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Effects of Hyperosmolarity on Ligand Processing and Receptor Recycling in the Hepatic Galactosyl Receptor System

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Binding, endocytosis, and degradation of asialo-orosomucoid (ASOR) mediated by the galactosyl (Gal) receptor were examined in isolated rat hepatocytes in complete media supplemented with an osmolite. The specific binding of ^{125}I-ASOR to cells at 4°C was unaffected by up to 0.4 M sucrose or NaCl. Unlike sucrose or NaCl, mannitol stimulated ^{125}I-ASOR binding at low concentrations but inhibited binding at higher concentrations. Continuous internalization at 37°C, which requires receptor recycling, was completely blocked at 0.2 M sucrose or 0.15 M NaCl, corresponding in each case to a total osmolality of about 550 mmol/kg. This effect was reversed and endocytic function was restored by washing the cells, indicating that cell viability was unaffected. The rate of degradation of internalized ^{125}I-ASOR was also inhibited by increasing sucrose concentrations. This inhibition is due to a block in the delivery of ligand to lysosomes and not an effect on degradation per se. In the presence of 0.2 M sucrose, the rate and extent of endocytosis of surface-bound ^{125}I-ASOR were, respectively, 33.0 ± 8.1% and 69.4 ± 10.5% (n=8) of the control without sucrose. Under these conditions, the dissociation of internalized receptor-ASOR complexes was completely inhibited. When sucrose was added, the effect on the endocytosis of surface-bound ^{125}I-ASOR was virtually immediate. Previous studies showed that about 40% of the surface-bound ^{125}I-ASOR which is internalized can return to the cell surface still bound to receptor (Weigel and Oka: J Biol Chem 259:1150, 1984). If 0.2 M sucrose was added after endocytosis occurred, ^{125}I-ASOR still returned to the cell surface, although the rate and extent of return were inhibited by more than 50%. Interestingly, hyperosmolarity is the only treatment we have found which can reversibly inhibit, although only partially, the endocytosis of surface-bound ^{125}I-ASOR.

Key words: asialoglycoproteins, endocytosis, ligand dissociation, sucrose, protein degeneration

The hepatic asialoglycoprotein or Gal receptor has been a good model system for studying receptor-mediated endocytosis and receptor recycling [1,2]. We have

Abbreviations used: Gal, galactosyl; ASOR, asialo-orosomucoid; BSA, bovine serum albumin; EGTA, ethyleneglycol bis (beta-aminoethyl ether) - N,N,N^1,N^1,-tetraacetic acid.

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been particularly interested in understanding the molecular mechanisms which control and regulate the separation and segregation of ligand and receptor into different cellular pathways [3]. Inhibitors which differentially affect various stages in these complicated processes are useful in elucidating and defining these pathways. For example, monensin and colchicine differentially affect, respectively, ligand dissociation [4–6] and ligand degradation [7]. These compounds have been very useful to investigators studying this and other receptor systems. In an effort to find other conditions which inhibit ligand processing or receptor recycling we have examined the effect of increasing osmotic strength on the function of the Gal receptor system. Daukas and Zigmond found that receptor-mediated but not fluid-phase endocytosis of chemotactic peptide in polymorphonuclear leukocytes was preferentially inhibited by increasing the osmolarity of the medium [8]. In a similar study we also found that endocytosis mediated by the Gal receptor system in hepatocytes is preferentially inhibited by hyperosmolarity, whereas fluid phase pinocytosis is unaffected (J.A. Oka and P.H. Weigel, J Cell Biol 105:311a, 1987).

Hyperosmolar conditions have been employed by other investigators as a perturbant to study a variety of cellular processes. When animal cells are exposed to hyperosmolar media, rapid changes can occur in a number of structural and functional properties including protein synthesis, cell volume and water content, and cell morphology [9,10]. Primary mouse bone marrow cultures grew better in medium of slightly higher osmolarity than plasma, which in turn is of greater osmolarity than the defined culture media normally used to grow these cells [11]. Cultured fibroblasts chronically exposed to hyperosmolar medium are able to adapt, to reestablish normal rates of protein synthesis, and to continue growth at normal rates [12,13]. Lysosomal enzyme defects in cultured skin fibroblasts from patients with I-cell disease were corrected when the medium osmolarity was increased by addition of 88 mM sucrose [14]. After 10 days in hyperosmotic medium, the activities of deficient hydrolases reached their normal levels and morphologically the cells were normal; inclusion materials usually seen in the defective cells were lost. Hyperosmotic medium containing sucrose has also been used to study the kinetics of cortical vesicle fusion during exocytosis in sea urchin eggs [15].

The present study was undertaken to further characterize the effect of hyperosmolarity on the Gal receptor system and to determine whether one or more steps in the overall process could be preferentially affected. A preliminary report of these results has been presented [16].

EXPERIMENTAL PROCEDURES

Materials

ASOR was prepared and iodinated using $Na^{125}I$ (Amersham, 10–20 mCi/μg iodine) and iodogen (Pierce Chem. Co.) as described previously [17]. Digitonin, from Sigma, was dissolved with heating to 1.4% (w/v) in 100% ethanol. Sucrose, mannitol, and sodium chloride were ACS reagent grade from Merck. Other chemicals were also reagent grade. Medium 1 contains a modified Eagle's medium (Gibco catalogue #420-1400) supplemented with 2.4 g/liter Hepes, pH 7.4, 0.22 g/liter $NaHCO_3$. Medium 1/BSA also contains 0.1% (w/v) BSA.

Cell Preparation

Hepatocytes were prepared from male Sprague-Dawley rats (150–250 g) by a modification of the collagenase perfusion procedure of Seglen [18]. Perfusion buffers

have been described by Seglen [18]. Animals are anaesthetized with ether; the abdominal cavity is exposed; and the portal vessel beyond the mesenteric branch is cannulated. After tying the cannula, perfusion of oxygenated buffer 1 at 37°C (52 ml/min) is immediately begun and the diaphragm, aorta, and inferior vena cava are severed to allow the animal to die and to provide an exit for the perfusate. During a 10-min perfusion of buffer 1, the liver is excised and placed on a stiff nylon mesh grid above a funnel designed to collect and recirculate the perfusate. Collagenase, preweighed to give 100–120 μg/g body weight, is dissolved in 5 ml of perfusion buffer 2 at 4°C just prior to beginning the surgery. The solution is filtered through a 0.45-μm filter and stored on ice for about 15 min while cannulation and excision are performed. The solution is then added to 55 ml of filtered buffer 2 at 37°C and recirculated through the liver for 10 min. Exact amounts of collagenase and the times of digestion are optimized for each batch of collagenase used. The temperature of the liver is kept between 32°C and 37°C. Cell viability is usually improved if the duration of the collagenase perfusion is $\leqslant$12 min. The liver is then placed in a 100-mm petri dish with buffer 1/BSA. All subsequent steps are done at room temperature using buffers at 30–37°C. Forceps are used to peel off the liver capsule and shake out cells. The liver is shaken to release cells three or four times in about 25 ml of buffer 1/BSA each time, and the pooled cell suspensions are successively filtered through 100-, 35-, and 27-μm nylon mesh filters (from Tetko, Inc., Elmsford, NY) to remove aggregates. The final cell filtrate is purified by four differential centrifugations at ~80g for 2 min. The last two washes are in buffer 3. The final cell pellets are resuspended in ice-cold medium 1/BSA. The cells are stored at 4°C until the experiment is begun. The cells were usually >90% viable and single cells. Cell viability was determined by trypan blue exclusion. Viability was not affected by up to 0.25 M sucrose, 0.2 M sodium chloride, or 0.8 M mannitol.

Experiments were performed in the absence of serum. Before use in most experiments, the cells were incubated at 37°C for 1 hr in medium 1/BSA to increase and stabilize the surface and total cell receptor content [19]. Cell incubations at 37°C were in Erlenmeyer flasks in a gyratory water bath shaking at 100 rpm. Cell suspensions were at 2–4 $\times$ 10^6/ml in medium 1/BSA. In experiments with surface-bound ligand, one flask was employed for each time point and the starting times for the incubation of a set of samples were staggered so that they finished together.

Determination of Receptor Activity, Endocytosis, and Degradation

Cell-surface receptor content was assessed by the binding of ^{125}I-ASOR (1.5 μg/ml) to suspensions of hepatocytes (4 $\times$ 10^6/ml in medium 1) at 4°C for 60 min. Greater than 95% occupancy of surface receptors was also achieved this way for experiments with cells containing prebound ligand. Cells were then washed two to three times by centrifugation [20]. Specific binding, which was usually 90%, was assessed in the presence of a 50-fold excess of unlabeled ASOR or by the ability of 7.5 mM EGTA to remove or prevent binding of ^{125}I-ASOR. Total cell receptor content, both internal and surface, was assessed in the presence of 0.055% (w/v) digitonin as described previously [21]. The amount of internalized glycoprotein was determined by diluting samples of cell suspension into 3 volumes of ice-cold medium 1/BSA containing a final concentration of 7.5 mM EGTA. After $\geqslant$ 10 min on ice, the cells were centrifuged, resuspended in Hanks, and centrifuged again. Degradation

of ^{125}I-ASOR was determined by measuring acid-soluble radioactivity by using 10% phosphotungstic acid dissolved in 2 N HCl [17].

Osmolarity

Osmolites were dissolved to a maximum concentration of 1.6 M in deionized distilled water. Medium 1 was concentrated twofold in a Buchi rotary evaporator. One volume of the concentrated media and an equal volume of 1.6 M osmolite were combined to give a stock solution of 0.8 M osmolite in medium 1. This medium was then added to a cell suspension to give the desired final concentration of osmolite. BSA was also included in most experiments at a final concentration of 0.1–0.4%. In this way the osmolarity of the medium was changed without altering the concentration of nutrients in medium 1. Osmolality was measured in units of mmol/kg by using a Wescore vapor pressure osmometer (model 5100C). The osmolality of medium 1/BSA was 264 mmol/kg; 0.8 M concentrations of the following osmolites increased the osmolality by the indicated amount: mannitol = 949 mmol/kg, sucrose = 1171 mmol/kg, and sodium chloride = 1,522 mmol/kg. Medium 1 contains 0.116 M sodium chloride and 0.06 M glucose.

General

A Packard multi-prias 2 gamma counter was used to determine ^{125}I radioactivity. Protein was determined by the method of Bradford [22] with BSA as the standard. Cells were centrifuged in a Sorvall GLC-1 at 800 rpm for 2 min.

RESULTS

Effect of Hyperosmolarity on the Binding of ^{125}I-ASOR

Several different osmolites were chosen in initial experiments to assess the effects of increasing osmolarity on the Gal receptor system. Sucrose, mannitol, and sodium chloride were picked since they have been used by numerous investigators for similar purposes. Mannitol was initially considered to be the best choice because it is both uncharged and is not metabolized by most eukaryotic cells. Sodium chloride is both an osmolite and an electrolyte, and therefore its effects are complicated. The binding of ^{125}I-ASOR to intact cells at 4°C was only moderately affected by increasing sucrose or sodium chloride concentrations (Fig. 1). However, binding was actually stimulated and then inhibited in a biphasic manner by mannitol. In the presence of digitonin to permeabilize the cells and gain access to intracellular receptors [21], sucrose and sodium chloride again showed little effect on binding with increasing concentrations. In this case mannitol caused an inhibition of ^{125}I-ASOR binding to a maximum of about 80% at 0.4 M (Fig. 1). In a related experiment the ability of these three compounds to displace previously bound ^{125}I-ASOR from either cell surface or internal receptors was examined at 4°C (Fig. 2). Sucrose and sodium chloride again were essentially without effect on the retention of bound ligand whereas mannitol apparently acted as a competitor releasing ligand bound to both surface and intracellular receptors.

Effect of Hyperosmolarity on the Endocytosis of ^{125}I-ASOR

Increasing concentrations of sucrose in the medium to achieve various levels of hyperosmolarity resulted in a decrease in the steady-state continuous rate of endocy-

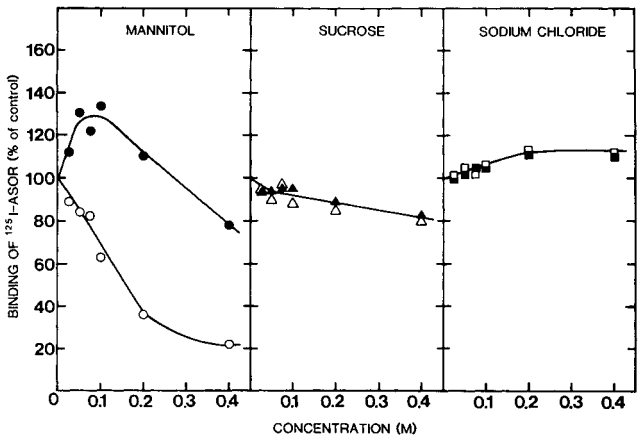

Fig. 1. Effect of osmolite concentration on the binding of ^{125}I-ASOR. Cells were treated with (○, △, □) or without (●, ▲, ■) 0.055% digitonin for 10 min at 4°C, centrifuged, and resuspended in medium 1/BSA without digitonin. They were allowed to bind ^{125}I-ASOR on ice for 60 min in the presence of mannitol (●, ○), sucrose (▲, △), or sodium chloride (■, □). The cells were washed free of nonbound ^{125}I-ASOR and bound radioactivity was determined.

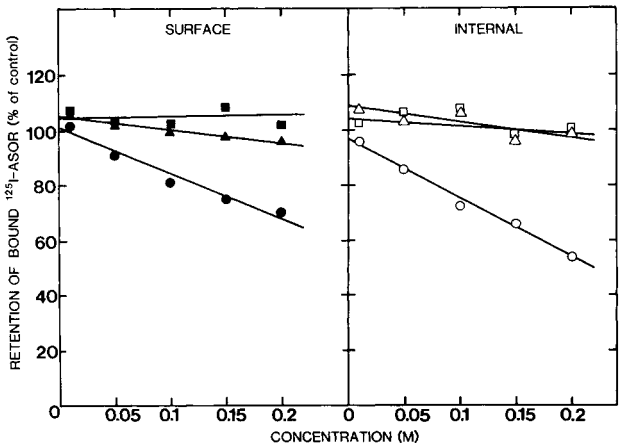

Fig. 2. Effect of osmolite concentration on the retention of ^{125}I-ASOR bound to surface or intracellular Gal receptors. Nonradioactive ASOR or ^{125}I-ASOR was first bound to the surface of intact cells at 4°C for 1 hr. All cells were then washed to remove nonbound ligand and treated with 0.055% digitonin to gain access to internal receptors. Cells with nonradioactive ASOR bound on the surface were incubated for 1 hr at 4°C with ^{125}I-ASOR (○, □, △) and those with ^{125}I-ASOR on the surface were incubated with nonradioactive ASOR (●, ■, ▲). The cells were washed to remove nonbound ligand. Aliquots of the cells were then incubated on ice for 30 min, washed, and again incubated 30 min with the indicated concentration of mannitol (●, ○), sucrose (▲, △), or sodium chloride (■, □). The cells were then washed and radioactivity remaining with the cell pellet was determined.

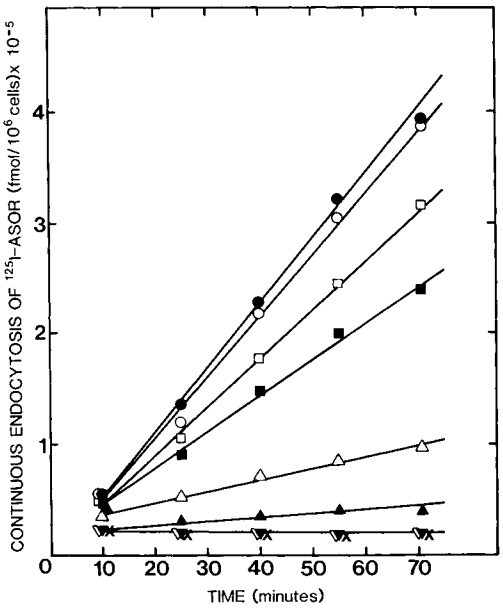

Fig. 3. Effect of sucrose on the continuous endocytosis of ^{125}I-ASOR. Cells were incubated in medium 1/BSA at 37°C with 7.1 μg/ml ^{125}I-ASOR in the absence (○) or presence of the following final concentrations of sucrose: (●) 0.025 M, (□) 0.075 M, (■) 0.1 M, (△) 0.15 M, (▲) 0.2 M, (▽) 0.25 M, (▼) 0.3 M, (x) 0.5 M. Samples were diluted into cold medium 1/BSA containing EGTA and washed, and cell-associated radioactivity was determined.

tosis of ^{125}I-ASOR at 37°C (Fig. 3). In all cases the rate of internalization was linear for more than 1 hr. Continuous endocytosis which requires receptor recycling was completely inhibited at concentrations of $\geqslant$0.2 M sucrose. Similar results were obtained with sodium chloride. When the rates of continuous endocytosis were plotted as a function of the osmolality of the medium, a sharp dropoff was observed for both sucrose and sodium chloride (Fig. 4). These values fell on identical curves. The coincidence of the dose-response curves with these two compounds suggests that their effects are related to increased osmolality and not some other property. In contrast, mannitol again behaved differently. Small increases in osmolality with mannitol caused a stimulation before a more gradual inhibition of the rate of endocytosis was observed (Fig. 4). The insert in Figure 4 shows the effect of the three osmolites on the rate of continuous endocytosis as a function of their concentration expressed in molarity. The inhibition of continuous endocytosis was very sensitive to increasing osmolality with sucrose or sodium chloride. Endocytosis was completely shut down when the osmolality of the medium was increased to approximately 550 mmol/kg, which is roughly twice that of the normal medium. In subsequent experiments mannitol was not used to vary the osmotic strength of the medium since it appears to interact with the Gal receptor. Sodium chloride was also not chosen because of the problems related to its charge. Therefore, in most of the following experiments hyperosmolarity was achieved with increasing concentrations of sucrose.

To determine whether the inhibition of endocytosis by hyperosmolarity represented irreversible damage to the cells, attempts were made to remove the osmolite from the medium and to determine whether the endocytic function was restored. Isolated hepatocytes were incubated at 37°C in the presence of 0.2 M sucrose,

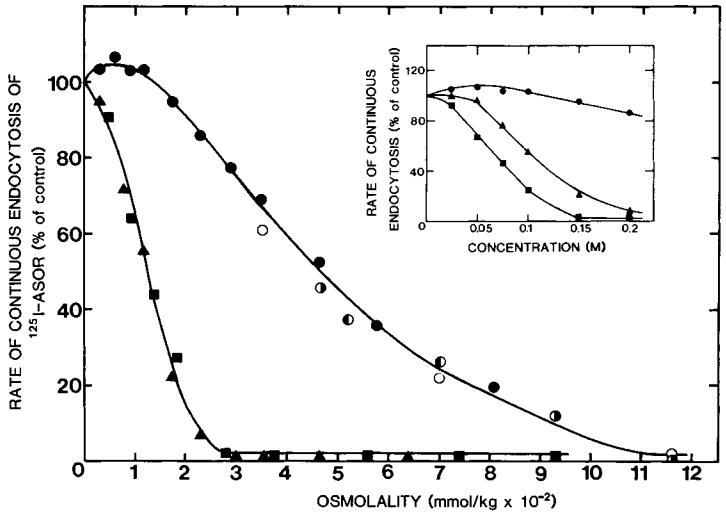

Fig. 4. Effect of osmolites on the rate of continuous endocytosis of ^{125}I-ASOR. Cells were allowed to endocytose ^{125}I-ASOR in the presence of various amounts of mannitol (○ ◐, ◑, ●), sucrose (▲), or sodium chloride (■). Each point represents the rate of endocytosis determined as in Figure 3. The x-axis is the increased osmolality above the osmolality of normal medium 1/BSA (264 mmol/kg). The inset is the endocytic rate plotted as a function of the molar concentration of the osmolite.

washed as described in Figure 5, put back at 37°C, and the ability of the cells to continuously internalize ^{125}I-ASOR was determined. After treatment with sucrose for 10–30 min, cells were unaffected in their subsequent ability to continuously endocytose ^{125}I-ASOR for at least 1 hr. We conclude that the effect of 0.2 M sucrose on the endocytic process is therefore reversible and that the hyperosmolar condition does not irreversibly damage the cells. The requirement for gentle manipulations, dilution into potassium chloride at 37°C, and slow cooling appeared to be necessary to retain cell viability and function. After treatment with sucrose, cells were more labile to the normal washing conditions and centrifugations usually employed. Possibly the cells are more fragile in hyperosmolar media.

Effect of Hyperosmolarity on the Endocytosis of Surface-Bound ^{125}I-ASOR

Other conditions, such as ATP depletion, will prevent cells from endocytosing ^{125}I-ASOR continuously by interfering with receptor recycling [23]. We, therefore, wanted to determine whether the hyperosmolar condition, which completely shut down receptor recycling and continuous uptake, also affected the internalization of one synchronous round of surface-bound ligand. In the presence of 0.2 M sucrose the initial rate of internalization at 37°C was 68% slower and the final extent of internalization was 37% less than in untreated cells (Fig. 6). In eight experiments these results were consistently obtained: in the presence of 0.2 M sucrose the rate of endocytosis of surface-bound ligand was 33.0% ± 8.1% of the control and the extent of uptake was 69.4 ± 10.5% of the control. Only about 70% of the surface-bound ligand was capable of being internalized in the presence of a concentration of sucrose in the medium which completely blocked continuous endocytosis.

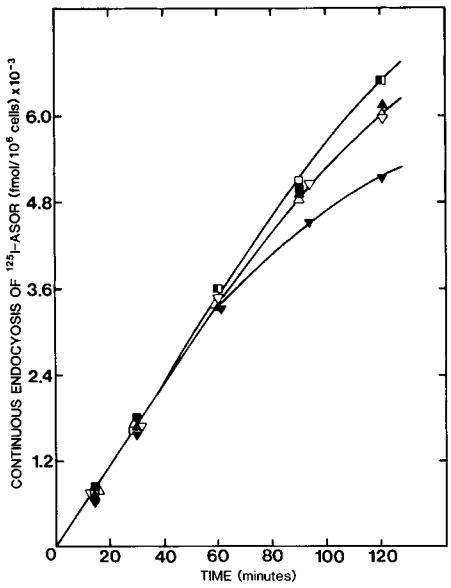

Fig. 5. Reversibility of the effect of sucrose on endocytosis. Cells were incubated at 37°C in the absence (□, △, ▽) or presence (■, ▲, ▼) of 0.2 M sucrose for 10 min (□, ■), 20 min (△, ▲), or 30 min (▽, ▼). Cells (6 ml) were diluted into 40 ml medium 1/BSA plus 300 mM KCl at 37°C and allowed to cool at room temperature for 10 min. Then the tubes were put on ice for 10 min. The cells were centrifuged, resuspended in medium 1/BSA with 7.1 μg/ml of ^{125}I-ASOR, and put at 37°C (this is the zero time on the graph). Samples were removed, washed, and cell-associated radioactivity was determined.

Effect of Hyperosmolarity on the Dissociation of Internalized ^{125}I-ASOR

In the same type of surface-bound uptake experiment the ability of the internalized ligand to dissociate was also assessed. As previously characterized [24,25], in the control situation the dissociation of internalized ligand proceeds in a biphasic manner (Fig. 6; left panel). Approximately half of the ^{125}I-ASOR dissociates very rapidly essentially immediately after internalization, and the remainder dissociates much more slowly. In the presence of 0.2 M sucrose, however, there was no evidence for dissociation of any internalized receptor-ASOR complexes (Fig. 6; right panel). An amount of sucrose which approximately doubles the osmolality of the medium partially inhibits the ability of surface-bound ligand to be internalized and completely blocks the dissociation of the ligand-receptor complexes which are internalized.

In a similar experiment we determined the speed with which a hyperosmolar condition inhibits the rate and extent of internalization of one round of surface-bound ^{125}I-ASOR. In this case cells were incubated at 30°C because the kinetics of uptake were too rapid at 37°C. At various times after the initiation of endocytosis, sucrose was added to the medium to a concentration of 0.2 M (Fig. 7). After sucrose was added, internalization continued for several minutes at a slower rate and then leveled off at a lower extent than what the cells were capable of internalizing. For example, when the osmolarity was increased 4 min after beginning internalization, the uptake of surface-bound ^{125}I-ASOR ceased within 3 min and reached an extent that was approximately 75% of the control. If sucrose was present at zero time or was added at 2 min, the extents of uptake and the rates of uptake were less. At 37°C the

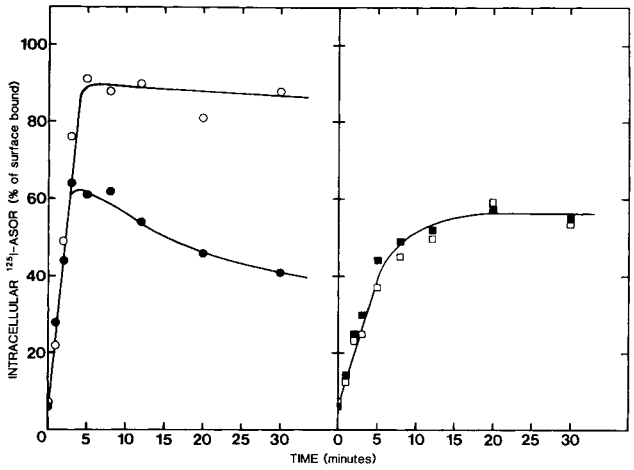

Fig. 6. Effect of sucrose on the endocytosis of surface-bound ^{125}I-ASOR. Cells were allowed to bind ^{125}I-ASOR at 4°C; nonbound ligand was removed by washing; and the cells were resuspended in medium 1/BSA. Sucrose in medium 1/BSA (to give a final concentration of 0.2 M; right panel) or an equal volume of medium 1/BSA (left panel) was added just before the cells were incubated at 37°C. All samples were diluted into ice-cold medium 1/BSA containing EGTA to assess only internalized radioactivity. Some cells were treated with 0.055% digitonin (●, ■) to release ^{125}I-ASOR which was not receptor-bound (ie, dissociated). Samples without digitonin (○, □) measure total internalized radioactivity. Cells were washed and bound radioactivity was determined.

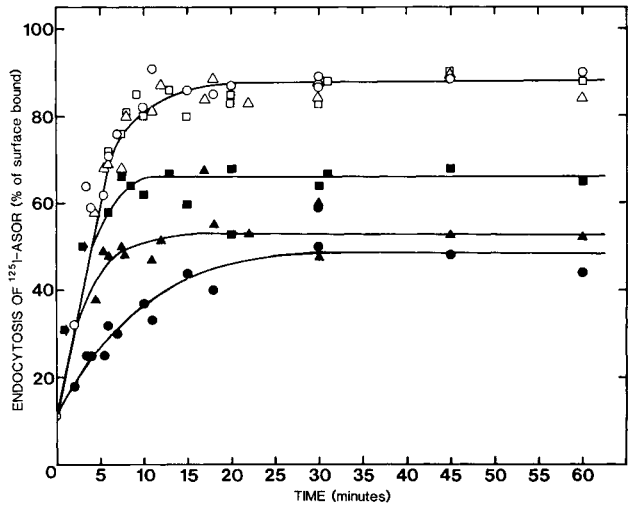

Fig. 7. Effect of time of addition of sucrose on the endocytosis of surface-bound ^{125}I-ASOR. ^{125}I-ASOR was prebound at 4°C to the cell surface as in Figure 6. To slow the process of endocytosis and to follow the internalization process more easily, the cells were then incubated at 30°C instead of 37°C. At 0 min (○, ●), 2 min (△, ▲), or 4 min (□, ■), sucrose in medium 1/BSA was added to a final concentration of 0.2 M (●, ▲, ■), or an equal volume of medium 1/BSA was added to the control samples (○, △, □). Cells were sampled and washed, and bound radioactivity was determined as in Figure 6. The arrows indicate the 2- and 4-min additions.

hyperosmotic effects on endocytosis were felt in < 1 min. Cells, therefore, do not need to be pretreated in hyperosmolar medium prior to the experiment in order to observe the partial inhibition of endocytosis of prebound ligand. Whatever effect is induced by the hyperosmolarity, it is felt by the cell and the Gal receptor system essentially immediately.

Effect of Hyperosmolarity on the Degradation of ^{125}I-ASOR

An experiment was performed to determine whether hyperosmolarity would affect the rate of processing of internalized asialoglycoproteins as measured by the appearance of acid-soluble degradation products. The above results showed that the continuous internalization and the dissociation of ^{125}I-ASOR from Gal receptors is inhibited in the presence of 0.2 M sucrose. Therefore, cells were first allowed to internalize ligand for different times and then the medium was made hyperosmolar in order to determine the effects on degradation per se and not on earlier steps in the receptor pathway (Fig. 8) When sucrose was added 60 min after cells had been continuously internalizing ^{125}I-ASOR, the rate of release of degradation products decreased by about 50% and then ceased within 1–2 hr. The amount of ligand degraded by the cells during that time represented approximately one-third of the ligand within the cells at the time of sucrose addition. This result suggests that the enzymes mediating degradation are not inhibited under hyperosmolar conditions but that the ability to deliver intracellular ^{125}I-ASOR continuously to the degradative compartment (eg, lysosomes) is affected. Hepatocytes were also pulse loaded at 37°C for different times with ^{125}I-ASOR, washed, and then reincubated at 37°C in the presence or absence of 0.2 M sucrose (Fig. 9). The inhibition of degradation under the hyperosmolar condition after exposure to ^{125}I-ASOR for 10, 20, or 30 min was

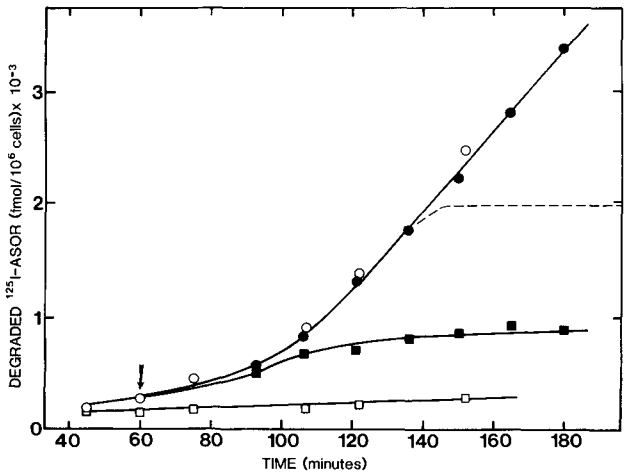

Fig. 8. Effect of sucrose on the continuous degradation of ^{125}I-ASOR. Cells were allowed to endocytose and degrade 1.5 μg/ml ^{125}I-ASOR at 37°C in medium 1/BSA. Sucrose in medium 1/BSA was added to a final concentration of 0.2 M at 0 min (□) or 60 min (■). The controls at 0 min (○) and 60 min (●) received medium 1/BSA only. Samples of cell suspension were removed and acid-soluble degradation products were determined as described in Experimental Procedures. The amount of degradation product in cells at any time was only 2–4% of the total amount degraded. The dashed line represents the amount of degradation products that would be present if all the endocytosed ^{125}I-ASOR present in the cells at the time of sucrose addition (60 min; arrow) had been degraded. Note that the X-axis begins at 35 min.

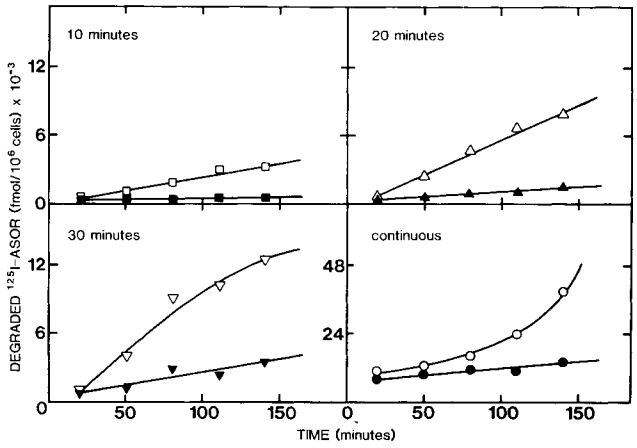

Fig. 9. Effect of sucrose on degradation of ^{125}I-ASOR. Cells were allowed to endocytose and degrade ^{125}I-ASOR at 5 μg/ml for 10 min (□, ■), 20 min (△, ▲), or 30 min (▽, ▼) as noted in the Figure. The samples were chilled, washed free of noninternalized radioactivity, and put back at 37°C with (■, ▲, ▼) or without (□, △, ▽) 0.2 M sucrose. Some cells were also at 37°C in the continuous presence of ^{125}I-ASOR with (●) or without (○) 0.2 M sucrose from the start of the experiment. Samples were removed and acid-soluble radioactivity was determined.

respectively 83, 84, and 73%. With longer times of continuous endocytosis a larger fraction of the ^{125}I-ASOR entering the cells was degraded in the presence of 0.2 M sucrose (Figs. 8,9). This again supports the conclusion that there is a blockade at some point in the delivery or processing pathway prior to the ligand actually being delivered to lysosomes.

Effect of Hyperosmolarity on Diacytosis

In previous studies we have shown that after internalization of a single round of surface-bound ^{125}I-ASOR, approximately half of the internalized receptor-ligand complexes are able to return to the cell surface prior to being dissociated [26]. These complexes appear to be in a dynamic exchange between an interior compartment and the cell surface until they finally dissociate intracellularly. In order to monitor the number of receptor-ligand complexes that return to the surface, the experimental protocol involves the use of EGTA in the extracellular medium so that when internalized complexes return to the cell surface the ^{125}I-ASOR is dissociated from the receptor and collects in the medium (Fig. 10). Using digitonin to permeabilize cells, one can then simultaneously follow the ligand that remains within cells still bound to receptor, the ligand that is within the cell and dissociated from receptor, and the ligand that accumulates in the medium containing EGTA. In the presence of 0.2 M sucrose, ^{125}I-ASOR was able to accumulate in the medium, suggesting that receptor-ligand complexes still returned to the cell surface although the kinetics were slower than in the absence of sucrose (middle panels, Fig. 10). In the absence of sucrose the amount of receptor-bound ligand normally decreases as it is dissociated and processed by the cells (top left panel, Fig. 10). In the presence of EGTA this decrease in receptor-bound internal ligand is accelerated. When 0.2 M sucrose is present in the medium in the absence of EGTA there is essentially no change over approximately 1 hr. In the presence of EGTA there is a decrease in the receptor-bound ligand as ^{125}I-

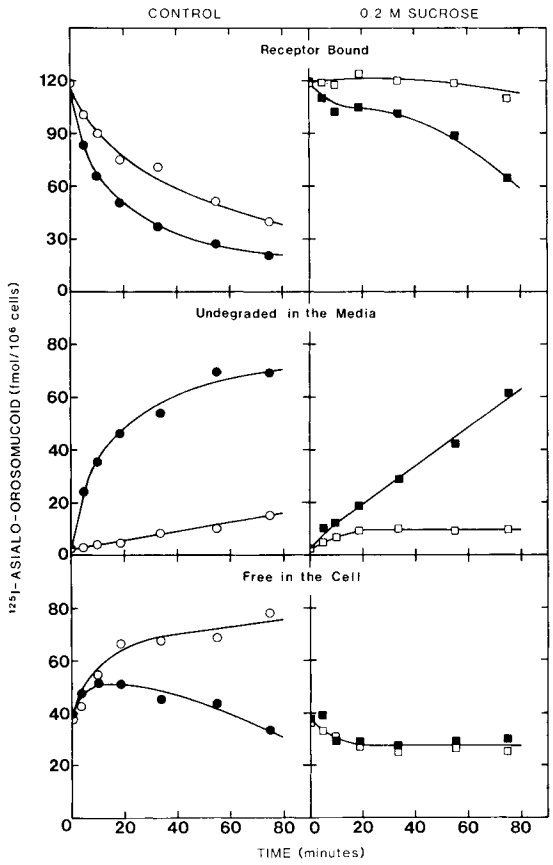

Fig. 10. Effect of hyperosmolarity on the return of ^{125}I-ASOR to the cell surface. Cells were allowed to bind ^{125}I-ASOR (at 1.5 μg/ml) at 4°C for 60 min. Nonbound ligand was removed by centrifugation and the cells were allowed to endocytose the surface-bound ^{125}I-ASOR for 7 min at 37°C. The cells were then chilled on ice and washed once with medium 1/BSA containing EGTA to remove any ligand still on the surface. Cells were then resuspended to 2 × 10^6/ml in medium 1/BSA and put at 37°C with (right panels; □, ■) or without (left panels; ○, ●) 0.2 M sucrose and with (■, ●) or without (□, ○) EGTA. The amount of ligand either bound to receptor (top panels), or released undegraded (acid insoluble) into the media (middle panels), or free in the cell (bottom panels) was determined at the indicated times.

ASOR accumulates in the medium (top right panel, Fig. 10). In the absence of sucrose or EGTA the amount of free ligand in the cell accumulates in a time-dependent manner as it dissociates from receptors and is routed to be degraded (lower left panel, Fig. 10). In the presence of EGTA this increase occurs for a shorter period of time, and then the amount of free ligand in the cell decreases. This decrease is due to the increased amount of intact ^{125}I-ASOR released into the medium in the presence of EGTA. When 0.2 M sucrose is present, however, there is an initial small decrease in the amount of free ligand within the cell, and then there is no further change (lower right panel, Fig. 10). The conclusion from this experiment is that the hyperosmolar condition prevents dissociation of receptor-ligand complexes (as shown in Fig. 6) and only partially inhibits but does not prevent the diacytosis of these complexes.

DISCUSSION

The overall process of receptor-mediated endocytosis involves numerous steps both in the ligand-processing and receptor-recycling pathways. In the hepatic Gal receptor system, previous studies have led to a proposal that there are actually two parallel pathways involved for ligand processing and receptor recycling [3,25]. Receptor-mediated endocytosis in this system involves at least 11 discrete stages. In this study the effect of hyperosmolarity was assessed on these various stages in the overall endocytic process.

Binding of ^{125}I-ASOR to Gal receptors was not affected by up to 0.4 M sucrose or sodium chloride at 4°C. Mannitol, however, exerted several effects different than the previous two agents and is much more complicated. Mannitol appears to act in at least three distinct ways: First, it is an osmolite and exerts similar effects to sucrose or sodium chloride. Second, it appears to be a weak competitive inhibitor of ^{125}I-ASOR binding to Gal receptors. Third, it appears to stimulate ^{125}I-ASOR binding to cell surface Gal receptors at low concentrations (Fig. 1). This latter effect was surprising and the basis for it is unknown. Experiments to determine whether surface vs internal receptors are preferentially affected by mannitol have thus far been inconclusive. One possibility is that at low concentrations mannitol can act like a weak nonionic detergent and stimulate the binding of ligand by increasing the valency of receptor [27]. Although Stowell et al [28] and Sarkar et al [29] have mapped the binding site of the mammalian hepatocyte lectin by using a variety of saccharides, glycoproteins, and neoglycoproteins, to our knowledge no one has examined alditols, such as mannitol, for inhibitory capability. The results with mannitol, therefore, cannot be interpreted solely on the basis of the effect of this compound as an osmolite.

Continuous endocytosis which requires receptor recycling was completely blocked by 0.2 M sucrose or 0.15 M sodium chloride, corresponding to a total osmolality of approximately 550 mmol/kg, roughly a doubling of the osmotic strength of the medium. Since both sodium chloride and sucrose give identical inhibition of receptor-mediated endocytosis as a function of their osmolal concentrations, it is very unlikely that their effects are related to a property other than the ability to act as an osmolite. The rate and extent of endocytosis of one round of surface-bound ^{125}I-ASOR were inhibited, respectively, by about 65 and 30% in the presence of 0.2 M sucrose. Ligand dissociation from receptors was completely inhibited. Degradation of ^{125}I-ASOR was also inhibited by 0.2 M sucrose but this appears to be due to a block in the delivery of ligand to lysosomes rather than to an inhibition of lysosomal function itself. In this respect the effect of hyperosmolarity on ligand degradation is similar to previous results with microtubule drugs [7]. In this case, inhibition of degradation was also due to a block in delivery of ligand to lysosomes and not degradation per se.

Ligand which has been internalized and is bound to receptor is still able to undergo diacytosis in the presence of 0.2 M sucrose. This characteristic return of ligand to the cell surface is a property of only one of the two Gal receptor pathways—the minor, state 1, pathway [3,26]. The effects of the osmolites, particularly sucrose or sodium chloride, were essentially immediate but were nonetheless reversible. Cell viability was maintained in the presence of all of the osmolites used even at high concentrations. Particular dilution conditions, temperature regulation, and the presence of extracellular potassium in the medium appeared to be necessary to achieve

successful reversibility of the effect of sucrose and to demonstrate the recovery of the endocytic function. The presence of a high concentration of external K^+ may facilitate the recovery of cell membrane potential. Other investigators have also observed that particular conditions for treating cells after exposure to hyperosmolarity are necessary to achieve cell recovery and to avoid cell injury [30,31].

The observation that a single round of endocytosis of prebound ligand is partially inhibited and that ligand dissociation is completely blocked by hyperosmolarity was somewhat surprising. Other treatments or inhibitors do not give a partial effect when one measures the ability of surface-bound ^{125}I-ASOR to be internalized. In initial experiments to correlate this partial inhibition of endocytosis with the presence of the two Gal receptor populations and pathways previously mentioned [3], we found no evidence that the basis for the partial effect is a preferential inhibition of one pathway or the other (data not shown). A likely alternative possibility is that conditions of hyperosmolarity immediately interfere with coated pit recycling and prevent the formation of new coated pits. Wall and Hubbard [32] reported that 58% of lactosyl-ferritin particles bound to Gal receptors were associated with coated pits in perfused intact liver that had first been treated with formaldehyde or at low temperature (<5°C). Therefore, approximately 60% of the receptor-ligand complexes at the start of an experiment may be in coated pits and are able to enter the cell even in the presence of 0.2 M sucrose. The hyperosmolor condition then could interfere with new coated pit formation. This would be consistent with the extent of internalization observed and also the rate at which the Gal receptor system slows down and stops after the medium is made hyperosmolar. Cessation of endocytosis occurs within minutes, which is appropriate for the time expected for the coated pit recycling pathway. This interesting possibility merits further examination since only a few treatments, such as depletion of intracellular potassium [33], are known to arrest coated pit formation and block receptor-mediated endocytosis.

Monensin is able to block the dissociation of ^{125}I-ASOR from intracellular receptor-ligand complexes [4]. Since hyperosmolarity also blocks ligand dissociation, this may prove to be a useful tool with which to sort out the ligand processing pathways that occur after internalization and prior to dissociation. It remains to be determined whether sucrose and monensin act in different ways or at different intracellular sites in the overall endocytic process.

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