C (●, ▲). At the indicated times, samples were removed and washed and the total cellular GalR activity in the cell pellet was determined. For clarity, the data for the azide-treated cells incubated at 4 °C are not included; no change in GalR activity occurred.

TABLE I

A dialyzable component of the cytosol is required for GalR inactivation

In two different experiments, hepatocytes (2×10^6 cells/ml) were incubated for 20 min at 4 °C in cytosolic buffer with or without 0.006% digitonin. After an aliquot (unwashed cells) was removed, the permeabilized cells were centrifuged and the cytosol was saved. The cell pellet was washed twice with cytosolic buffer and resuspended at 4×10^6 cells/ml in cytosolic buffer. The cytosol was either used immediately, stored at 4 °C for 20 h, or dialyzed against $0.01 \times$ cytosolic buffer at 4 °C for 16 h followed by dialysis against $1 \times$ cytosolic buffer for 4 h at 4 °C. Aliquots of intact cells, permeabilized unwashed cells, or permeabilized washed cells were resuspended at 2×10^6 cells/ml in either cytosolic buffer, cytosol, dialyzed cytosol, or stored cytosol and incubated at 37 or 4 °C for 30 min. Total cellular GalR activity was then determined as described under "Experimental Procedures." Results are expressed as a percentage of the control activity remaining after incubation at 4 °C.

Condition	GalR activity	
	Experiment 1	Experiment 2
	% of control	
Intact cells	85.8 \pm 7.6	
Permeabilized cells, unwashed	42.7 \pm 3.4	
Permeabilized washed cells plus cytosolic buffer	101.5 \pm 10.5	95.1 \pm 4.7
Permeabilized washed cells plus cytosol	41.1 \pm 3.5	51.4 \pm 1.8
Permeabilized washed cells plus dialyzed cytosol		106.8 \pm 7.7
Permeabilized washed cells plus stored cytosol		61.2 \pm 1.4

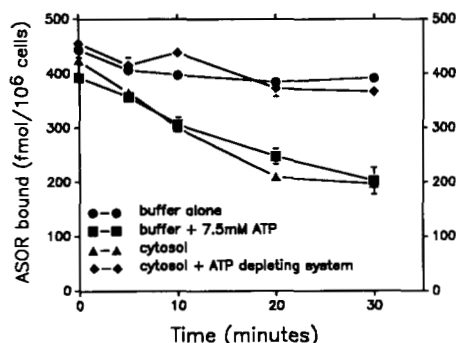


FIG. 6. Effect of cytosolic ATP depletion on GalR inactivation in permeable cells. Hepatocytes were permeabilized as in Fig. 1 and centrifuged, and the cytosol was removed. The cells were washed twice with and resuspended at 4×10^7 cells/ml in cytosolic buffer. The cells were then diluted to 2×10^6 cells/ml in cytosolic buffer (●), cytosol (▲), cytosol preincubated at 37 °C with 25 units/ml hexokinase, and 5 mM glucose for 10 min (◆) or in cytosolic buffer containing 7.5 mM ATP (■) and incubated at 37 °C. At the indicated times, samples were removed and total cellular GalR activity was determined.

This second incubation caused inactivation of only a small fraction of the residual active GalRs. The final GalR activity was still about 43% of the initial amount before the first incubation. This result indicates that depletion of an inactivating factor is not responsible for the ability of only roughly half of the total GalRs to maintain their activity, and it is consistent with the above conclusion that the sensitive and insensitive GalRs are different.

To address the possible involvement of ATP, GalR inactivation was assessed in the presence of either ATP alone or ATP-depleted cytosol (Fig. 6). As expected, incubation of permeabilized hepatocytes at 37 °C with cytosol resulted in a rapid loss of 125 I-ASOR binding activity with ~50% inactivation by 30 min. However, when the cytosol was first depleted of ATP by using hexokinase and glucose and then incubated

with the permeabilized washed cells, still in the presence of the ATP depleting system, the cytosol was unable to support GalR inactivation. Addition of glucose alone did not significantly alter the extent of GalR inactivation. However, hexokinase alone was able to partially block GalR inactivation (not shown). This can be explained because the K_m of hexokinase for glucose is low (~ 8 – 30μ M), and the endogenously available glucose is probably sufficient to support ATP depletion by hexokinase. ATP levels in cytosol were quantitated in the presence and absence of hexokinase and confirmed the ability of hexokinase to deplete ATP. Since ATP-depleted cytosol is no longer capable of supporting GalR inactivation, the absolute requirement for ATP by this process was suggested. When permeabilized washed hepatocytes were incubated at 37 °C in the absence of cytosol but in the presence of 7.5 mM ATP, GalR activity loss occurred with kinetics similar to that obtained with cytosol (Fig. 6). Thus, ATP alone was sufficient to cause GalR inactivation. These results, taken together, indicate that ATP may be the only cytosolic component required for GalR inactivation.

The specificity of the requirement for ATP was tested by substituting other nucleoside triphosphates for ATP. Incubation of permeabilized washed hepatocytes at 37 °C with 3 mM ATP resulted in inactivation of 40–50% of total cellular GalRs by 20–30 min. However, incubation of the permeabilized washed cells with any of the other NTPs, ADP, or AMP did not result in any significant change in GalR activity (Fig. 7). Thus, other nucleotides cannot support GalR inactivation, and the ATP requirement for GalR inactivation in permeabilized cells is specific and absolute.

The extent and rate of ATP-induced GalR inactivation were dependent on the concentration of ATP (Fig. 8). The ATP dose-response curves for the rate and extent of GalR inactivation are almost identical. In the absence of ATP, permeabilized washed hepatocytes bound ~800 fmol of ASOR/ 10^6 cells. At each ATP concentration tested, GalR inactivation continued at a linear rate for up to 45 min. The maximal rate of inactivation, obtained at ~3.0 mM ATP, was 8 fmol of ASOR binding sites (1%) lost/min/ 10^6 cells. A half-maximal rate of GalR inactivation was obtained at ~0.3 mM ATP. The extent of GalR inactivation, measured after 45 min of incubation at 37 °C, also increased with increasing concentrations of ATP. A half-maximal extent of GalR inactivation was obtained at ~0.3 mM ATP. Maximal extent of inactivation, obtained at ~3.0 mM ATP was 55% of the total cellular activity. Further increasing the ATP concentration to 7.5 mM did not significantly increase the extent or rate of GalR

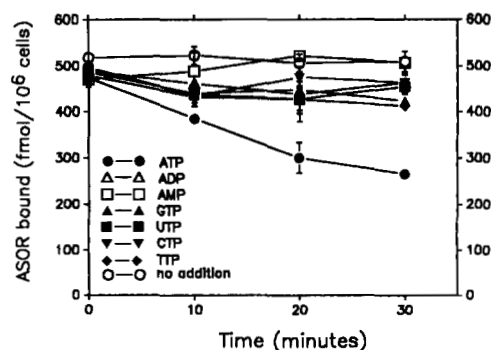


FIG. 7. Specificity of the ATP requirement for GalR inactivation in permeable hepatocytes. Cells were permeabilized at 4 °C for 20 min and then washed twice in cytosolic buffer and resuspended at 2×10^6 cells/ml either in cytosolic buffer alone (○) or with 3 mM ATP (●), ADP (△), AMP (□), GTP (▲), UTP (■), CTP (▼), or TTP (◆) and incubated at 37 °C. Samples were removed at the indicated times, and total cellular GalR activity was determined.

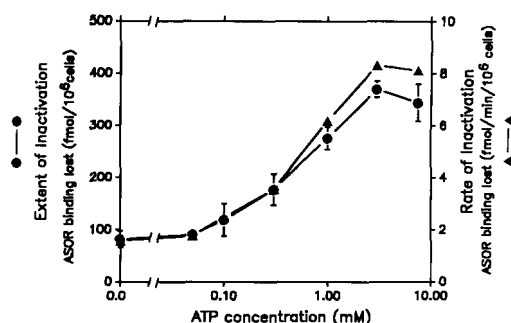


FIG. 8. Effect of ATP concentration on the rate and extent of Gal receptor inactivation in permeable hepatocytes. Cells were permeabilized, washed, and incubated in cytosolic buffer at 37 °C with the indicated concentrations of ATP. Samples were removed at different times and assessed for total ¹²⁵I-ASOR binding activity. For the extent of GalR inactivation (●), the data are expressed as the amount of ligand binding activity lost by 45 min. The results are averages of duplicate determinations ± sample standard deviation. For the determination of rate at each concentration of ATP, ¹²⁵I-ASOR binding activity was graphed *versus* time. The slopes of these lines were calculated by least squares linear regression analysis using 3–4 points, to determine the rate of inactivation (▲). Correlation coefficients ranged from –0.91 to –0.99.

inactivation. Thus, analogous to the results with cytosol, ~50% of the total GalR population is inactivated and ~50% is resistant to ATP-induced inactivation.

Cytosol Decreases the ATP Concentration Needed for GalR Inactivation—From the above results, it is clear that cytosolic ATP is essential for GalR inactivation. However, the concentration of ATP in cytosol, determined by a luciferin-luciferase assay, is only ~5–6 μM, and ATP by itself, at such low concentrations, does not cause any significant loss of GalR activity in washed permeabilized cells (Fig. 8). Nonetheless, the extent of GalR inactivation induced by cytosol was comparable to that caused by 3.0 mM ATP. Thus, in the absence of other cytosolic components, a ~500-fold greater ATP concentration is required to attain similar levels of GalR inactivation. When washed, permeable hepatocytes are incubated with millimolar amounts of ATP at 37 °C, the ATP is hydrolyzed, and, consequently, its concentration steadily decreases with time until a much lower steady state value is reached (not shown). These observations suggest that the ATP-dependent GalR inactivation in the presence of cytosol may be enhanced by other cytosolic components.

Preincubation of cytosol at 37 °C for 60 min resulted in the complete hydrolysis of cytosolic ATP as determined by the subsequent quantitation of ATP. However, such ATP-depleted cytosol still supports GalR inactivation (Table II), and ATP resynthesis is seen within minutes at 37 °C. This indicates that in the presence of cytosol, the permeable cells are capable of replenishing cytosolic ATP by either glycolysis or oxidative phosphorylation. This was found to be the case, since GalR inactivation by ATP-depleted cytosol was almost completely blocked when oxidative phosphorylation was inhibited by the addition of 1 mM atractyloside (Table II) or 2 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. Similarly, GalR inactivation was achieved in the absence of either ATP or cytosol by supplying the substrates for oxidative phosphorylation; *viz.* inorganic phosphate, ADP, glutamate, and malate (Table II). None of these substrates by itself was able to support GalR inactivation. It is also worth noting that the extent of GalR inactivation obtained with 0.5 mM ADP in the presence of these substrates was comparable to that obtained with 5 mM ATP. Thus, the continued resynthesis of ATP in digitonin-permeabilized hepatocytes is efficient and explains why lower concentrations of ATP are

TABLE II
Oxidative phosphorylation facilitates GalR inactivation in permeabilized hepatocytes

Isolated hepatocytes were permeabilized as in Table I, cytosol was saved, and the cells were washed twice and resuspended at 4×10^7 cells/ml in cytosolic buffer. The cells were then diluted to a density of 2×10^6 cells/ml in cytosol or cytosolic buffer containing the indicated additions. After incubation at 4 (control) or 37 °C for 40 min (Experiment 1) or 30 min (Experiment 2), aliquots of the cell suspension were removed, washed, and assessed for total cellular GalR activity as described under "Experimental Procedures." The results obtained for cells incubated at 37 °C are expressed as a percentage of the corresponding cells incubated at 4 °C (control). (P_i is inorganic phosphate.)

Condition or addition	GalR activity % of control
Experiment 1	
Buffer	99.9 ± 8.0
Cytosol, fresh	51.8 ± 2.1
Cytosol, 60 min, 37 °C	62.4 ± 10.0
Buffer + 1 mM atractyloside	98.8 ± 2.5
Cytosol, 60 min, 37 °C + 1 mM atractyloside	90.2 ± 5.5
Experiment 2	
Buffer	95.1 ± 4.7
Buffer + 5 mM ATP	56.5 ± 5.3
Buffer + 2.5 mM P_i	91.5 ± 1.9
Buffer + 0.5 mM ADP	87.0 ± 7.0
Buffer + 5 mM glutamate + 2.5 mM malate	87.1 ± 6.7
Buffer + 0.5 mM ADP + 2.5 mM P_i	90.6 ± 6.4
Buffer + 0.5 mM ADP + 2.5 mM P_i + 5 mM glutamate + 2.5 mM malate	66.9 ± 2.8

required in the presence of cytosol to achieve the same level of GalR inactivation compared to ATP alone.

DISCUSSION

The activities of several class I (1) receptors have been shown to be reversibly regulated. Vasopressin and angiotensin II receptors on rat hepatocytes are inactivated by a Ca^{2+} -dependent cytosolic protein. This inactivation is reversed by lowering the pH to 5.5 but not by EGTA (53). The aromatic hydrocarbon receptor in Hepa-1 cells is reversibly inactivated in a time- and temperature-dependent manner in the presence of cytochalasin B. The extent of the loss of ligand binding activity correlates with the depletion of cellular ATP. Reactivation is readily achieved by incubation of cells at 37 °C after removing cytochalasin B (54). The activity of the pregnenolone receptor in guinea pig adrenocortical cytosol requires phosphorylation by a cytosolic kinase. Alkaline phosphatase treatment at pH 9.0 causes its reversible inactivation (55). The calf uterus estradiol-17 β-receptor is also active in the phosphorylated form and inactive when it is dephosphorylated. A nuclear phosphatase and a cytosolic kinase are responsible for the reversible inactivation and reactivation process, respectively (56).

In addition to our reports on inactivation of the hepatic GalR (6, 19, 52), evidence has also been presented by others for the presence or generation of inactive transferrin (57, 58) and mannose (59) receptors. In the case of these class II recycling receptors, which mediate the continuous endocytosis of large amounts of ligand, receptor inactivation may be an important cellular strategy to ensure the efficient segregation of receptor and ligand after their internalization and dissociation. Internalized ligand can be concentrated ~10⁴-fold in endosomes relative to the extracellular concentration. This high ligand concentration could drive the reassociation of ligand and receptor, even though the lower endosomal pH decreases the affinity constant for receptor-ligand complex formation. In other words, the decreased affinity of receptor

for ligand at lower pH could be offset by the dramatically increased ligand concentration in the endosome. This would result in the return of occupied receptors to the cell surface; such receptors would, essentially, be nonfunctional. Transient inactivation of receptors during the segregation of free receptor and ligand would ensure that this separation is efficient. Additionally, receptor inactivation may be an alternative mechanism to mediate receptor-ligand dissociation. Receptor inactivation during constitutive recycling could also provide a mechanism for cells to regulate their endocytic and recycling processes.

The molecular mechanism responsible for the reversible inactivation of GalRs during constitutive recycling (6, 19) is not clear. GalR internalization is a prerequisite for its inactivation, since hyperosmolarity, which stops internalization, also protects against GalR inactivation by chloroquine and monensin (23). The intracellular accumulation of inactive GalRs in ATP-depleted intact cells indicates that both the GalR reactivation and GalR externalization processes require ATP. When cellular ATP levels are restored, the intracellular GalR activity returns to normal levels prior to the cell surface GalR activity, suggesting that GalR reactivation also occurs intracellularly (19). In order to understand the physiological significance of these observations, it is important to identify the biochemical events contributing to the regulation of GalR activity.

Here, we have used permeabilized cells as an *in vitro* system to reconstitute the GalR inactivation observed in intact cells. Incubation of permeabilized hepatocytes at 37 °C in the presence of cytosol results in a reduction in GalR activity. This activity loss represents a real rather than trivial GalR inactivation for several reasons. The activity loss occurs without affecting the ability of the cells to bind anti-GalR antibody. A battery of protease inhibitors did not prevent GalR activity loss. Western blot analysis also shows that equal amounts of GalR with identical subunit sizes are immunoprecipitated from permeabilized control cells or cells in which 60% of all GalRs are inactivated. The extent of reduction in GalR activity does not correlate with the extent of release of lysosomal enzymes. Since GalR activity loss does not occur with dialyzed cytosol, which still contains most of the soluble cell proteins, degradation by proteases is not likely responsible for the decreased GalR activity. These results provide convincing evidence that this loss of ligand binding activity is due to GalR inactivation rather than GalR protein degradation.

Further evidence that the observed GalR inactivation is physiologically relevant is the finding that ATP is required for inactivation. GalRs on permeabilized washed hepatocytes are inactivated without cytosol by incubation in the presence of ATP at 37 °C; other cytosolic components are not essential to reconstitute this GalR inactivation. Other membrane-bound enzymes and/or proteins must interact with ATP and GalR to cause its inactivation, since ATP by itself does not affect the activity of purified GalR.²

Even though ATP could cause GalR inactivation independently of the presence of cytosol, a ~500-fold higher concentration of ATP than present in the cytosol was required to cause maximal GalR inactivation. This apparent inconsistency is explained by the demonstration that permeabilized washed hepatocytes are able to resynthesize ATP continuously in the presence of cytosol. Since mitochondria in mildly permeabilized rat hepatocytes are still intact (25), they are functional and can support ATP synthesis by oxidative phosphorylation. The cytosol provides enzymes, substrates, and cofactors to support glycolysis and oxidative phosphorylation.

This was shown by reconstituting GalR inactivation in permeabilized washed hepatocytes in the absence of cytosol or added ATP by providing substrates for oxidative phosphorylation. In the absence of cytosol or in the presence of dialyzed cytosol, there is no oxidative phosphorylation because of the unavailability of substrates. As a result, much higher concentrations of ATP are required for a comparable extent of GalR inactivation when ATP regeneration cannot occur. Our results are consistent with those of Katz and Wals (27), who reported that mitochondria in hepatocytes permeabilized with 0.1% digitonin for 1–2 min at room temperature are capable of oxidative phosphorylation. All of these results suggest that cytosolic components facilitate GalR inactivation only by maintaining an appropriate cytosolic ATP concentration and that ATP alone is necessary and sufficient for GalR inactivation in permeabilized rat hepatocytes.

In intact hepatocytes, GalR reactivation appears to require ATP (6, 19). However, it is possible that both processes, GalR inactivation and its reactivation, require ATP, but with different K_m values for ATP. Thus, in intact cells, if GalR inactivation has a very low K_m , the process will still occur even in azide-treated, ATP-depleted cells. A residual cellular ATP content of 5% still represents ~150 μ M ATP. The reactivation process may have a substantially higher K_m for ATP and would then be blocked in ATP-depleted intact cells. Efforts to reconstitute GalR reactivation in the permeable cells have so far been unsuccessful. This could be explained if GalR reactivation requires cytosolic components that are either inactive or present at lower concentrations than required.

In the *in vitro* permeable system to reconstitute GalR inactivation, there is always only a 40–60% loss of GalR activity. This is not due to limiting amounts of ATP. This resistance of ~50% of the total cellular GalRs to inactivation is consistent with earlier studies demonstrating the presence of two subpopulations of GalR on rat hepatocytes (49–51). In intact cells exposed to various drugs and metabolic poisons, only ~50% of the total cellular GalRs, corresponding to the State 2 GalR subpopulation, are inactivated (52). The partial inactivation of GalRs in the permeable cells is also due to these State 2 GalRs. Only if active State 2 GalRs are present does GalR inactivation in the *in vitro* system occur. When azide-treated intact hepatocytes, which express only active State 1 GalRs, were permeabilized and incubated at 37 °C in the presence of cytosol no further GalR inactivation occurred.

We propose that the *in vitro* inactivation of GalRs in permeabilized cells reconstitutes part of a normally occurring GalR inactivation and reactivation process. The biochemical changes induced by ATP that result in GalR inactivation still remain to be explained. Possible mechanisms may include phosphorylation of either the GalR or other regulatory membrane components. The ATP requirement may also be to support energy-dependent mechanisms, such as endosome-endosome fusion (60) or the maintenance of normal proton gradients across endosomal membranes (61). The *in vitro* system described here should be extremely useful in answering these and other questions regarding the regulation of GalR activity and function.

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² P. H. Haynes and P. H. Weigel, unpublished observation.

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