Triamcinolone Acetonide Regulates Glucocorticoid-receptor Levels by Decreasing the Half-life of the Activated Nuclear-receptor Form*

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Glucocorticoid-receptor activation in GH1 cells results from the conversion of a 10 S oligomeric cytosolic form to a 4–5 S nuclear-binding species (Raaka, B. M., and Samuels, H. H. (1983) J. Biol. Chem. 258, 417–425). In this study, we report that triamcinolone acetonide (9α-fluoro-11β,16α,17α,21-tetrahydroxy-pregna-1,4-diene-3,20-dione 16,17-acetonide) elicits a time- and dose-dependent reduction of total-cell (nuclear + cytoplasmic) receptor. The mechanism of receptor regulation was studied by dense amino acid labeling of receptor using media containing 3H, 14C, and 15N-labeled amino acids. Total cell receptor was extracted with 0.4 M KCl and newly synthesized dense receptor and pre-existing receptor of normal density. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Isolated cytosol preparations in vitro at 15–30 °C or without hormone by high ionic strength (3, 4). The details of glucocorticoid hormone-mediated receptor activation have also been examined in vivo in several different cell types (5–7). In GH1 cells, a growth-hormone-producing rat pituitary cell line, glucocorticoids act synergistically with thyroid hormone to increase growth-hormone synthesis and mRNA accumulation (5, 8, 9). A previous study from this laboratory analyzed the influence of [3H]triamcinolone acetonide on the receptor forms generated as a consequence of hormone-mediated receptor activation (7).

In GH1 cells, cultured without hormone and harvested in hypotonic buffer, receptor is localized to the cytosol and sediments as 10 S in low-salt gradients (7). When GH1 cells are incubated at 37 °C with [3H]TA, the 10 S cytosolic receptor decreases and a 4–5 S cytosolic receptor reciprocally increases along with the extent of nuclear translocation. Only the 4–5 S cytosolic receptor is a DNA-binding protein, suggesting that the 4–5 S and not the 10 S species represents the activated cytosolic receptor form (7). In the absence of ligand, 0.4 M KCl also converts the 10 S receptor to the 4–5 S form in cytosol in vitro (7). Dense amino acid labeling, followed by sedimentation of cytosol in sucrose/D2O gradients containing 0.4 M KCl, identified discrete 4–5 S forms of newly synthesized dense receptor and pre-existing receptor of normal density. In contrast, sedimentation in low-salt sucrose/D2O gradients demonstrated no discrete density or normal 10 S species but showed as a progressive density shift indicating density heterogeneity of the 10 S form.

These studies support the view that the unactivated 10 S receptor is an oligomer (most likely a tetramer) which is in rapidly exchanging equilibrium in intact cells with a 4–5 S activated hormone-binding subunit (7). The binding of hormone to 10 S receptor appears to markedly shift the oligomer-subunit equilibrium in favor of the 4–5 S component which reversibly associates with chromatin (7). This model is also supported by the observation that when hormone is removed from cells, the 4–5 S cytosolic receptor and receptor bound to nuclei rapidly regenerate the unactivated 10 S form without requirement for new receptor synthesis (7).

In this study, we utilized dense amino acid labeling to analyze the effects of TA on receptor synthesis and degradation in GH1 cells. We examined this since glucocorticoids have been reported to decrease glucocorticoid-receptor levels in vivo (10) as well as in cultured cells (11, 12). Long-term incubation of GH1 cells with TA causes about a 50% reduction...
in both nuclear and cytoplasmic steady-state receptor levels. Receptor half-life was about 20 h in cells cultured without hormone. \( ^3 \)H\[TA \]rapidly labeled the half-life to about 10 h without affecting receptor synthesis and accounts for the decrease in the steady-state amount of receptor. Removal of hormone, which regenerates the 10 S receptor from the activated 4–5 S form, results in prolongation of the half-life to a level observed in control cells. These studies further support a reversible model for receptor activation in which \( ^3 \)H\[TA \]converts the 10 S receptor to the 4–5 S form which is degraded at an increased rate by the cell.

**EXPERIMENTAL PROCEDURES**

**Materials**—[6,7–\( ^3 \)H\[TA \]] (32 Ci/mol) was obtained from New England Nuclear. A mixture of amino acids enriched in the dense isopes \( ^3 \)H, \( ^13 \)C, and \( ^15 \)N to 98, 80, and 90 %, respectively, were obtained from Merck, Sharp and Dohme, Canada. Amino acids with normal density, D, TA, protamine sulfate, and other chemicals were obtained from Sigma. Cell-culture media and serum were supplied by Gibco. The preparation of medium containing dense amino acids (dense medium) was as described previously (7), except that no serum component was added. Each 100 ml of dense medium contained 120 mg of amino acids enriched in the dense isotopes\( ^3 \)H, \( ^13 \)C, and \( ^15 \)N. Dense medium was sterilized by filtration through a 0.25- \( \mu \)m filter.

**Cell-culture Conditions**—GH cells were grown in monolayer cultures at 37 °C in 75-cm² plastic flasks as described previously. Before each experiment, cells were cultured for at least 24 h in Ham's F-10 medium supplemented to 10% (v/v) with hormone-depleted calf serum (14) followed by 24 h in serum-free Ham's F-10. At times ranging from 1-48 h prior to harvesting the cells, the medium was replaced with serum-free medium supplemented with various concentrations of \( ^3 \)H\[TA \]. For density-labeling experiments, the medium was replaced with dense medium, with or without hormone, at various times prior to harvesting the cells. Rat HTC cells and Reuber H-35 hepatoma cells were maintained in Dulbecco’s minimal essential medium supplemented with penicillin (50 \( \mu \)g/ml) and streptomycin (50 \( \mu \)g/ml), 15 mM Heps, pH 7.4, pyruvate (110 milligrams/liter) and 10% fetal bovine serum (v/v). Prior to experiments, the HTC and Reuber H-35 hepatoma cells were cultured for at least 24 h in Dulbecco’s minimal essential medium prepared as above, except that the serum component was 10% (v/v) hormone-depleted fetal bovine serum prepared as described (14).

**Preparation of Nuclei**—The culture medium was removed and the cells were washed three times with isotonic saline at 4 °C and allowed to drain thoroughly to remove the last drops of saline. The cells were harvested with a rubber policeman at 4 °C in 0.25 ml of buffer, pH 7.4 at 25 °C, containing 20 mM Tris-HCl, 5 mM sodium molybdate, 2 mM dithiothreitol, and 0.02% Triton X-100 (low-salt buffer). After mixing for 10 Min at 37 °C, the remaining pelleted nuclei was determined by the method of Burton (15). In all sedimentation experiments, parallel flasks in duplicate were incubated with \( ^3 \)H\[TA \] ± 1,000-fold excess of nonradioactive steroid to assess receptor levels.

**Gradients and Centrifugation**—In every case, discontinuous sucrose gradients were run in a sucrose density gradient of various compositions as described below. The remaining pellet fraction, which contained nuclear material, was washed twice by resuspension in 1 ml of low-salt buffer. The washed nuclear pellet was incubated with 0.5 ml of absolute ethanol for 30 min at 37 °C to extract \( ^3 \)H\[TA \]. Radioactivity in the ethanol extract was determined by scintillation counting at 45% efficiency. The DNA content of the nuclei was determined by the method of Burton (15). In all sedimentation experiments, parallel flasks in duplicate were incubated with \( ^3 \)H\[TA \] ± 1,000-fold excess of nonradioactive steroid to assess receptor levels.

**RESULTS**

**Quantitation of Glucocorticoid Receptor by Protamine Sulfate Precipitation and Sucrose-gradient Sedimentation**—Additional evidence that the 10 S receptor form represents unactivated receptor comes from the protamine sulfate-precipitation study illustrated in Fig. 1. Kalimi (17) reported that a 10 S receptor from rat liver. Fig. 1 shows that protamine sulfate precipitates the 10 S form and not the 4–5 S form which we have previously shown to be a DNA-binding protein. GH cells, cultured in glucocorticoid-free medium for 48 h, were incubated for 1 h (Fig. 1, A and B) or 24 h (Fig. 1, C and D) with 10 nM \( ^3 \)H\[TA \]at 37 °C. Half of each isolated cytosol was precipitated with protamine sulfate and the resultant supernatant (Fig. 1, B and D) and the remaining control cytosol preparations (Fig. 1, A and C) were sedimented in low-salt sucrose/H\( _2 \)O gradients to separate 10 S and 4–5 S receptor forms (7).

In the control cytosols (Fig. 1, A and C), both 10 S and 4–5 S receptor forms are found in the gradients. The percentage of cytosolic \( ^3 \)H\[TA \]-receptor complexes in the 10 S form was 35 ± 1% as shown on this and 34 other gradient profiles (not illustrated). Protamine sulfate treatment removes approximately 90% of the 10 S receptor without affecting the abundance of the slower-sedimenting form but slightly decreases its sedimentation coefficient (Fig. 1, B and D). Radioactivity corresponding to the 10 S form lost from the gradients was quantitatively recovered in the protamine sulfate precipitate. Although the nuclear-bound receptor was about 65% of total-cell receptor at both the 1-h and 24-h \( ^3 \)H\[TA \]-incubation times, receptor levels were reduced at 24 h compared to the 1-h \( ^3 \)H\[TA \]incubation. This is also reflected by the decreased abundance of cytosolic receptor forms at the 24-h incubation time both before and after protamine sulfate precipitation (Fig. 1, C and D).

**Time Course of Receptor Depletion in GH\( _2 \) Cells Incubated with 10 nM \( ^3 \)H\[Triamcinolone Acetonide**—To examine the time course of receptor depletion, GH\( _2 \) cells previously cultured in glucocorticoid-free medium were incubated with 10 nM \( ^3 \)H\[TA \]at 37 °C for various times (Fig. 2). Since the equilibrium dissociation constant for binding of TA to the
glucocorticoid receptor is approximately 1 nM (7), 10 nM \([\text{H}]\)TA occupies greater than 90% of the receptor-binding sites. In GH1 cells exposed to hormone for 1 h, total cytosolic and nuclear binding of \([\text{H}]\)TA to receptor was approximately 260 fmol/100 \(\mu\)g of DNA. Cells exposed to 10 nM hormone for longer times show an initial rapid decrease in the amount of nuclear and cytosolic receptor that by 6 h is about 75% of the 1-h value. By 24 h, total glucocorticoid receptor is 130 fmol/100 \(\mu\)g of DNA which is 50% of the control value. By 48 h, the amount of receptor appears to reach a new steady-state level which is typically 40–50% of the value in cells grown without hormone. Receptor reduction occurs in both the nuclear and cytosolic fractions and, at all times, 60–70% of the total receptor is localized in the nuclear compartment.

**Influence of \([\text{H}]\)Triamcinolone Acetonide on Receptor Abundance in HTC and Reuber H-35 Hepatoma Cells**—The time-dependent depletion of glucocorticoid receptor levels by \([\text{H}]\)TA has been confirmed in two related cell types. HTC and Reuber H-35 hepatoma cells have been extensively studied as model systems for glucocorticoid-hormone action (18–22). A previous study in HTC cells demonstrated that \([\text{H}]\)TA converts the glucocorticoid receptor from a 10 S to a 4–5 S form and that, in the absence of hormone, the receptor in HTC cells has approximately the same half-life as in GH1 cells (7). Table I illustrates that incubation with 10 nM \([\text{H}]\)TA decreases the receptor level within 24 h in both HTC and Reuber H-35 cells. After a 48-h incubation, the amount of receptor decreased by 63 and 32% in HTC and Reuber H-35 cells, respectively.

**Table I**

| Cell type     | [\(\text{H}\)]TA (mol/100 \(\mu\)g DNA) | Receptor Decrease (%) |
|---------------|------------------------------------------|-----------------------|
| Reuber H-35   | 325                                      | 19                    |
| 24            | 265                                      | 32                    |
| 48            | 230                                      | 62                    |
| HTC           | 289                                      | 63                    |
| 24            | 110                                      | 63                    |
| 48            | 108                                      | 63                    |

**Distribution of Pre-existing and Newly Synthesized Glucocorticoid Receptor in the Nuclear and Cytosolic Compartments of GH1 Cells**—In order to perform density-labeling studies, it was necessary to extract the maximum amount of glucocorticoid receptor from the nuclear fraction prior to sucrose/D2O centrifugation. Glucocorticoid hormone-receptor complexes were layered onto sucrose/H2O gradients to separate the time-dependent depletion of glucocorticoid receptor levels by the amount of receptor appears to reach a new steady-state total receptor is localized in the nuclear compartment.

Panels A and C (designated Control) were not precipitated with protamine sulfate. In Panels B and D, the samples were precipitated with protamine sulfate and the supernatants, along with the control cytosols, were layered onto sucrose/H2O gradients to separate the 10 S and 4–5 S receptor forms as described under “Experimental Procedures.” Sedimentation was from right to left and only the bottom 29 fractions are shown.
can be extracted from isolated nuclei with high concentrations of KCl (21), with pyridoxal phosphate (23, 24), or by digestion with micrococcal nuclease (25). An analysis was made of the efficiency of extraction of nuclear glucocorticoid receptor using KCl concentrations from 0.4-0.6 M, micrococcal nuclease digestion followed by extraction with high salt, and, finally, 5 mM pyridoxal phosphate alone and in combination with high salt (data not shown). In all cases, the degree of extraction of nuclear-bound receptor was approximately 55% and was independent of the [3H]TA concentration or incubation time. Therefore, extraction with 0.4 M KCl was chosen for all subsequent studies. This also had the additional advantage of converting the oligomeric 10 S receptor to the 4–5 S form to allow for estimation of receptor half-life and synthesis rates by dense amino acid labeling (7).

Previous short-term incubation studies (3 h) (7) indicated that the nuclear and cytoplasmic forms of the glucocorticoid receptor are in a state of rapidly exchanging equilibrium. To examine this with long-term hormonal exposure and to assess whether the nuclear and cytoplasmic pools have similar turnover rates, dense amino acid labeling of nuclear and cytosolic receptor was examined. Cells were incubated with 10 nM [3H]TA for a total of 44 h and the culture medium was exchanged for dense medium containing 10 nM [3H]TA at the times indicated prior to harvesting the cells. Nuclear and cytosolic fractions were isolated and the receptor was extracted from the nuclear fraction with 0.4 M KCl. After adjusting the cytosolic fraction to 0.4 M KCl, normal and density-labeled receptors from each compartment were separated by velocity sedimentation in sucrose/D2O gradients (Fig. 3). The amounts of normal and dense receptor were determined by summing the appropriate regions of each gradient, which were corrected for the overlap of the two components (7). Cells cultured for 6 h in dense medium had approximately 30% of the receptor in the newly synthesized dense form. After 10 h in dense medium, the amount of dense receptor increased to approximately 50%. By 14 h, the nuclear receptor form is 75% dense and the cytosolic form of the receptor appears to be predominantly dense. The similar distribution of newly synthesized and pre-existing receptor in both cytoplasmic and nuclear compartments after various times of dense amino acid labeling suggests that receptor rapidly equilibrates between these two compartments.

In a related study, GH1 cells previously depleted of glucocorticoid hormones were grown in dense medium for 6–12 h. Exactly 1 h prior to harvesting the cells, 10 nM [3H]TA was added to the culture medium to occupy and elicit nuclear translocation of receptor. The nuclear and cytosolic fractions were separated by centrifugation, adjusted to 0.4 M KCl, and sedimented in sucrose/D2O gradients (data not shown). After each time period of density labeling, the ratio of dense to normal receptors was essentially identical to the nuclear and cytoplasmic compartments. Since the relative abundance of dense and normal receptor was the same in both compartments after short- or long-term incubations with [3H]TA, subsequent analysis of receptor half-life and synthetic rates was made with receptor extracted from broken-cell preparations with 0.4 M KCl.

**Figure 3.** Distribution of pre-existing and newly synthesized glucocorticoid receptor in the nuclear and cytosolic compartments of GH1 cells. GH1 cells were cultured for a total of 44 h in serum-free Ham's F-10 medium supplemented with 10 nM [3H]TA. The medium was replaced with dense medium, 6, 10, and 14 h prior to harvesting the cells. After separating the nuclear and cytosolic fractions by centrifugation, the receptor was extracted from the nuclei with 0.4 M KCl and the cytosol was adjusted to 0.4 M KCl. Dense and normal forms of the receptor were separated by centrifugation in sucrose/D2O gradients containing 0.4 M KCl as described under "Experimental Procedures" and quantitated by summing the appropriate regions of the gradient profiles. Sedimentation was from right to left and only the bottom 26 fractions collected from each gradient are shown.
density remaining as a percentage of total receptor content (not illustrated).

Effect of $[^3H]$Triamcinolone Acetonide on Receptor Half-life and Synthetic Rate during Steady-state Conditions—Dense amino acid labeling was used to compare receptor half-life in control cells cultured without glucocorticoid and in cells cultured with $10 \text{ nM} [^3H]$TA for 44 h to reduce the steady-state amount of receptor (Fig. 5). Receptor half-life was 20 h in control cells and 11 h in cells cultured with $10 \text{ nM} [^3H]$TA for 44 h. The steady-state amounts of receptor were 200 and 110 fmo1/100 μg of DNA, respectively. The half-life ratio of 11/20 = 0.55 is in good agreement with the observed receptor ratio in the presence and absence of TA (110/200 = 0.55). Thus, the change in receptor half-life appears to fully account for the reduction in receptor levels, suggesting that TA had little or no effect on the receptor synthetic rate. Since the receptor levels represent steady-state values, the synthetic rate constant ($k_d$) derived from the above equation was used to calculate receptor half-life ($t_{1/2} = 0.693/k_d$) (26, 27). Receptor half-life estimated under these conditions decreased from about 20 h in the absence (Fig. 6A) to about 9 h in the presence of hormone for 7 h (Fig. 6B). Cells cultured in parallel flasks for 30 h with $[^3H]$TA (Fig. 6C) showed a receptor half-life of 8 h. The estimated half-life of 9 h found after a 7-h exposure to hormone is in excellent agreement with the average value of 9.5 ± 0.3 h derived from 24 separate determinations in receptor-depleted cells treated for 24 h or longer with $10 \text{ nM} [^3H]$TA. It can be calculated that for this excellent agreement to occur, $[^3H]$TA would have to shorten receptor half-life from 20 h to 9 h within 1 h of incubation.

Effect of Cycloheximide on the Hormone-induced Change in Receptor Half-life—To evaluate the role of protein synthesis in hormone-mediated shortening of receptor half-life, GH$_1$ cells were incubated with 25 μM cycloheximide for 10 h with or without $10 \text{ nM} [^3H]$TA. This concentration of cyclohexi-
cycloheximide, received control cells incubated without cycloheximide to KCl and sedimented in sucrose/H2O gradients containing supplemented with receptor in cells incubated with cycloheximide mals glucocorticoids and then incubated with 25 nM [3H]TA. Receptor half-life of receptor complexes. Receptor half-life was estimated using the formulas, \( R(t = 0) \) as the receptor in the cells incubated for the 10-h period which is in agreement with the dense glucocorticoid-receptor levels.

The regulation of glucocorticoid-receptor levels is an important aspect of cellular function. Cycloheximide inhibits protein-synthetic rates by greater than 95% (28). Cells incubated with cycloheximide alone and a set of control cells were grown in medium free of serum and glucocorticoid for 44 h. Normal medium was exchanged with dense medium 7 h before the cells were harvested. In Panel A, the cells received 10 nM [3H]TA 1 h prior to harvesting, while, in Panel B, the cells received [3H]TA for the 7-h dense amino acid-labeling period. In Panel C, cells were incubated for 30 h with [3H]TA including the 7-h dense amino acid-labeling period. Whole cells were extracted with 0.4 M KCl buffer and normal and dense receptors were separated and quantitated as described in Fig. 4. The receptor half-life was estimated using the formulas, \( R(t) = R_{(t = 0)} e^{-kt} \) and \( k_t = 0.693/t_t \), where \( R_t \) is the amount of receptor of normal density remaining at 7 h (t) of dense amino acid labeling. For Panels A and B, \( R_{(t = 0)} \) was determined by summing the dense and normal receptor in Panel A, which received [3H]TA for 1 h. Since Panel C represents a steady-state value, the sum of dense + normal receptor was used as the \( R_{(t = 0)} \) value for Panel C. The gradient profiles illustrated are fractions 1–26 of a total of 40 fractions collected from each gradient. For Panels A and B, the cells received [3H]Triamcinolone Acetonide during approach to steady-state conditions. GH1 cells were grown for 44 h in medium without serum or glucocorticoids and then incubated with 25 μM cycloheximide for 10.5 h. Either 10 or 10 h prior to harvesting the cells, the medium was supplemented with 10 nM [3H]TA. Control cells, incubated without cycloheximide, received 10 nM [3H]TA 1 h prior to harvesting. The receptor was extracted from the broken-cell preparation with 0.4 M KCl and sedimented in sucrose/H2O gradients containing 0.4 M KCl to quantitate receptor. To estimate receptor half-life, the amount of receptor in cells incubated with cycloheximide (\( R_t \)) was compared to control cells incubated without cycloheximide (\( R_{(t = 0)} \)) using the formula \( R_t = R_{(t = 0)} e^{-kt} \) and \( k_t = 0.693/t_t \).

Cycloheximide inhibits protein-synthetic rates by greater than 95% (28). Cells incubated with cycloheximide alone and a set of control cells were grown in medium free of serum and glucocorticoid for 44 h. Normal medium was exchanged with dense medium 7 h before the cells were harvested. In Panel A, the cells received 10 nM [3H]TA 1 h prior to harvesting, while, in Panel B, the cells received [3H]TA for the 7-h dense amino acid-labeling period. In Panel C, cells were incubated for 30 h with [3H]TA including the 7-h dense amino acid-labeling period. Whole cells were extracted with 0.4 M KCl buffer and normal and dense receptors were separated and quantitated as described in Fig. 4. The receptor half-life was estimated using the formulas, \( R(t) = R_{(t = 0)} e^{-kt} \) and \( k_t = 0.693/t_t \), where \( R_t \) is the amount of receptor of normal density remaining at 7 h (t) of dense amino acid labeling. For Panels A and B, \( R_{(t = 0)} \) was determined by summing the dense and normal receptor in Panel A, which received [3H]TA for 1 h. Since Panel C represents a steady-state value, the sum of dense + normal receptor was used as the \( R_{(t = 0)} \) value for Panel C. The gradient profiles illustrated are fractions 1–26 of a total of 40 fractions collected from each gradient.

Table II

| Incubation conditions | Hours of [3H]TA | Total cpm | Per cent of control | Half-life |
|-----------------------|----------------|-----------|---------------------|----------|
| Control               | 1              | 9310      | 100                 |          |
| Cycloheximide         | 1              | 6687      | 72                  | 22 h     |
| Cycloheximide         | 10             | 4605      | 49                  | 10 h     |

The rapid shortening of receptor half-life caused by [3H]TA (Fig. 6) can be reversed by removing the hormone (Fig. 7). Cells were cultured in medium containing 3 nM [3H]TA for 1 h, a concentration which will activate approximately 75% of the receptors (7). After removing hormone from cells by exchanging the culture medium several times over a 210-min period with hormone-free medium, the cells were cultured in dense medium for 11 h with or without 3 nM [3H]TA. This washout procedure removes greater than 99% of cell-associated [3H]TA. Receptor half-life was calculated from the amount of normal-density receptor found in gradients (Fig. 7, B and C) using the total receptor (dense + normal) as \( R_{(t = 0)} \) in the gradient shown in Fig. 7A. Fig. 7A represents control cells incubated with dense amino acids for 11 h and with 3 nM [3H]TA for the final h to identify and quantitate the receptor. The half-life changed from 17 h to 7 h (Fig. 7A) to 10 h in cells exposed to 3 nM [3H]TA continuously (Fig. 7B). In cells exposed to hormone for 1 h (Fig. 7C), receptor half-life was calculated as 15 h. Although in close agreement with the control cells in Fig. 7A, the 15 h half-life (Fig. 7C) may be an underestimate of the actual value. The 1-h preincubation with 3 nM [3H]TA would be expected to reduce the receptor by about 10% prior to the 11-h incubation with dense amino acids. This would result in a slight decrease in the \( R_t/R_{(t = 0)} \) ratio and therefore yield a half-life which is slightly less than the actual value. The results of Fig. 6 along with Fig. 7 suggest that the receptor half-life decreases rapidly after exposure to hormone and that, after removal of hormone, receptor half-life approaches the value found in cells cultured without hormone. Following the removal of hormone from cells, the rate of synthesis of receptor was 9.8 fmol/100 μg of DNA/h (Fig. 7C). This was calculated under nonsteady-state conditions using the formula, \( k_t = k/k_t/(1 - e^{-kt}) \) (26, 27), where the rate is similar to the average in cells cultured without hormone. Following the removal of hormone from cells, the rate of synthesis of receptor was 9.3 fmol/100 μg of DNA/h (Fig. 7C). This was calculated under nonsteady-state conditions using the formula, \( k_t = k/k_t/(1 - e^{-kt}) \) (26, 27), where the rate is similar to the average in cells cultured without hormone.
Fig. 7. Recovery of receptor half-life after removal of [3H]triamcinolone acetone from cells. GH4c1 cells were grown in medium without serum or glucocorticoids for 30 h and two sets of cells (panels B and C) were then incubated with 3 nM [3H]TA for 1 h. A third set of cells (Panel A) was incubated for 1 h in medium without glucocorticoids. Hormone was removed from cell cultures (B and C) by replacing the culture medium four times over a 210-min period with hormone-free medium. After this washing procedure, cells were incubated with dense medium for 11 h either in the presence (B) or absence (A and C) of 3 nM [3H]TA. Cells which did not receive hormone (A and C) during the dense amino acid labeling period were incubated with 3 nM [3H]TA for 1 h prior to harvesting. Normal and dense receptors were extracted and quantitated as described in Fig. 4. Estimates of receptor half-life were made using the formulas indicated in the legend to Fig. 6. $R_{1/2}$ was determined by summing the dense and normal receptor in Panel A. The gradient profiles illustrated are fractions 1–28 of a total of 40 fractions collected from each gradient.

Fig. 8. Relationship of receptor occupancy and receptor half-life to receptor depletion. GH4c1 cells were grown in medium without serum or glucocorticoid hormone for 30 h. The medium in duplicate flasks was supplemented with the concentrations of [3H]TA indicated in the figure 44 h prior to harvesting the cells. Normal medium was replaced with dense medium 11.5 h before harvesting the cells and the concentration of the hormone in the medium was maintained. The concentration of [3H]TA was adjusted to 10 nM in each culture and the dense and normal forms of the receptor were resolved in sucrose/D2O gradients. Degradation rate constants and half-life values were calculated using $R_w$ as the amount of normal density receptor, $t = 11.5$ h, and $R_{1/2}$ as the steady-state amount of receptor (dense + normal) for each [3H]TA concentration.

As illustrated in Fig. 8, half-maximal depletion of total receptor occurred at about 0.5 nM TA. This value is similar to the concentration required for half-maximal occupancy of receptor (Fig. 8) (7), suggesting that receptor activation is necessary for receptor depletion. Receptor half-lives show a dose-dependent decrease (0.2 nM, 16.7 h; 0.5 nM, 14.8 h; 1.0 nM, 11.3 h; 2 nM, 10.6 h; 10 nM, 10.2 h) which parallels the reduction in steady-state glucocorticoid receptor levels. The half-life values are in the expected range as the average values for cells cultured without hormone is $9.15 \pm 1.9$ h ($n = 22$) and $9.5 \pm 0.3$ h ($n = 24$) in receptor-depleted cells under steady-state conditions. The rate of receptor synthesis, calculated from the $k_s$ values and the steady-state amounts of receptor, over the hormone-concentration range was $10.1 \pm 0.24$ fmol/100 µg of DNA/h. This rate is in the expected range found in 12 separate determinations of $3.7 \pm 0.3$ fmol/100 µg of DNA/h.

**DISCUSSION**

Dense amino acid labeling has been used to analyze the influence of [3H]TA on the level of glucocorticoid receptor in
was synthesized at a rate of approximately 10 fmo1/100 pg of DNA/h. These rates establish the steady-state amount of receptors in cells cultured without glucocorticoid at about 10/0.693(19) = 275 fmo1/100 pg of DNA. When GH cells are cultured with 10 nM [3H]TA, receptor half-life is about 10 h and the average synthetic rate was also approximately 10 fmo1/100 pg of DNA/h. These rates account for a new steady-state level of receptor of 10/(0.693/10) = 140 fmo1/100 pg of DNA, which is about 50% of the value found in control cells cultured without hormone.

The amount of glucocorticoid receptor in GH cells begins to decrease within 3 h after addition of 10 nM TA in cells previously cultured without glucocorticoid (Fig. 2). This decrease appears to be secondary to a rapid effect of [3H]TA on shortening receptor half-life without altering receptor synthetic rates (Fig. 6). Based on the dense amino acid-labeling time of 7 h shown in Fig. 6, it can be calculated that [3H]TA shortens receptor half-life within 1 h of incubation from the control value (20 h) to the value determined under steady-state depletion conditions (9 h). Therefore, [3H]TA rapidly elicits a receptor half-life which determines the new steady-state levels of receptor observed after 24-48 h of [3H]TA incubation (Fig. 2).

In this study, estimation of receptor half-life and the influence of [3H]TA on receptor turnover was performed using serum-free conditions. In a previous study, our laboratory reported (7) a 10-h half-life for the glucocorticoid receptor in GH cells which was determined by dense amino acid labeling with media containing 10% hormone-depleted calf serum (v/v). The 2-fold difference in receptor half-life in cells cultured without hormone in serum-free (20 h) versus serum-containing media (10 h) has been consistently reproduced in our laboratory. [3H]TA also elicits receptor depletion in GH cells cultured in media supplemented with hormone-depleted serum. However, the maximal depletion is approximately 35% compared to 50% with serum-free conditions.2 We do not know the reason for these differences. However, since we were primarily interested in studying receptor regulation by glucocorticoid, and serum contains a variety of undefined factors, we performed our studies under serum-free conditions.

In GH cells, the mechanism of glucocorticoid-receptor activation and nuclear translocation is consistent with a model in which the unactivated receptor is a 10 S oligomer (tetramer) that is in rapid equilibrium with a 4–5 S subunit. Glucocorticoid agonists shift the 10 S to 4–5 S equilibrium in the direction of the 4–5 S form, which appears to be activated receptor and shows high affinity for DNA (7). Evidence to support this model comes from: 1) dense amino acid-labeling studies of the 10 S and the 4–5 S receptor forms, 2) glucocorticoid agonist-dose response conversion of the 10 S to the 4–5 S form which parallels nuclear translocation of receptor and, 3) the loss of nuclear receptor and the reappearance of 10 S cytosolic receptor after removal of hormone from intact cells without requirement for new receptor synthesis (7). Additional evidence that the activated receptor in cytosol is in equilibrium with nuclear-bound receptor comes from the dense amino acid-labeling studies illustrated in Fig. 3. In this study, cells were incubated with 10 nM [3H]TA for 44 h followed by dense amino acid labeling in the presence of [3H] TA for 6, 10, and 14 h prior to harvesting. The ratio of newly synthesized dense to pre-existing receptor of normal density in the cytosolic and nuclear compartments were virtually identical, indicating that nuclear-bound receptor is in constant exchange with receptor in the cytosol.

Several lines of evidence suggest that [3H]TA decreases receptor half-life as a consequence of ligand-mediated conversion of the 10 S cytosolic receptor to the 4–5 S nuclear-binding form. First, glucocorticoid receptor half-life and steady-state receptor levels are inversely related to the extent of receptor occupancy and activation in the intact cell (Fig. 8). Secondly, we estimate that [3H]TA shortens receptor half-life from 20 h to 9 h within 1 h of incubation (Fig. 6), at which time receptor is fully occupied and nuclear translocation is complete (7). Finally, removal of hormone, which rapidly regenerates the 10 S unactivated receptor from the 4–5 S nuclear-binding form (7), restores receptor half-life to the value observed in cells cultured without hormone (Fig. 7). Since reduction of receptor half-life by [3H]TA occurs when protein synthesis is inhibited by cycloheximide (Table II), receptor degradation is not secondary to hormonal induction of a receptor-specific protease but appears to reflect ligand-mediated formation of the 4–5 S receptor form which is degraded at an increased rate by the cell.

Although these studies suggest that the 4–5 S receptor form is degraded more rapidly than the 10 S receptor by the cell, dense amino acid-labeling techniques cannot distinguish whether the 4–5 S receptor form is degraded more rapidly in the nuclear or cytosolic compartment. However, since the nuclear and cytosolic receptor pools are in a rapidly exchanging equilibrium (Fig. 3) (7), the ligand-mediated reduction of receptor is evident in both the cytosolic and nuclear compartments. Although [3H]TA translocates about 65% of the glucocorticoid receptor to the nucleus in GH cells, only 55% of nuclear [3H]TA receptor complexes are extracted with 0.4 M KCl. Pyridoxal phosphate or nuclease digestion, alone or in combination with 0.4–0.6 M KCl, did not increase the efficiency of nuclear-receptor extraction. Since all cytosolic receptors can be examined, our dense amino acid-labeling studies analyzed approximately 70% (35% cytosol + (0.55)(65% nuclear)) of total-cell receptor while the remainder represents unextracted nuclear receptor.

The partial extraction of nuclear [3H]TA receptor complexes may reflect the trapping of receptor in chromatin during the extraction process (21, 29) or the binding of [3H]TA to receptor complexes to a non-binding number of high-affinity nuclear-binding sites as has been suggested for the estrogen receptor (30). Nevertheless, the effect of [3H]TA on receptor half-life appears to influence both the 0.4 M KCl extractable and nonextractable receptor pools since the ratio of salt-sensitive to salt-resistant nuclear-bound glucocorticoid receptor complexes was constant in control and receptor-depleted cells. In addition, the receptor half-lives derived from dense amino acid-labeling analysis (extracted nuclear + cytosol receptor) accounts for the extent of receptor depletion in the salt-resistant fraction as well.

As an alternative explanation for hormone-mediated receptor depletion is reversible inactivation of the receptor-binding site rather than acceleration of the rate of receptor degradation. In this case, inactivation refers to loss of glucocorticoid binding rather than inability to interact with DNA or other nuclear components. Wheeler et al. (31) have shown that when IM-9 lymphocytes are incubated in glucose-free medium without oxygen, the glucocorticoid-receptor-binding capacity decreases approximately 2–3-fold. When glucose and oxygen were reintroduced, the receptor-binding activity returned and was independent of protein synthesis. Therefore, TA-mediated receptor depletion in GH cells could result from an effect of hormone which interferes with some metabolic events and

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2 W. R. McIntyre and H. H. Samuels, unpublished observation.
While receptors have suggested that identification of the receptor in the inactive form after hormone removal (Figs. 3 and 7) (7) (7). To account for our observations, we proposed a model in which unactivated and activated receptor are in equilibrium and hormone acts to shift the equilibrium to the activated DNA-binding form (7). Munck and Holbrook (45) recently proposed an alternative cyclic but irreversible model of receptor activation which requires energy for regeneration of unactivated receptor. Although our current study supports the view that activated receptor is degraded more rapidly by the cell, it is not distinguish between the reversible or irreversible models for receptor activation. Mineralocorticoids (46), progesterone (47, 48), and estrogen (49) have been reported to elicit rapid decreases in total-cell homologous-receptor content. Recently, Eckert et al. (50) reported that estrogen can shorten the half-life of the estrogen receptor in MCF7 cells. Therefore, a decrease in receptor abundance and rapid shortening of receptor half-life may be a general phenomena which occurs as a consequence of steroid-hormone-mediated receptor activation in the cell.

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Regulation of Glucocorticoid-receptor Levels

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