Temperature Dependence of Endocytosis Mediated by the Asialoglycoprotein Receptor in Isolated Rat Hepatocytes

EVIDENCE FOR TWO POTENTIALLY RATE-LIMITING STEPS* (Received for publication, December 15, 1980, and in revised form, January 8, 1981)

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The rate of endocytosis of cell surface-bound [3H]asialo-orosomucoid was determined as a function of temperature. Freshly isolated rat hepatocytes were allowed to bind [3H]asialo-orosomucoid at 4°C, washed to remove nonbound ligand, and internalization was then assessed by the resistance of cell-associated radioactivity to release by the Ca2+ chelator EDTA. At 10°C or below, endocytosis is negligible. Above 10°C, the rate of endocytosis is proportional to temperature but the increase of the rate of endocytosis with increasing temperature changes sharply at about 20°C. From 10 to 20°C, the apparent activation energy for endocytosis, calculated from an Arrhenius plot, is 45.9 kcal/mol and the temperature coefficient, Q10, is 15.8. However, between 20 and 41°C, the calculated activation energy is 17.0 kcal/mol and the Q10 is 2.6. Although the rate of endocytosis of previously bound [3H]asialo-orosomucoid is very dependent on the temperature, the final extent of endocytosis is essentially temperature-independent between 14 and 37°C.

The results suggest that there are at least two steps in the overall process of endocytosis mediated by the asialoglycoprotein receptor on isolated hepatocytes which can be potentially rate-limiting, one at 10°C and another at approximately 20°C.

Receptor-mediated endocytosis may be important for the biological activity of many molecules which interact with the cell surface and elicit cellular responses including insulin (1, 2), low density lipoprotein (3), nerve growth factor (4), and epidermal growth factor (5). In mammalian liver, an asialoglycoprotein receptor (6) mediates the endocytosis, leading to subsequent degradation, of desialylated glycoproteins. This receptor is believed to function normally in vivo in the removal of asialoglycoproteins from serum and has been shown recently to be reutilized or recycled both in vivo (7, 8) and in vitro in isolated hepatocytes (9). Several laboratories have reported that reutilization of cell surface receptors may also occur in other systems (10, 11). For many reasons, the asialoglycoprotein receptor in rat hepatocytes appears to be a good model system for the study of endocytosis and receptor reutilization. We are studying several aspects of the asialoglycoprotein receptor system in an effort to elucidate how the receptor functions at the molecular level during endocytosis, receptor reutilization, and during the specific binding of hepatocytes to synthetic carbohydrate substrata (12, 13). We recently showed, for example, that a large patch of asialoglycoprotein receptors forms at the cell-substratum junction when hepatocytes bind to synthetic galactoside culture surfaces (14).

Numerous investigators have shown in many systems that endocytosis does not occur below 10°C (15). In the present study, we examine the effect of temperature on the rate of internalization of asialo-orosomucoid by freshly isolated intact hepatocytes and demonstrate the occurrence of at least one other process which becomes rate-limiting at or below 20°C.

A preliminary report of some of the results reported here has appeared (16).

EXPERIMENTAL PROCEDURES

Materials—Human orosomucoid (α, acid glycoprotein) was generously provided by Dr. M. Wickerhauser of the Plasma Derivatives Laboratory of the American Red Cross. [3H]Asialo-orosomucoid was prepared as recently described (17). Triton X-100, collagenase, and BSA (Fraction V) were from Sigma. Male Sprague-Dawley rats were obtained from Timco Breeding Laboratories, Houston, Texas. Scintillation fluid (3a70B) was from Research Products International Corp. All other chemicals were reagent grade.

Cell Preparation—Hepatocytes were prepared by the collagenase perfusion technique of Seglen (18) with minor modifications (13). Final cell pellets were suspended on ice in Medium 1 which contained a modified Eagle's medium (Grand Island Biological Co., catalogue 420-1400) supplemented with 2.4 g/liter of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, and 0.22 g/liter of NaHCO3. Cells were routinely 85 to 95% viable as judged by trypan blue exclusion and 85 to 95% single cells. All experiments were performed in the presence of 0.1% (w/v) BSA and the absence of serum.

Determination of Intracellular [3H]Asialo-orosomucoid—Cells were allowed to bind [3H]asialo-orosomucoid at 4°C, for 75 to 110 min, and then washed twice by centrifugation to remove nonbound radioactivity (17). Cells were resuspended to 4 × 106 cells/ml in ice-cold Medium 1 plus 0.1% BSA and then incubated at the desired temperature. At various times, samples (0.8 or 0.9 ml) were removed and diluted to 7 ml of ice-cold Medium 1 plus 0.1% BSA with or without 20 mM EDTA, in disposable borosilicate tubes (13 × 100 mm). The samples were mixed, incubated for at least 5 min, and then centrifuged. Previous experiments (17) showed that within 5 min on ice 99% of the specifically bound [3H]asialo-orosomucoid was released by this EDTA treatment. The supernatant fluid was removed by aspiration and the cell pellet was dissolved in 1.2 ml of 0.2% Triton X-100. One ml of the lysate was mixed with 9 ml of scintillation fluid and radioactivity was determined using a Beckman LS 7500 liquid scintillation spectrometer. The total internal and surface-bound [3H]asialo-orosomucoid is determined in the absence of EDTA; only internal glycoprotein is measured in the presence of EDTA. The initial amount of material bound to the cell surface was determined on the washed cells at 4°C.

General—The method of Lowry et al. (19) was used to determine protein using BSA as the standard. All centrifugations of cell suspensions were performed for 2 min at 800 rpm in a GLC-table top centrifuge (Sorvall Instrument Co.).

RESULTS AND DISCUSSION

In the present study, we have eliminated effects due to the 

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1 The abbreviation used is: BSA, bovine serum albumin.
the amount of ligand available for internalization to what is nonbound ligand before beginning an experiment. This limits continuous binding of [3H]asialo-orosomucoid by removing experiments indicated by different symbols were performed with different cell preparations as described in Fig. 1 and under "Experimental Procedures." The temperatures were: ○, 11.5 °C; ●, 17 °C; △, 23.5 °C; ▲, 29 °C; □, 35 °C; ■, 41 °C. B, each symbol represents an experiment such as shown in A, in which the rate of internalization was determined at different temperatures. The amount of [3H]asialo-orosomucoid bound ranged from 56 to 69 fmol/10⁶ cells.

![Fig. 2. Effect of temperature on the extent of endocytosis of surface-bound [3H]asialo-orosomucoid by hepatocytes. Three experiments indicated by different symbols were performed with different cell preparations as described in Fig. 1 and under "Experimental Procedures." Samples from suspensions at different temperatures were taken at various times until the extent of endocytosis was maximal (e.g. about 10 min at 37 °C, 20 min at 30 °C, 1.5 to 2 h at 20 °C, and 3 to 4 h at 14 °C). These plateau values are expressed as the percentage of specifically bound [3H]asialo-orosomucoid which could initially be released from the cells at 4 °C with EDTA. At temperatures below 14 °C, the rate of endocytosis is so slow that the experiments cannot be taken to completion because cell viability is compromised.](#)

![Fig. 3. Arrhenius plot of the rate of endocytosis of surface-bound [3H]asialo-orosomucoid by hepatocytes. Endocytosis rates from three different experiments were determined at different temperatures as in Fig. 1. The lines, calculated by linear regression analysis from the points below and above 20 °C, had correlation coefficients of −0.97 and −0.98, respectively.](#)

continuous binding of [3H]asialo-orosomucoid by removing nonbound ligand before beginning an experiment. This limits the amount of ligand available for internalization to what is on the cell surface and essentially synchronizes endocytosis. Under these conditions, endocytosis of [3H]asialo-orosomucoid bound to the surface of isolated rat hepatocytes is essentially not detectable at 10 °C or below (Fig. 1), whereas at 37 °C a maximal uptake of about 70 to 90% of the specific surface-bound glycoprotein usually occurs within 5 to 10 min.² In many other systems, endocytosis has also been found to occur poorly or not at all below 10 °C (15), indicating that below this temperature some process(es) necessary for uptake becomes severely rate-limiting. Above 10 °C, the endocytosis of previously bound [3H]asialo-orosomucoid is proportional to temperature and initially linear with time (Fig. 1A). The rate of endocytosis increases linearly between 10 and 20 °C, but at about 20 °C there is a discontinuity in the rate of increase, which becomes severalfold greater and is then linear between 20 and 41 °C (Fig. 1B). Although there is a sharp break in the rate of change of the rate of endocytosis at 20 °C, the final extent of internalization of surface-bound [3H]asialo-orosomucoid appears relatively independent of temperature (Fig. 2) between 14 and 37 °C.

These results suggest that another process directly or indirectly necessary for endocytosis to occur becomes rate-limiting at or below 20 °C. In agreement with this, an Arrhenius plot of the rate of endocytosis at temperatures between 11.5 and 41 °C shows a single inflection point at approximately 20 °C (Fig. 3). Above 20 °C, the apparent activation energy, calculated from the slope of the line, for the endocytosis of [3H]asialo-orosomucoid is 17.0 kcal/mol.² Below 20 °C, the

² Several papers have appeared recently in this journal (20, 23) in which the units for the activation energy (Eₐ) determined from Arrhenius plots were reported as kcal mol⁻¹ degree⁻¹. This is incorrect. Since k = Ae⁻Eₐ/RT, the units of Eₐ are the same as those of RT and the degree unit cancels out. The correct units for Eₐ are kcal/mol.

³ P. H. Weigel and J. A. Oka, manuscript in preparation.
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The extent of vesicle fusion below this temperature. The result was dramatically inhibited at 52°C, at which temperatures alveolar macrophages may also show an inflection point at (20).

The fusion of pinocytotic vesicles and lysosomes did not occur possibility that either endocytosis

The activation energy of 17.0 kcal/mol for the endocytosis of [125I]asialo-orosomucoid is lower than the value of 26.5 kcal/mol reported by Dunn et al. (20) for the uptake of [125I]asialo-fetuin in intact perfused rat liver. However, other workers (15, 21-23) have reported activation energies ranging from 17 to 25 kcal/mol for fluid phase pinocytosis or receptor-mediated endocytosis in single cell preparations. The Q10 value of 2.6 reported here for endocytosis above 20°C is also in close agreement with that obtained in different systems (15, 22).

Unlike the results presented here for isolated hepatocytes (Fig. 3), Dunn et al. (20) did not observe a discontinuity in an Arrhenius plot of the rate of cellular entry of radioactive glycoprotein into hepatocytes in situ. However, there are numerous differences between the two studies. It is possible, for example, that endocytosis in vivo in the highly polarized hepatocyte (24) may be less susceptible to decreased temperature than in the isolated hepatocyte. The Arrhenius plot presented by Kaplan and Nielsen (Fig. 6 in Ref. 23) for the internalization of 6-macroglobulin-trypsin complexes by rabbit alveolar macrophages may also show an inflection point at about 20°C.

The degradation of [125I]asialo-fetuin in perfused rat liver was dramatically inhibited at ≈20°C, at which temperatures the fusion of pinocytic vesicles and lysosomes did not occur (20). Isolated rat hepatocytes also fail to degrade insulin at temperatures below 20°C (25). We find that isolated hepatocytes cease to degrade internalized [125I]asialo-orosomucoid at or below about 20°C. The rate of endocytosis for the lipids in eukaryotic membranes appears to be in the vicinity of 20°C (26) and may explain the decrease in the extent of vesicle fusion below this temperature. The result reported here, that the rate of endocytosis in isolated hepatocytes is greatly reduced at or below 20°C, suggests the possibility that either endocytosis per se is affected or that endocytosis is coupled to or dependent on subsequent intracellular vesicle fusion events which are temperature-sensitive.

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Calculated apparent activation energy for endocytosis is 45.9 kcal/mol. The Q10, or temperature coefficient, for endocytosis above 20°C is 2.6, whereas below 20°C the Q10 is 15.6.