Monensin Inhibits Intracellular Dissociation of Asialoglycoproteins from Their Receptor

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ABSTRACT Treatment of short-term monolayer cultures of rat hepatocytes with the proton ionophore, monensin, abolishes asialoglycoprotein degradation, despite little effect of the drug on either surface binding of ligand or internalization of prebound ligand. Centrifuging cell homogenates on Percoll density gradients indicates that, as a result of monensin treatment, ligand does not enter lysosomes but sediments instead in a lower density subcellular fraction that is likely an endocytic vesicle. Analyzing the degree of receptor association of intracellular ligand revealed that monensin prevents the dissociation of the receptor-ligand complex that normally occurs subsequent to endocytosis. The weak base, chloroquine, also blocks this intracellular dissociation. Evidence from sequential substitution experiments is presented, indicating that monensin and chloroquine act at the same point in the sequence of events leading to ligand dissociation. These data are discussed in terms of a pH-mediated dissociation of the receptor-ligand complex within a prelysosomal endocytic vesicle.

Streptomyces cinnamonensis produces a carboxylic acid ionophore called monensin that can mediate proton exchange for monovalent cations, preferably sodium (1). Monensin, when added to eucaryotic cells, has been shown to have a variety of effects on membrane dynamics. Tartakoff and Vassalli (2, 3) demonstrated that the transit of membrane vesicles from the Golgi complex to the plasma membrane was disrupted by monensin or the functionally related ionophore, nigericin. Consequently, the normal cellular traffic of both secretory proteins (2, 4, 5) and newly synthesized membrane proteins (5, 6) is perturbed. In addition to these processes that involve introducing membrane to the cell surface, monensin also appears to interfere with a number of events related to endocytosis including both fluid-phase pinocytosis (7) and the receptor-mediated uptake of low density lipoproteins (8). Monensin also protects cells from killing by diphtheria toxin (9) and infection with certain enveloped viruses (10, 11), both of which are receptor-mediated endocytic processes.

On the basis of these observations, we have examined the effect of monensin on the uptake and catabolism of asialoglycoproteins by cultured hepatocytes. This process is among the most extensively studied examples of receptor-mediated endocytosis (12, 13). By virtue of these studies, the system affords certain unique advantages that may facilitate the understanding of monensin action and, in turn, the process of receptor-mediated endocytosis itself. The hepatic receptor for asialoglycoproteins has been isolated and its binding properties have been characterized (12, 13). Antibodies are available that recognize topographically distinct portions of the receptor molecule (14), and we have recently prepared and characterized monoclonal antibodies to the receptor (15). In addition, an assay has been developed that can distinguish intracellular ligand that is bound to the receptor from that which has dissociated from the receptor subsequent to endocytosis (16). We have applied this assay in the current study to demonstrate that monensin markedly inhibits receptor-ligand dissociation. The results of complementary hepatocyte fractionation experiments indicate that monensin treatment results in the failure of ligand to enter lysosomes. Instead, it accumulates in an intermediate subcellular fraction that likely represents an endocytic vesicle.

MATERIALS AND METHODS

Rat hepatocytes were isolated as described (16) and cultured at 3 × 10⁶ hepatocytes per 60-mm Lux Contur dish (Lux Scientific Inc., Newbury Park, CA) in 3 ml of medium consisting of Waymouth's 752/1 (Gibco Laboratories, Gibco Div., Lawrence, MA), 25 mM Hepes pH 7.2, 5% heat inactivated fetal bovine serum (Gibco Laboratories), 1.7 mM additional CaCl₂, 5 μg/ml bovine insulin (Gibco Laboratories), 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Cultures were maintained in a 5% CO₂ atmosphere at 37°C. At 4 h into the culture, the medium was changed, and at the time of experiments, we judged adherent cells >90% viable by trypan blue exclusion. 1 h prior to use in experiments, the medium was replaced with serum-free medium. In this kind of experiment, little qualitative variation was observed in different preparations of hepatocytes.

125I-asialo-orosomucoid was prepared as described (17). The ligand (1 μg) was added to the hepatocyte monolayers in 1 ml of ice-cold serum-free medium. After 60 min at 4°C, unbound ligand was removed by four washes with 1.5 ml of cold serum-free medium. The third wash contained 0.5 mM N-acetylgalactosamine.
This concentration of GalNAc removed <10% of the bound ligand but decreased variability in the solubilization-precipitation assay for receptor-bound versus unbound ligand (16). Washed cultures of hepatocytes were warmed by adding 1.5 ml of serum-free medium at 37°C. To assess ligand degradation, we added an aliquot of the medium to an equal volume of ice-cold 20% trichloroacetic acid, 4% phosphotungstic acid, and after 10 min on ice, the amount of acid-soluble radioactivity was determined. Radioactivity that could be released from the cells by washing with ice-cold 0.15 M NaCl, 0.01 M Tris-HCl pH 7.6, containing either EGTA (20 mM) or GalNAc (50 mM) was taken as a measure of surface-bound ligand. To determine the degree of association of intracellular 

125I-asialo-orosomucoid with receptor, we solubilized EGTA-washed cells with Triton X-100 and we precipitated the receptor-ligand complex with ammonium sulfate as described (16). Evidence for the validity of this assay in distinguishing receptor-bound and unbound ligand has been presented (16, 18). Monensin (Calbiochem-Behring Corp., San Diego, CA) and chloroquine (Boehringer Mannheim Biochemicals, Indianapolis, IN) were dissolved in absolute ethanol and H2O, respectively, at 50 mM, and these stock solutions were stored at -20°C. These agents were diluted (≤500-fold) into serum-free medium and added to the cells along with the 125I-asialo-orosomucoid. All subsequent washes and incubation media contained the same concentrations (0–100 μM) of the agents. In reversibility experiments, we found that washing with complete medium (containing 5% serum) markedly facilitated monensin removal.

For density gradient centrifugation, cells were homogenized in 0.28 M sucrose, 2 mM CaCl2, 0.01 M Tris-HCl, pH 7.6, (homogenization buffer) in a Dounce homogenizer by 20 complete strokes of a tight-fitting pestle (Kontes Co., Vineyard, NJ). The homogenates were centrifuged at 280 g for 10 min, and the supernatant was made 20% in Percoll (Pharmacia Fine Chemicals. Div. of Pharmacia, Inc., Piscataway, NJ) by adding one-third volume of 80% Percoll in homogenization buffer. After thorough mixing, the samples were centrifuged at 10,000 g in a Beckman 65 rotor (Beckman Instruments, Inc., Fullerton, CA) to a final ω2 value of 4.55 × 108 (-45 min). Ten-drop fractions (∼0.3 ml) were collected from the bottom of the resultant gradients and the radioactivity content of each fraction was determined. The lysosomal marker enzyme hexosaminidase was assayed according to Hall et al. (19) in aliquots of the gradient fractions in the presence of 0.5% Triton X-100.

### RESULTS

The effect of monensin on the binding of 125I-asialo-orosomucoid to cultured hepatocytes was examined by incubating the cells at 4°C with the ligand in the presence of increasing concentrations of monensin. As shown in Fig. 1, no appreciable effect on ligand binding was seen over a monensin-concentration range of 10^{-8} to 10^{-4} M. After washing to remove unbound ligand, cells were warmed to 37°C in medium containing the monensin concentration present during binding (0–10^{-4} M). In control cultures, this elevation in temperature resulted in the internalization of >80% of the prebound 125I-asialo-orosomucoid within 15 min. The extent of internalization was not significantly altered by monensin at the concentrations tested (Fig. 1). Warming the cell cultures without monensin for longer periods of time (>30 min) led to the appearance of the acid-soluble radioactivity in the medium, indicating lysosomal degradation of the ligand. Although we observed no appreciable effect of monensin on ligand binding or internalization of prebound ligand, acid-soluble radioactivity in the culture medium was completely abolished ≥10 μM, and was reduced by ~50% at 1 μM. Thus, whereas the hepatocytes bind and internalize ligand, endocytosis in the presence of monensin (≥10 μM) does not result in catabolism of the asialo-glycoprotein.

We next endeavored to determine which step(s) in the overall process of ligand catabolism was blocked by the ionophore. Monensin, by collapsing proton gradients (1), would be expected to raise the pH of lysosomes. Because many lysosomal hydrolases have acidic pH optima, monensin might act by preventing lysosomal degradation of ligand. To address this possibility, we fractionated cell homogenates on self-forming Percoll gradients (Fig. 2). Radio-labeled asialo-orosomucoid was bound to cells in the absence or presence of 50 μM monensin, and the cells were washed free of unbound ligand and then warmed to 37°C for 15 or 45 min. The material associated with the homogenates of unwarmed cells banded in the Percoll gradient as a symmetrical peak of radioactivity with its maximum in fraction 6 (Fig. 2 A). This peak was not affected by the presence of 50 μM monensin. All of this radioactivity could be displaced from the intact cells by washing with medium containing 20 mM EGTA, indicating a cell surface localization of this radioactivity. After the cells had been warmed to 37°C for 45 min, <20% of the cell-associated ligand was released by the EGTA or 50 mM GalNAc wash. Hepatocytes containing this intracellular (50 mM GalNAc-resistant) radioactivity were subjected to homogenization and centrifugation on Percoll density gradients (Fig. 2 B). The major peak of radio-labeled ligand had a modal density (fraction 3) lower than the cell surface radioactivity seen in 2 A. A second broader peak in fractions 20–30 was also seen in the warmed cultures. This pattern was not affected by exposing the cells to 50 μM monensin. Also shown in Fig. 2 B is the distribution of the lysosomal enzyme, β-hexosaminidase. Lysosomal localization to the bottom of the gradients is independent of monensin treatment. Clearly, neither of the peaks of radioactivity coincides with this lysosomal marker, and little if any ligand is in the lysosome-rich portions of the gradient. After 45 min at 37°C, appreciable ligand degradation had occurred in the control hepatocyte cultures. The residual cell-associated radioactivity was coincident with the lysosomal enzyme in the bottom of the gradient (Fig. 2 C). In sharp contrast to control cultures, the hepatocytes, warmed for 45 min in the presence of 50 μM monensin, yielded a gradient pattern essentially unchanged from the patterns of the 15-min cultures in 2 B. From these results, we concluded that the monensin inhibition of asialo-glycoprotein degradation could not be ascribed to reduced ability of lysosomal hydrolases to degrade the ligand.

![Figure 1](https://example.com/figure1.png)

**Figure 1** Effect of monensin on the pathway of asialo-glycoprotein catabolism. The surface receptors of hepatocytes in monolayer culture were occupied by incubation with 125I-asialo-orosomucoid (1 μg/ml) in the presence of the indicated concentration of monensin. After being washed with serum-free medium containing the same concentration of monensin, surface-bound ligand was assessed by determining the radioactivity released by a wash with ice-cold buffer containing 20 mM EGTA. Other cultures were warmed for 15 min to 37°C and the decrease in EGTA-releasable radioactivity was taken as a measure of the internalization of ligand. Degradation of ligand was assessed by determining acid-soluble radioactivity in the medium after 45 min at 37°C. Data for surface binding ([C]), ligand internalization ([O]), and ligand degradation ([X]) represent the average of duplicate culture dishes, and are expressed as a percentage of the value obtained with no monensin present. Analogous determinations were performed using cultures to which a 100-fold excess of unlabelled asialo-orosomucoid had been added. Inclusion of the unlabelled ligand resulted in >90% reduction in the values obtained. The residual nonspecific values were subtracted in the calculations.
Rather, the $^{125}$I-asialo-orosomucoid never entered the lysosomes but remained instead in what are likely lower-density endocytic vesicles.

Previously, we have presented evidence that the intracellular dissociation of asialoglycoproteins from their receptor is an intermediate step in the overall process of ligand catabolism (16). This conclusion was based on data obtained using an assay capable of distinguishing receptor-bound $^{125}$I-asialo-orosomucoid from the unbound ligand. In this assay, cells are solubilized in nonionic detergent and the receptor-ligand complex is selectively precipitated with ammonium sulfate. On the basis of known acid lability of the interaction of asialoglycoproteins with their receptor, we suggested that intracellular dissociation might occur via an encounter with an acid environment. If this hypothesis was correct, a proton ionophore would be anticipated to raise the pH of such an environment and consequently might prevent the dissociation step in the pathway leading to asialoglycoprotein degradation. To test this possibility, we first allowed cells to internalize $^{125}$I-asialo-orosomucoid and washed free of unbound ligand. Cultures were subjected to homogenization and Percoll density gradient centrifugation, either unwarmed (A), or after 15 min (B) or 45 min (C) at 37°C. All solutions either contained no monensin (O) or monensin at a concentration of 50 μM (O). The cultures represented in B and C had been washed with an ice-cold buffer containing 50 mM N-acetylgalactosamine prior to homogenization and hence depict the distribution of intracellular ligand. The enzyme, β-hexosaminidase, was measured in control (O) and monensin-treated (A) cultures by the ability of gradient fractions to catalyze hydrolysis of p-nitrophenyl-β-D-N-acetylglucosaminide with 0.5% Triton X-100 present (19). The p-nitrophenol produced was measured at 400 nm.

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The inhibition of ligand dissociation is reversible. Hepatocytes with $^{125}$I-asialo-orosomucoid that had been internalized in the presence of 50 μM monensin were washed in ice-cold, monensin-free medium. The intracellular ligand remained receptor-bound at 4°C, but upon warming again to 37°C, the ligand dissociated from the receptor (Fig. 4). Degradation of ligand, evidenced by acid-soluble radioactivity in the media, also resumed after removal of monensin (data not shown). The reversibility of the monensin inhibition afforded an opportunity to examine the acidification of the nonlysosomal compartment in which the receptor-ligand complex accumulates during the monensin block. A proton translocating ATPase has been implicated in the acidification of lysosomes (20, 21). To determine whether cellular energy stores were required for the intracellular release of $^{125}$I-asialo-orosomucoid, 0.1% sodium azide and 50 mM 2-deoxyglucose were added to the cells 15 min prior to removal of monensin. After 15 min of exposure to these concentrations of the metabolic inhibitors, total cellular

![Figure 2](image_url) Subcellular fractionation of cultured hepatocytes: effect of time at 37°C and monensin. Monolayer cultures of hepatocytes were incubated with $^{125}$I-asialo-orosomucoid and washed free of unbound ligand. Cultures were subjected to homogenization and Percoll density gradient centrifugation, either unwarmed (A), or after 15 min (B) or 45 min (C) at 37°C. All solutions either contained no monensin (O) or monensin at a concentration of 50 μM (O). The cultures represented in B and C had been washed with an ice-cold buffer containing 50 mM N-acetylgalactosamine prior to homogenization and hence depict the distribution of intracellular ligand. The enzyme, β-hexosaminidase, was measured in control (O) and monensin-treated (A) cultures by the ability of gradient fractions to catalyze hydrolysis of p-nitrophenyl-β-D-N-acetylglucosaminide with 0.5% Triton X-100 present (19). The p-nitrophenol produced was measured at 400 nm.

![Figure 3](image_url) Effect of monensin on intracellular dissociation of the receptor-asialoglycoprotein complex. Experimental design was as described in Fig. 1. After 45 min at 37°C the amount of acid-soluble radioactivity in the medium was determined. The hepatocyte monolayers were then washed with ice-cold buffer containing 20 mM EGTA and the EGTA-resistant (intracellular) radioactivity subjected to the solubilization-precipitation assay for distinguishing receptor-bound and unbound ligand. Data for degraded ligand (X) and receptor-bound ligand (O) represent average of duplicate dishes and are expressed as percentages of the internalized ligand. Values obtained including a 100-fold excess of unlabeled asialo-orosomucoid have been subtracted.

![Figure 4](image_url) Reversibility of the monensin inhibition of ligand dissociation. Cells were incubated with $^{125}$I-asialo-orosomucoid at 4°C in the absence or presence of 50 μM monensin. After being washed with serum-free medium containing the same concentration of monensin, the hepatocytes were warmed for the indicated times to 37°C. The culture dishes were then washed with ice-cold buffer containing 20 mM EGTA and the intracellular (EGTA-resistant) radioactivity subjected to the solubilization-precipitation assay for receptor-bound ligand. In the unwarmed cultures (0 min), the EGTA wash was omitted. After 45 min at 37°C (arrow), monensin was removed from a portion of the culture dishes by washing with ice-cold medium containing 5% fetal bovine serum. Incubation was then continued either at 37°C or at 4°C in serum-free medium with or without monensin. Results are expressed as the percentage of assayed ligand that was receptor-bound in the solubilization-precipitation assay. O, no monensin; O, with 50 μM monensin, sham-washed at 45 min; V, monensin removed at 45 min and incubation continued at 4°C; X, monensin removed at 45 min and incubation continued at 37°C; O, 0.1% sodium azide, 50 mM 2-deoxyglucose added at 30 min and monensin removed at 45 min, followed by continued incubation at 37°C with azide/2-deoxyglucose present.
Our results show that the proton ionophore, monensin, prevents a specific molecular event in the pathway of asialoglycoprotein endocytosis, i.e., the dissociation of ligand from receptor. This dissociation normally follows the endocytosis of the receptor-ligand complex by hepatocytes (16). Chloroquine, a diffusible weak base, has the same effect and appears to act at the same point in the dissociation process. Both agents would be expected to result in elevation of the pH of acidic compartments within the cell, although distinctly different mechanisms are involved. In view of the acid lability of the interaction between asialoglycoproteins and their hepatic binding protein (18, 24), we have interpreted our results in terms of a low pH-mediated intracellular dissociation. The involvement of an acidic endocytic vesicle may be common to a number of endocytic processes. Based on fluorescence excitation spectra of fluorescein-conjugated ligands, it has been shown that α2-macroglobulin (25, 26) and transferrin (27) enter environments of below pH 6 in mouse fibroblasts and human erythroleukemia cells, respectively. Moreover, monensin and weak bases elevate the pH of these endocytic vesicles as judged by an increase in fluorescein-α2-macroglobulin fluorescence (26). Diminished interaction at low pH with their respective receptors has been reported for α2-macroglobulin (26), insulin (28), epidermal growth factor (26, 29), lysosomal enzymes (30), and neutrophil chemotactic peptide (31). The delivery into the cytoplasm of diphtheria toxin A subunit (9, 32, 33) and the nucleocapsid of Semliki Forest virus (11, 36, 37) may occur from an acidic endocytic vesicle. Lowered pH appears to be critical in the conformational change required for the diphtheria toxin fragment’s penetration of membranes and the membrane fusion event involved in delivery of the viral nucleocapsid. Weakly basic amines as well as monensin have been shown to protect cells from diphtheria toxin (9, 32-35) and certain enveloped viruses (10, 11, 36, 37). It may well be that the bacteria and viruses utilize the cellular mechanism for acid-mediated dissociation of endocytosed proteins from their receptors.

It is clear from the density gradients of Fig. 2 that monensin, in addition to inhibiting ligand dissociation, prevents delivery of ligand to lysosomes. It is possible that failure to dissociate results in the lack of movement of ligand to lysosomes. Alternatively, the elevation of the intravesicular pH may, in itself, prevent vesicular movement to, and/or fusion with, lysosomes irrespective of the state of association of receptor and ligand.

Our results suggest that, following entry of the receptor-ligand complex, dissociation of this complex occurs in a non-lysosomal cellular locus and is pH-mediated. This may be a critical event in sparing the asialoglycoprotein receptor for reutilization (38, 39). Monensin (8) and amines (30, 40-46) appear to affect receptor recycling in a number of systems. Recently, Baenziger (47) has reported that delivery of asialoorosomucoid to lysosomes is not required for receptor reutilization. In that study, exposing cells to a high potassium/low sodium buffer resulted in internalization but no degradation of ligand. Radio-labeled ligand appeared to be associated with a subcellular fraction of density similar to that of plasma membranes. In another related study, Dunn et al. (48) have shown that at reduced temperature (18°C), ligand is not delivered to lysosomes. The authors found lactosaminated ferritin particles juxtaposed to the inner surface of endocytic vesicles, and suggested that this ligand remained bound to the asialoglyco-

### Table I

*Equivalence of Monensin and Chloroquine in the Inhibition of Intracellular Dissociation of Ligand*

| Incubation 1 | Incubation 2 | Internal ligand unbound % |
|-------------|-------------|---------------------------|
| Control     | None        | None                      |
| Chloroquine | Chloroquine | Chloroquine               |
| Monensin    | Monensin    | 9.3                       |
| Reversibility | Monensin | Monensin                  |
| Chloroquine | Chloroquine | 10.4                      |
| Substitution | Chloroquine | Chloroquine              |
| Monensin    | Monensin    | 9.0                       |
| Chloroquine | Chloroquine | 9.0                       |

Experimental design was as described in Fig. 4. Cells were incubated for 60 min at 4°C with asialoorosomucoid in the presence of the agents listed under Incubation 1, and unbound ligand was removed by washing in serum-free media containing the indicated agent. Monensin and chloroquine were each used at a concentration of 50 μM. After being washed, hepatocytes were warmed for 10 min to 37°C (Incubation 1) and then washed with complete media containing the agent listed under Incubation 2. These agents were also included in the serum-free media used to warm the cells to 37°C for an additional 10 min (Incubation 2). Following this incubation, cells were washed with ice-cold buffer containing 20 mM EGTA and the internal (EGTA-resistant) radioactivity subjected to the solubilization-precipitation assay. Data represent average of duplicate cultures dishes and are expressed as the percentage of the assayed ligand that was receptor-bound. Analogue determinations were performed using cultures to which a 10-fold excess of unlabeled asialoorosomucoid had been added. These values were subtracted in the calculation.
protein receptor at the lower temperatures. Thus, there are apparently a number of ways to perturb the endocytic pathway such that ligand does not reach the cell’s degradative machinery. The use of our assay for distinguishing receptor-bound and unbound ligand has enabled us to identify a molecular event in the pathway that is inhibited by monensin.

Perturbants of organelle pH, especially diffusible weak bases, are often referred to as lysosomotropic (22). This term can be neutralized by weak bases or ionophores. Clearly, inhibiting such a number of ways to perturb the endocytic pathway apparently a number of ways to perturb the endocytic pathway such that ligand does not reach the cell’s degradative machinery.

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