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LOSS OF SURFACE GALACTOSYL RECEPTOR ACTIVITY ON ISOLATED RAT HEPATOCYTES INDUCED BY MONENSIN OR CHLOROQUINE REQUIRES RECEPTOR INTERNALIZATION *VIA* A CLATHRIN COATED PIT PATHWAY

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We studied the effect of hyperosmotic inhibition of the clathrin coated pit cycle on the monensin- and chloroquine-dependent loss of surface galactosyl (Gal) receptor activity on isolated rat hepatocytes. Cells treated for 60 min without ligand at 37°C with 25 μM monensin or 300 μM chloroquine in normal medium (osmolality ≈275 mmol/kg) bound 40-60% less ^{125}I-asialo-orosomucoid (ASOR) at 4°C than untreated cells. Cells exposed to monensin or chloroquine retained progressively more surface Gal receptor activity, however, when the osmolality of the medium was increased above 400 mmol/kg (using sucrose as osmolite) 10 min prior to and during drug treatment. Cells pretreated for 10 min with hyperosmolal media (600 mmol/kg) alone internalized ≤10% of surface-bound ^{125}I-ASOR. Thus, the ligand-independent loss of surface Gal receptor activity on monensin- and chloroquine-treated hepatocytes requires internalization of constitutively recycling receptors *via* a coated pit pathway.

Mammalian hepatocytes reduce their surface Gal receptor activity in the absence of ligand by ≈50% following treatment with monensin or chloroquine[1] (1-3). Although they lose surface Gal receptor activity, hepatocytes retain surface receptor protein, and accumulate inactive Gal receptors on their surfaces after chloroquine or monensin (2) treatment[2]. There are at least two explanations for these observations. i) Chloroquine- and monensin-treated hepatocytes could inactivate surface Gal receptors by a transmembrane mechanism not requiring receptor internalization, as suggested by Fiete *et al* (2). ii) Chloroquine- and monensin-treated hepatocytes could internalize and inactivate Gal receptors intracellularly, and then re-express these inactive receptors back on the plasma membrane. If the latter explanation is true,

[1] McAbee, D.D., Clarke, B.L., Oka, J.A., and Weigel, P.H. (Manuscript submitted).
[2] McAbee, D.D., Lear, M.L., and Weigel, P.H. (Manuscript submitted).

Abbreviations: ASOR, asialo-orosomucoid; BSA, bovine serum albumin; EGTA, ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid; Gal, galactosyl; HBSS, Hank's balanced salt solution; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid.

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then cells prevented from internalizing Gal receptors would maintain their surface receptor activity despite monensin or chloroquine treatment. Hyperosmolarity preferentially blocks receptor-mediated endocytosis in mammalian cells (4,5) by disrupting the clathrin coated pit cycle (6). Therefore, to distinguish between these two possibilities, we determined whether or not hepatocytes without a functional coated pit pathway (inhibited by hyperosmotic medium) modulated their surface Gal receptor activity following monensin or chloroquine treatment.

METHODS

Materials. Orosomucoid obtained from Sigma Chemical Co. was desialylated, and ASOR was iodinated as described (7). $Na^{125}I$ (10-20 mCi/μg iodine) was from Amersham. Sucrose (grade 1), monensin, and chloroquine were from Sigma. BSA (CRG-7) was from Armour Biochemicals. Collagenase was from Serva (cat. #17449) or from Boehringer Mannheim (Type D). Medium 1/BSA contains modified Eagle's medium (Grand Island Biological Co.) supplemented with 2.4 g/L HEPES, pH 7.4, 0.22 g/L $NaHCO_3$ and 0.1% (w/v) BSA. HBSS was prepared according to the GIBCO catalogue.

Cell Preparation. Hepatocytes were prepared from male Sprague-Dawley rats (≈250 g) by a collagenase liver perfusion procedure (8). Final cell pellets were suspended in Medium 1/BSA. Cells were routinely >90% single cells and viable as judged by trypan blue exclusion. Experiments were performed in the absence of serum. Cells were first incubated at 37°C for 60 min to increase and stabilize the cell surface Gal receptor number (9).

Osmolality. Sucrose was dissolved to a concentration of 1.6 M in double distilled water. Medium 1 was prepared at twice the normal concentration in double distilled water. Concentrated Medium 1, 1.6 M sucrose and distilled water were mixed to give normal Medium 1 containing various sucrose concentrations. The osmolality of the medium was therefore changed without altering the concentration of nutrients and salts. Osmolality was measured using a Wescore Vapor Pressure Osmometer (Model 5100C).

Determination of Gal Receptor Activity. Cells were rapidly chilled in 5 volumes of ice-cold HBSS, collected by centrifugation, and incubated with 1.5 μg/mL ^{125}I-ASOR on ice for 60 min with occasional mixing. The cells were washed twice by centrifugation with HBSS at 4°C, dissolved in 0.3 N NaOH, then assayed for radioactivity and protein. Specific binding of ^{125}I-ASOR was ~90%.

General. Centrifugations of cell suspensions were at 800 rpm for 2 min in a Beckman GPR refrigerated centrifuge. Protein was determined using the Coomassie Protein Assay (Pierce Chemical Co.) with BSA as standard. Radioactivity was determined on a Packard Multiprias 2 gamma spectrometer.

RESULTS AND DISCUSSION

When treated with monensin or chloroquine at 37°C in normal medium, hepatocytes lost 57 and 43%, respectively, of their surface ^{125}I-ASOR binding capacity at 4°C (Table I), as previously reported (1-3). These values represented the loss of 150,000-200,000 ^{125}I-ASOR binding sites/cell. Media with osmolalities ≥500 mmol/kg block the continuous receptor mediated uptake of

Table I

Effect of Hyperosmolality on Monensin- and Chloroquine-induced Loss of Surface Gal Receptor Activity¶

Sucrose Present	Inhibitor Added	Osmolality (mmol/kg)*	^{125}I-ASOR Bound (fmol/mg protein)§	% of Control
no	none	279	528 ± 27	100
yes	none	519	471 ± 35	89
no	monensin	282	228 ± 35	43
no	chloroquine	273	302 ± 23	57
yes	monensin	504	517 ± 32	98
yes	chloroquine	499	459 ± 31	87

¶In the absence of ligand, cells were incubated in Medium 1/BSA with or without 0.2 M sucrose for 10 min, then treated with or without monensin (25μM) or chloroquine (300 μM) for an additional 60 min, all at 37°C.

*Media measured following removal of cells by centrifugation.

§Receptor activity was measured at 4°C as described in Methods. Values represent the mean ± mean absolute deviation of duplicate samples.

ASOR (5,6) and reduce the number of coated pits (6) on isolated rat hepatocytes. Therefore, we determined whether or not a hyperosmotic block of the clathrin coated pit cycle prevented the loss of surface Gal receptor activity on hepatocytes treated with monensin or chloroquine. We used sucrose as an osmolite in these experiments. Hepatocytes in Medium 1/BSA with an increased osmolality showed no alteration in surface binding of ^{125}I-ASOR (Table I). Hepatocytes incubated in hyperosmotic media at 37°C for 10 min prior to and during treatment with monensin or chloroquine lost only $\leq$13% of their surface ^{125}I-ASOR binding. We obtained similar results with cells incubated in hyperosmotic media 2.5-30 min prior to and during drug treatment (not shown). Thus, doubling the osmolality of Medium 1/BSA with sucrose did not affect surface Gal receptor activity but prevented any significant loss of surface receptor activity induced by either chloroquine or monensin.

We then titrated the effect of increasing osmolality on the loss of surface receptor activity induced by monensin or chloroquine. Hepatocytes incubated in medium with an osmolality of 270-510 mmol/kg either maintained (Fig. 1A) or increased slightly (Fig. 1B) their surface Gal receptor activity. Hepatocytes treated with monensin (Fig. 1A) or chloroquine (Fig. 1B) in normal medium lost about 50% of their surface Gal receptor activity. Media with osmolalities <375 mmol/kg did not prevent the loss of surface Gal receptor activity when cells were subsequently treated with monensin or chloroquine. Cells in media with osmolalities >375 mmol/kg, however, retained proportionately more surface ^{125}I-ASOR binding in spite of drug treatment. Cells treated with monensin or chloroquine in media with an osmolality of $\simeq$500 mmol/kg lost $\leq$19% of their surface ^{125}I-ASOR binding. Thus, media with osmo-

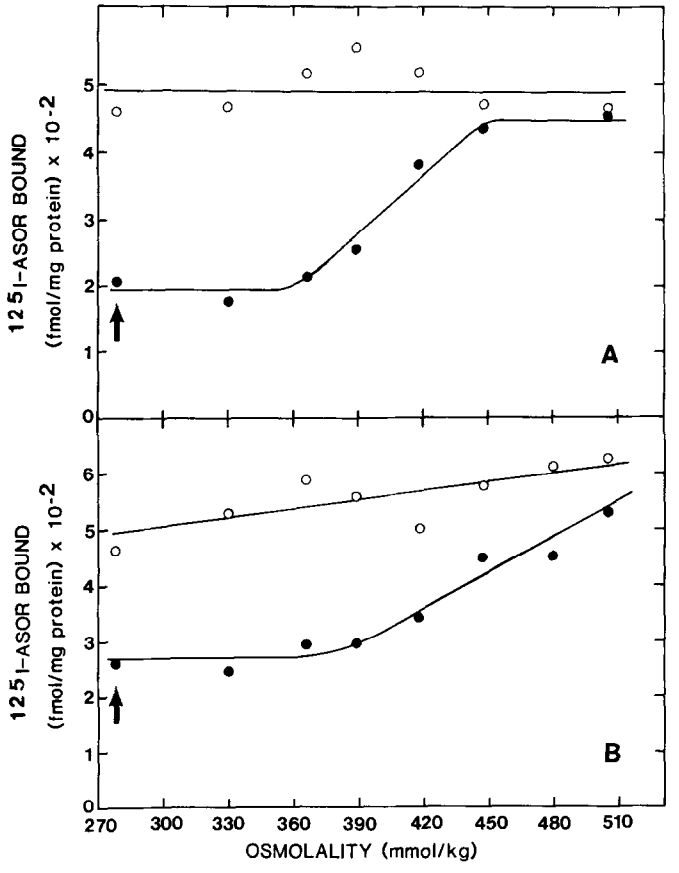

Figure 1. Effect of increasing osmolality on surface Gal receptor activity in monensin- and chloroquine-treated hepatocytes. Cells were incubated for 10 min at 37°C without ligand in Medium 1/BSA supplemented with 0-0.2 M sucrose to give the designated osmolality, and then incubated with (●) or without (○) 25 μM monensin (A) or 300 μM chloroquine (B) for an additional 60 min. Cells were assayed for ^{125}I-ASOR binding as described in *Methods*. Symbols represent the mean of duplicate determinations which differed by <10%.

lalities between 375-500 mmol/kg essentially blocked the effect of monensin or chloroquine on surface Gal receptor activity. Notably, this osmotic range reduces the rate of continuous ^{125}I-ASOR uptake by isolated rat hepatocytes by up to 95% (5).

The preceding results are consistent with the notion that hyperosmotic media protect surface Gal receptors from the effects of monensin and chloroquine treatment by preventing receptor internalization *via* a clathrin coated pit pathway. To test this, we incubated hepatocytes for 10 min at 37°C in media with osmolalities of 260-600 mmol/kg. The cells were chilled rapidly, allowed to bind ^{125}I-ASOR at 4°C, washed, and then placed back at 37°C in medium at the appropriate osmolality. Total cell associated and internalized (EGTA-resistant) ^{125}I-ASOR were measured as a function of time (Fig. 2). Total ^{125}I-ASOR bound by cells treated in hyperosmotic media was ≥80% of cells in normal medium. The treatment with hyperosmotic media blocked proportion-

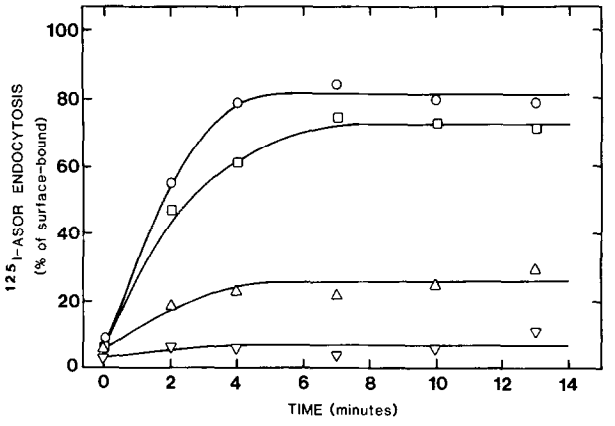

Figure 2. Effect of hyperosmolality on uptake of surface-bound ^{125}I-ASOR by hepatocytes. Cells were incubated for 10 min at 37°C in Medium 1/BSA in the absence or presence of sucrose at osmolalities of 261 (no sucrose, ○), 377 (0.1 M sucrose, □), 483 (0.2 M sucrose, △), or 606 (0.3 M sucrose, ▽) mmol/kg. The cells were chilled rapidly, allowed to bind ^{125}I-ASOR at 4°C, washed, then incubated at 37°C at the appropriate osmolality. At the designated times, cell samples were chilled rapidly in 5 volumes of HBSS with or without 7.5 mM EGTA, washed, then assayed for radioactivity. EGTA at 7.5 mM strips ≥95% of surface-bound ligand from hepatocytes and distinguishes between surface and internal ligand. Results are expressed as the percentage of total cell associated ^{125}I-ASOR internalized by cells incubated in different media.

ately the subsequent uptake of surface-bound ^{125}I-ASOR. An osmolality of 606 mmol/kg inhibited ^{125}I-ASOR uptake by >90%. These data confirm that hepatocytes incubated briefly in hyperosmotic medium to disrupt coated pits, cannot subsequently internalize surface Gal receptors.

An intracellular transmembrane modulation of surface Gal receptor activity (2) following monensin or chloroquine treatment is not the likely explanation for receptor inactivation. The results show that the loss of surface Gal receptor activity on hepatocytes treated with monensin and chloroquine requires receptor internalization. Although monensin (10) and chloroquine (11) act on cells by different mechanisms, they both cause hepatocytes to accumulate inactive Gal receptors on their surfaces (2) and internally[2]. We have shown previously that a subpopulation of Gal receptors on hepatocytes constitutively recycles and is reversibly inactivated and then reactivated intracellularly before recycling back to the cell surface (12,13). Hyperosmotic disruption of the clathrin coated pit cycle prevents the loss of surface Gal receptor activity normally induced by monensin or chloroquine. Monensin- and chloroquine-treated hepatocytes, however, still accumulate inactive Gal receptors intracellularly, regardless of the osmolality of the medium (not shown). This suggests that receptors which have migrated past the hyperosmotic-sensitive step during constitutive recycling are not reactivated normally after inactivation when cells are treated with monensin or chloroquine. Hyperosmotic medium, which decreases the number of surface coated pits on

hepatocytes (6), completely blocks receptor mediated endocytosis of ASOR by isolated rat hepatocytes, but does not affect fluid phase endocytosis (6). Similarly, hyperosmotic medium prevents the receptor mediated uptake of f-Met-Leu-Phe, but not fluid phase uptake of ^{3}H-sucrose, by polymorphonuclear leukocytes (4). Therefore, this study also indicates that Gal receptor inactivation during constitutive receptor recycling occurs along a clathrin coated pit pathway, not a fluid phase pathway.

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