Glucocorticoid Regulation of Amino Acid Transport in Anucleate Rat Hepatoma (HTC) Cells

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ABSTRACT The transport of α-aminoisobutyric acid (AIB) by rat hepatoma tissue culture (HTC) cells is rapidly and reversibly inhibited by dexamethasone and other glucocorticoids. To investigate the role of the nucleus in the regulation of transport and to determine whether steroid hormones or steroid-receptor complexes may have direct effects on cytoplasmic or membrane functions, we have examined the regulation of transport by dexamethasone in anucleate HTC cells. Cytoplasts prepared from suspension cultures of HTC cells fully retain active transport of AIB with the same kinetic properties as intact cells. However, the uptake of AIB is not inhibited by dexamethasone or other corticosteroids. Neither is the inhibited rate of transport, manifested by cytoplasts prepared from dexamethasone-treated cells, restored to normal upon removal of the hormone. Anucleate cells exhibit specific, saturable binding of [3H]dexamethasone; however, the binding is reduced compared with that of intact cells. The nucleus is thus required for the glucocorticoid regulation of amino acid transport in HTC cells.

MATERIALS AND METHODS

Cells and Media
HTC cells, an established line of rat hepatoma cells, were grown in spinner culture, without antibiotics, in Eagle's Minimal Essential Medium (autoclavable powder; Grand Island Biological Co., Grand Island, New York), modified as described by Heaton and Gelehrter (11). For experiments, cells were resuspended at 10^6 cells/ml in Induction Medium--bovine serum albumin (IM-BSA; serum-free growth medium supplemented with 1 mg/ml bovine serum albumin and 50 mg/liter neomycin) and incubated in a gyratory shaker water bath at 37°C for 6–18 h before further treatment. Routinely, 94–98% of the cells excluded trypan blue after 24–48 h of incubation in IM-BSA.

Enucleation of HTC Cells
HTC cells were enucleated by centrifuging cytochalasin B–treated cells through a Ficoll gradient, using a procedure modified from Wigler and Weinstein (24, 25). An aqueous 50% stock solution of Ficoll type 400 was diluted with IM-BSA to prepare the components of the gradient solutions; a refractometer was used to check the final concentration of the gradient solutions. The gradients were prepared in 3 × 10 cm cellulose nitrate tubes (Beckman Instrument Co., Spinco Div., Palo Alto, Calif.) by carefully layering the following solutions of Ficoll: 2 ml of 30%; 2 ml of 22%; 0.5 ml of 20%; 0.5 ml of 18%; and 2 ml of 15%. All components of the gradients contain 10 μg/ml cytochalasin B (21 μM)
dissolved in 0.5% dimethyl sulfoxide (DMSO). The gradients were then incubated at 37°C for 16 h.

For six gradients, 3 x 10^6 cells were resuspended in 18 ml of 15% Ficoll containing 10 μg/ml cytochalasin B, and 3 ml of this suspension were carefully layered onto each gradient (5 x 10^6 cells/gradient). Each tube was overlaid with 2 ml of IM-BSA containing cytochalasin B. The gradient tubes were loaded onto a 5W-41 rotor, prewarmed to 37°C. The cells were then centrifuged for 1 h at 25,000 rpm (~100,000 g) in a Beckman L5-50 ultracentrifuge (Beckman Instrument Co.) without refrigeration; the temperature in the chamber during the run was 31°-34°C. After centrifugation, the cytoplasts, which banded primarily in the 20% region of the gradients, were removed, diluted with IM-BSA and centrifuged to remove Ficoll and cytochalasin B, and resuspended in growth medium or IM-BSA as required. The concentration of cytoplasts was determined by counting a sample of suspension in a hemocytometer. A sample of cytoplast suspension was also heat-fixed on a microscope slide and stained with 0.5% acetoorcein to determine the efficiency of the enucleation procedure; 1,500-2,000 nuclei were scored for the numbers of nucleated and nonnucleated cells.

### Assays

Uptake of AIB was measured in a 1.1-ml sample of cell suspension in IM-BSA, to which was added 0.5 μCi [1-14C]aminoisobutyric acid (51.6 mCi/mmol) plus unlabeled AIB to a final concentration of 0.51 mM. After 10 min at 37°C, duplicate 0.5-ml samples were removed, and the cells were pelleted by centrifugation for 40 s at 8,000 g in an Eppendorf 2200 microcentrifuge (Brinkman Instruments, Westbury, N. Y.). The cell pellets were hydrolyzed with KOH and analyzed for radioactivity (11, 16). Extracellular water in the cell pellets was calculated from inulin space (trapped water) measurements; pellet radioactivity was then corrected for trapped-supernate counts, and AIB transport velocity was expressed as nmols per minute per milligram of protein. Protein content of KOH-hydrolyzed cell pellet samples was measured by the method of Lowry et al. (14).

DNA and protein synthesis were measured by incubating cells or cytoplasts in serum-free medium for 1 h at 37°C with 1 μCi/ml [3H]thymidine (6.7 Ci/mmol) or [1-14C]amino acid mixture (159.1 mCi/mg). Thymidine or amino acid incorporation into TCA-precipitable material was measured and expressed as counts per minute per milligram of protein per hour.

Tyrosine aminotransferase was assayed as described by Spencer and Gelehrter (23). Activity is expressed as nmols of p-hydroxyphenylpyruvate formed per minute per milligram of protein.

### Binding of Dexamethasone to Cells and Cytoplasts

Steroid binding to cells and cytoplasts was measured by a procedure adapted from Sibley and Tomkins (22). To tubes containing a total of 5-7 x 10^6 cells or cytoplasts suspended in 0.5 ml of serum-free medium for 1 h at 37°C with 1 μCi/ml [3H]dexamethasone (20.9 Ci/mmol), or with or without 50 μM unlabeled dexamethasone. The cell suspensions were incubated for 40 min at 37°C, then diluted with 3 ml of ice-cold phosphate-buffered saline and centrifuged at 1,200 g for 1 min in an SW-41 rotor (Beckman Co., Morristown, N.J.) centrifuge (24). The cell pellets were washed free of the drug at the same time as the cytoplasts were being prepared for use. During the course of experiments, both cells and cytoplasts were maintained in suspension in IM-BSA supplemented as required with steroids or other compounds.

### RESULTS

HTC cells can be readily enucleated by centrifuging the cells through Ficoll gradients containing cytochalasin B. This drug causes disruption of microfilaments, resulting in extrusion of the nucleus; upon centrifugation, nuclei are separated from cytoplasts on the basis of differences in buoyant density (24). Cytoplasts form a discrete band in the 20% Ficoll region of the gradients; intact cells, centrifuged through gradients without cytochalasin B, are found in a less discrete area in the 22% region of the gradients (Fig. 1). The yield of enucleated cells is usually 60% but ranged from 40 to 80% (Table I). The efficiency of enucleation is >90% as determined by counting cells and cytoplasts in acetoorcein-stained preparations (Fig. 2) and by measuring the incorporation of [3H]thymidine by cells and cytoplasts (Table I). Properties of intact cell controls (amino acid incorporation, AIB transport, and tyrosine aminotransferase induction) were the same whether they were centrifuged through Ficoll gradients without cytochalasin B or were incubated with cytochalasin in 15% Ficoll but not centrifuged in the gradients. Thus, for most experiments, controls were cells treated with cytochalasin B but not centrifuged; they were washed free of the drug at the same time as the cytoplasts were being prepared for use. During the course of experiments, both cells and cytoplasts were maintained in suspension in IM-BSA supplemented as required with steroids or other compounds.

### Characteristics of HTC Cytoplasts

Cytoplasts of HTC cells are rounded and are about half the size of intact cells (Fig. 2). The protein content of cytoplasts was 47% of that of whole cells: 83 ± 4 pg/cytoplast vs. 177 ± 12 pg/cell, mean ± SE (Table I). Enucleated cells readily attach to the surface of plastic tissue culture plates within 1-2 h but fail to take on the polygonal shape assumed by intact cells.

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**FIGURE 1** Schematic diagram of preparation of cytoplasts from HTC cells.

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**PREPARATION OF CYTOPLASTS FROM HTC CELLS**

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Cytoplasts, both attached to plastic and in suspension, maintain their membrane integrity for up to 24 h, as judged by trypan blue exclusion. Protein synthesis is reduced in enucleated cells; the incorporation of \(^3\)H-amino acids into acid-precipitable material by cytoplasts was 35% that of intact cells, and this rate decreased to <20% after 20–22 h (Table I). A similar decrease in the rate of protein synthesis was reported by Ivarie et al. (12) in HTC cells enucleated as monolayer cultures on cover slips.

AIB Transport in HTC Cytoplasts

In initial studies with cytoplasts, we established that the kinetic parameters of transport were the same as in intact cells. Cells and cytoplasts incubated in serum-free medium with 0.5 mM AIB showed the same time-course and extent of accumulation of AIB during 180-min incubation (data not shown). To determine the kinetic parameters of transport by cells and cytoplasts, we incubated duplicate suspensions of each for 10 min with concentrations of AIB ranging from 0.1 mM to 10 mM. The AIB saturation curves obtained were virtually identical for both intact cells and cytoplasts. Fig. 3 shows the data rearranged as a Hofstee plot. The \(V_{\text{max}}\) and apparent \(K_m\) for AIB transport in both cells and cytoplasts are the same: 12 nmol \(\cdot\) min\(^{-1}\) \(\cdot\) mg of protein\(^{-1}\) and 2.4 mM, respectively. Thus, AIB transport appears to be preserved intact in enucleated cells.

To determine whether AIB transport in enucleated cells was inhibited by dexamethasone, we exposed cells and cytoplasts in suspension to 0.1 \(\mu\)M or 50 \(\mu\)M dexamethasone for 8 h and 18 h and we measured the initial rates of AIB uptake. Cytoplasts are capable of active transport of AIB for as long as 18–20 h, at a rate equal to that found in intact cells or two to three times higher when expressed on a per milligram of protein basis (Table II). In the presence of 0.1 \(\mu\)M dexamethasone, AIB transport by intact cells was inhibited 80–90%, whereas the steroid had no effect on the rate of transport by cytoplasts (Table II and Fig. 4). Even 50 \(\mu\)M dexamethasone (a \(>100 \times\) saturating concentration of steroid) had little or no inhibitory effect on transport in cytoplasts (Table II). When similar

| % Yield | % Enucleation (acetoorcein stain) | Thymidine incorporation | % Excluding trypan blue | Protein content* | Amino acid incorporation |
|---------|-----------------------------------|------------------------|------------------------|-----------------|------------------------|
| 63 ± 4 (9) | 98 ± 0.4 (7) | 7.4 ± 0.7 (12) | 98 ± 0.4 (10) | 83 ± 4 (12) | 35 ± 3 (11) | 25 ± 1 (4) | 17 ± 7 (3) |

Cytoplasts were prepared as described in Materials and Methods. Values for each entry are the mean ± SE, with the number of experiments in parentheses. * Intact cell control: 177 ± 12 pg/cell.
experiments were carried out in monolayer cultures, 0.1 μM dexamethasone inhibited AIB transport in intact cells by 45 ± 3% after an 18-h incubation, whereas transport was inhibited only 3 ± 1% in cytoplasts (mean ± SE, n = 7).

The time-course of the effect of steroids on transport in cytoplasts was examined by incubating cells and cytoplasts in serum-free medium with 0.1 μM dexamethasone, 50 μM 11β-hydroxyprogesterone, and 50 μM deoxycorticosterone. The results illustrated in Fig. 4 show that whereas all three steroids inhibit transport ~80% in intact cells by 21 h, AIB uptake in enucleated cells was either not inhibited or only minimally inhibited by any of the three steroids. The nucleus appears to be required to mediate the inhibition of transport in HTC cells by dexamethasone as well as other steroids.

It had been reported previously that dexamethasone did not induce tyrosine aminotransferase activity in rat hepatoma cells enucleated while attached to plastic plates, demonstrating the essential role of the nucleus in the steroid-mediated induction of this enzyme (7, 10, 12). The data of Table II illustrate the effect of dexamethasone on tyrosine aminotransferase induction in our cytoplast preparations and confirm these earlier reports. In this and five other experiments, dexamethasone induced a sixfold or greater increase in transaminase in intact cells, whereas in cytoplasts only a twofold or smaller increase was observed. In our experiments, as in those previously re-

**Figure 3** Kinetics of AIB transport in HTC cells and cytoplasts. Duplicate suspensions of cells (10⁶/ml) or cytoplasts (5 x 10⁶/ml) were incubated for 10 min in serum-free medium with 0.5 μCi/ml [¹⁴C]AIB and unlabeled AIB added to final concentrations ranging from 0.1 to 10 mM. Data from the experiment were rearranged for a Hofstee plot and the best-fit lines were obtained by linear regression analysis (r² is 0.94 for cytoplasts, 0.97 for intact cells).

**Figure 4** Time-course of the effect of dexamethasone and other steroids on AIB transport in cells and cytoplasts; cytoplasts with 0.1 μM dexamethasone (●), or 50 μM 11β-hydroxyprogesterone (▲), or 50 μM deoxycorticosterone (▼); intact cells with dexamethasone (▲), or 11β-hydroxyprogesterone (▼), or deoxycorticosterone (▲). Suspensions of cells or cytoplasts were incubated in serum-free medium with steroid and, at the times indicated, initial rates of uptake of AIB were determined. Each point represents the mean of duplicate samples from a single suspension culture.

**Table II** Dexamethasone Regulation of Transport and Enzyme Induction in HTC Cells and Cytoplasts

| Incubation time | Control | 0.1 μM Dex | 50 μM Dex | Control | 0.1 μM Dex |
|-----------------|---------|------------|-----------|---------|------------|
|                 | nmol AIB·min⁻¹·mg⁻¹ |             | nmol product·min⁻¹·mg⁻¹ |             |             |
| Cells           |         |            |           |         |            |
| 8 h             | 1.98    | 0.80 (60%)* | 0.46 (77%)* | 34      | 214 (529%)‡ |
| 18 h            | 1.54    | 0.29 (81%) | 0.20 (87%) |          |            |
| Cytoplasts      |         |            |           |         |            |
| 8 h             | 4.34    | 4.63 (--)  | 4.17 (4%) | 7       | 17 (143%)  |
| 18 h            | 5.40    | 5.81 (--)  | 4.72 (13%)|          |            |

Each datum represents the mean of duplicate samples from a single culture. Dex, dexamethasone.

* % Inhibition.
‡ % Increase.

Reversibility of Dexamethasone Inhibition of Transport

When intact HTC cells previously incubated with dexamethasone are washed and resuspended in steroid-free medium, the velocity of AIB transport recovers from the dexamethasone-inhibited level to that of untreated cells after 12–18 h. The recovery of transport is blocked by addition of cycloheximide (16). Tyrosine aminotransferase activity of cells from which dexamethasone has been removed decreases with a half-time of ~2.5 h (7). We therefore examined the reversibility of the dexamethasone-inhibited AIB transport in cytoplasts prepared from dexamethasone-treated cells. Cells were incubated for 18 h in IM-BSA containing 0.1 μM dexamethasone and enucleated by centrifuging through Ficoll gradients also containing 0.1 μM steroid. The cytoplasts so obtained were resuspended in conditioned IM-BSA containing dexamethasone, washed twice with, and resuspended in, conditioned IM-BSA with or without dexamethasone. As shown in Fig. 5a, transport velocity in dexamethasone-treated and washed intact cells increased in 2–4 h to ~50% of control rate and reached control values by 12–
20 h. By contrast, cytoplasts prepared from steroid-treated cells, there was only a modest increase in the velocity of transport over that in cytoplasts maintained in steroid-containing medium even after a 20-h incubation in steroid-free medium (Fig. 5a). In the same experiments, transaminase activity of washed intact cells declined 2.4-fold after 5 h, and sevenfold after 12 h; the enzyme activity in cytoplasts subjected to the same treatment did not vary significantly from that in cytoplasts maintained in dexamethasone-containing medium (data not shown). We conclude that the nucleus is required to mediate the reversal of dexamethasone regulation of transport velocity and transaminase activity in HTC cells.

**Mechanism of the Loss of Dexamethasone Regulation of Transport in Anucleate Cells**

The inability of dexamethasone to inhibit AIB transport in enucleated cells could be attributed to several factors. Cytochalasin B itself might interfere in the sequence of events of dexamethasone action (4); cytoplasts might be unable to take up the steroid or to bind it specifically; and the reduced level of protein synthesis in enucleated cells might prevent dexamethasone from producing inhibition. The following experiments were performed to investigate these possibilities.

Incubation of cells with cytochalasin B for 6 h had no effect on transport. The results presented in Fig. 6 show that cytochalasin at 10 μg/ml (21 μM) or 50 μg/ml (105 μM) neither significantly inhibited AIB transport itself nor affected the inhibition of transport by dexamethasone.

Cytoplasts incubated with [3H]dexamethasone show specific, cell-associated binding of the steroid that is blocked by a 1,000-fold excess of unlabeled dexamethasone. However, the specific binding of dexamethasone to cytoplasts was lower than that found in whole cells, i.e., 41% of that in intact cells when expressed per milligram of protein (Table III). Specific, saturable binding of [3H]dexamethasone is also demonstrable in lysates prepared from anucleate cells. The apparent Kd of dexamethasone binding in cytoplast lysates was the same as in lysates of intact cells, but the maximal binding capacity was reduced to ~30% of that in control cell lysates (Table IV).

We have shown previously that the steroid regulation of AIB transport in HTC cells is blocked by high concentrations of cycloheximide (16); thus the reduced rate of protein synthesis observed in cytoplasts might account for the inability of dexamethasone to inhibit transport in these preparations. To further investigate this possibility, we incubated HTC cells with a concentration of cycloheximide (1 μM) that decreased protein synthesis in intact cells to the level observed in cytoplasts, and we measured AIB transport in the presence of this concentration of cycloheximide, with and without 0.1 μM dexamethasone. When protein synthesis was inhibited by 70% in intact cells, the transport of AIB was still fully inhibited by dexamethasone. Thus, the loss of sensitivity of cytoplasts to dexamethasone and other corticosteroids probably cannot be accounted for simply by the reduced level of protein synthesis in enucleated cells.

**DISCUSSION**

Enucleated cells provide a particularly good means to investigate the role of the nucleus in the regulation of cellular transport. The inability of dexamethasone to inhibit AIB transport in enucleated cells could be attributed to several factors. Cytochalasin B itself might interfere in the sequence of events of dexamethasone action (4); cytoplasts might be unable to take up the steroid or to bind it specifically; and the reduced level of protein synthesis in enucleated cells might prevent dexamethasone from producing inhibition. The following experiments were performed to investigate these possibilities.

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functions. In particular, they are useful to examine the possibility that dexamethasone, or the dexamethasone-receptor complex, might have direct effects in the cytoplasm or on membrane functions such as transport. HTC cells in suspension can be conveniently and readily enucleated, with good yield and a high degree of purity, and they retain many of the properties of intact cells. They maintain their membrane integrity, as judged by their ability to exclude trypan blue for at least 24 h and to adhere to plastic. They synthesize protein, as measured by incorporation of amino acids into acid-precipitable material, though at a rate reduced from that of intact cells. We have shown that cytoplasts actively transport AIB and that the kinetic parameters of transport are unchanged from those of whole cells. Enucleated cells also show plasminogen activator activity, another membrane-associated property; glucocorticoid regulation of this property is also lost in cytoplasts (1).

However, steroids fail to regulate AIB transport in enucleated cells. The velocity of AIB transport is not inhibited by dexamethasone even at concentrations as high as 50 μM, and only slightly by deoxycorticosterone or 11β-hydroxyprogesterone at concentrations that produce maximal inhibition in intact cells. Furthermore, AIB uptake by cytoplasts prepared from cells previously incubated with dexamethasone does not increase to its uninhibited level when the cytoplasts are washed free of the steroid, in contrast to the case with intact cells. In these same experiments, we have also shown that tyrosine aminotransferase activity of cytoplasts is neither induced by dexamethasone nor deinduced by removal of steroid from the incubation medium, confirming previous reports (7, 10, 12).

We have examined a number of possibilities to explain the failure of dexamethasone to regulate AIB transport in cytoplasts. Cytochalasin B disrupts microfilaments (24) and is known to inhibit carbohydrate transport in a number of cell lines (6, 17). However, we have shown that this agent by itself has no effect on AIB transport, nor does it alter regulation of transport by dexamethasone in intact cells. Enucleated cells exhibit a decreased level of protein synthesis, and it has been demonstrated that the dexamethasone inhibition of transport can be blocked by cycloheximide (16). However, intact cells incubated with sufficient cycloheximide to inhibit protein synthesis to 30% of control level still show maximal inhibition of transport by dexamethasone. It seems unlikely, then, that the decreased level of protein synthesis in cytoplasts per se can account for the failure of dexamethasone to inhibit transport in these preparations.

Cytoplasts might be unresponsive to corticosteroids because of decreased steroid binding activity resulting from the enucleation procedure. Receptor proteins could be lost during enucleation or could be degraded or altered in some way that might change their steroid binding properties. Intracellular proteases released during the procedure could inactivate or destroy existing receptors, and the reduced level of protein synthesis might prevent synthesis of additional receptors. However, we have demonstrated saturable, specific binding of dexamethasone by cytoplasts and by cell-free lysates prepared from cytoplasts, although the amount bound is less than that bound by intact cells. In HTC cells, glucocorticoid responsiveness is directly proportional to receptor occupancy, i.e., there is no evidence for "spare receptors" (21). Therefore, we might expect decreased dexamethasone binding to be reflected in a decreased magnitude of response, but a 60–70% decrease in binding should not explain a total loss of responsiveness. It is also possible that cytochalasin B and/or the enucleation procedure interferes with the normal activation of receptors after steroid binding. The observations shown in Fig. 6 appear to exclude the former possibility. Cytochalasin B, even at a concentration five times greater than that used for enucleation, does not interfere with the dexamethasone inhibition of AIB transport in intact cells, indicating that receptor activation and translocation into the nucleus remain intact.

We have previously suggested that dexamethasone may induce the synthesis of a labile protein that enhances the degradation of a rate-limiting component of the transport mechanism (8, 16). There is evidence that protein degradation in HTC cells is dependent upon protein synthesis and can be blocked by cycloheximide (5, 9). In addition, in HTC cells enucleation results in a reduction in the rate of degradation of tyrosine aminotransferase and a small number of other unstable proteins (7). These results imply the involvement of some nuclear component in the turnover of HTC cell proteins. Thus the loss of glucocorticoid regulation of amino acid transport in cytoplasts could result from the absence of a nuclear component required for degradation.

We conclude that an intact nucleus is required for the glucocorticoid regulation of amino acid transport in HTC cells. This may reflect a direct nuclear action of the steroid and/or a requirement for the nucleus for protein degradation.

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