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Unique long-acting antiglucocorticoid in whole and broken cell systems

(cortisol 21-mesylate/irreversible antagonists/tyrosine aminotransferase/HTC cells/glucocorticoid receptors)

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ABSTRACT **The biological properties of cortisol 21-mesylate (CM), an alkylating derivative of cortisol, were investigated in a line of rat hepatoma tissue culture (HTC) cells. CM appears to bind to glucocorticoid receptors in cell-free extracts because CM inhibits the specific binding of [^{3}H]dexamethasone. However, in whole cells CM not only fails to induce the enzyme tyrosine aminotransferase (TyrATase) but also inhibits the induction of TyrATase by dexamethasone. Thus CM is an antiglucocorticoid. This is not caused by cell death, because CM is relatively nontoxic up to concentrations of 10 μM. The concentration of CM needed for half maximal inhibition of TyrATase induction is an order of magnitude lower than that predicted from the apparent cell-free affinity of CM for the glucocorticoid receptors of HTC cells, which suggests that the cell-free binding data does not reflect an equilibrium situation. In fact, the reactive α-keto mesylate group was intentionally incorporated into cortisol in hopes of obtaining a steroid capable of undergoing irreversible reactions. When HTC cells were preincubated with either CM or the reversible antiglucocorticoid progesterone and then washed to remove free steroid, only the CM-treated cells failed to show subsequent induction of TyrATase by dexamethasone. Furthermore, preincubation of HTC-cell cytosol with CM blocked ≈75% of the subsequent exchange binding of [^{3}H]dexamethasone to glucocorticoid receptor sites. Thus, the actions of CM in whole and broken cells either require an exceptionally long time for reversal or are not reversible. Together, these results indicate that CM is a unique antagonist and could be an irreversible antiglucocorticoid *in vitro*.**

Steroid antagonists have been quite useful in studying the mechanism of steroid hormone action both *in vivo* and *in vitro* and have had practical clinical applications. The antiestrogens nafoxidine and tamoxifen have been extensively employed in studies of estrogen action (1–6) and are used in the treatment of estrogen-dependent breast cancer (7, 8). Investigations with the antiglucocorticoid progesterone led to the allosteric model of glucocorticoid action (9). The antimineralocorticoid spironolactone is used in the treatment of hypermineralocorticoid syndromes and as a diuretic (10).

After the initial observations of steroid hormone antagonism (9, 11–15), radioactively labeled antagonists have been employed to try to establish their mechanism of action. Although the details are far from understood, it does appear that all of the above steroid antagonists exert their effects through the appropriate steroid receptor (1–6, 9, 16). However, this is not the only possible mode of action of an antagonist. In theory, an antagonist could affect any of the known and unknown steps involved in steroid hormone action—from entry of the steroid into the target cell to the production of the observed biological end products. Long-acting steroid antagonists (e.g., refs. 1–6) are of particular interest in mechanistic studies and clinical applications; an "irreversible" antagonist would be exceptionally useful. Irreversibility could be defined as permanent inhibition of the steroid-induced effect(s), but because of the regenerative properties of biological systems, this is an improbable result. A more likely definition is that an irreversible antagonist covalently inactivates one or more steps in the steroid-specific chain of events. Restoration of steroid sensitivity in the affected system would thus require renewal of the blocked elements, which could occur rapidly or slowly.

We have prepared several derivatives of active glucocorticoids for the purpose of affinity-labeling glucocorticoid receptors (17, 18). In the course of these studies, we observed some unique biological properties for one of these steroids, cortisol 21-mesylate (CM). The results described below indicate that CM, which is an alkylating steroid (18, 19), is an exceptionally long-acting antiglucocorticoid in whole and broken cells and could be an irreversible inhibitor of glucocorticoid action.

EXPERIMENTAL

[^{3}H]Dexamethasone (24 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels) was purchased from Amersham. Dexamethasone (16α-methyl-9α-fluoro-1,4-pregnadiene-11β, 17α, 21-triol-3,20-dione) was a gift from T. Y. Shen of Merck Sharp & Dohme. Cortisol (4-pregnene-11β, 17α, 21-triol-3,20-dione) was purchased from Sigma. The growth of rat hepatoma tissue culture (HTC) cells in spinner (9) or monolayer cultures (20) has been described. The spinner culture cells were centrifuged in a 0°C rotor (700 × *g* for 15 min), washed twice with phosphate-buffered saline (700 × *g* at 0°C for 5 min), and stored at −20°C for up to 3 months before use. The induction of tyrosine aminotransferase (TyrATase) was studied in whole cells at 37°C; steroid binding to cell-free receptors was conducted at 0–4°C. Details of the cell-free competition assay (21), the cell-free exchange assay (18), and the whole-cell TyrATase assays with monolayer cultures (21) are to be found elsewhere. Quantitations of protein and TyrATase activity were achieved by the methods of Lowry *et al.* (22) and Gopalakrishnan and Thompson (20), respectively. CM was prepared as described elsewhere (18).

RESULTS

CM prevents the cell-free formation of receptor–dexamethasone complexes. Thus, in a cell-free competition assay with crude receptors from a well-characterized (9, 17, 18, 21, 23) line of rat hepatoma tissue culture (HTC) cells, the apparent affinity of CM for glucocorticoid receptors was found to be $^1/_{15}$th that of the parent steroid cortisol (Fig. 1). This result was not

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Abbreviations: HTC cells, rat hepatoma tissue culture cells; TyrATase, tyrosine aminotransferase, L-tyrosine:2-oxoglutarate aminotransferase (EC 2.6.1.5); CM, cortisol 21-mesylate, 4-pregnene-11β, 17α, 21-triol-3,20-dione-21-methanesulfonate.

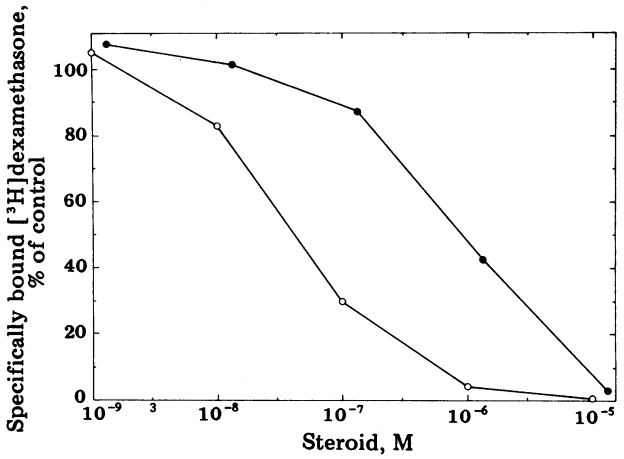

FIG. 1. Determination of the relative affinities of cortisol and CM for HTC-cell glucocorticoid receptors in a cell-free competition assay. Duplicate crude HTC-cell receptor solutions were incubated for 3 hr at 0°C with 5.6 nM [^{3}H]dexamethasone and varying concentrations of cortisol or CM (final protein concentration = 5.1 mg/ml). A 500-fold excess of dexamethasone was used to determine the level of nonspecific binding of the ^{3}H-labeled steroid. The average specifically bound [^{3}H]dexamethasone in the presence of varying concentrations of cortisol (○) or CM (●) was plotted as percentage of the control (variation < ±4%) (21).

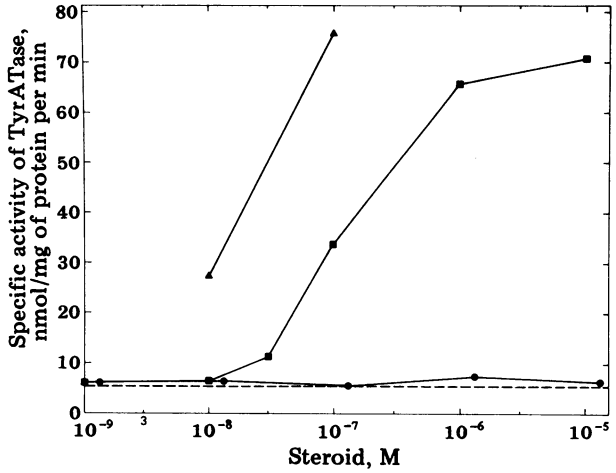

FIG. 2. Biological activity of dexamethasone, cortisol, and CM in whole HTC cells. Duplicate monolayer cultures of HTC cells were treated with fresh medium containing 1% EtOH with or without dexamethasone (▲), cortisol (■), or CM (●) for 18 hr at 37°C, followed by harvesting and determination of the specific enzyme activity of TyrATase as described (21). The basal level of TyrATase activity is shown by the dashed line.

unexpected because, in general, the introduction of bulky substituents on the C-21 of glucocorticoids results in derivatives with a reduced affinity for glucocorticoid receptors (18, 24). What was unexpected was the effect of CM on the induction of TyrATase.

HTC cells display several biological responses to glucocorticoids (9, 25). TyrATase is one specific glucocorticoid-inducible enzyme in HTC cells; extensive studies by Tomkins *et al.* argue that TyrATase induction in HTC cells is a primary response to glucocorticoids that is initiated by the binding of steroid to a specific cytoplasmic receptor (23). Because the whole-cell dose–induction curve usually agrees quite well with the cell-free binding of steroid agonists to receptors (21, 23, 26), it was surprising that the whole-HTC-cell biological activity of CM did *not* parallel the above determined cell-free affinity of CM for HTC-cell receptors. Instead of being $^1/_{15}$th as active as cortisol in inducing TyrATase in whole HTC cells, CM was completely inactive (Fig. 2). One explanation for this lack of biological activity is that the reactive CM is converted to an inactive steroid. Alternatively, CM could be an anti-inducer or an inhibitor of glucocorticoid action. The inhibition by CM of TyrATase induction by the glucocorticoid dexamethasone in whole cells (Fig. 3) showed that the second interpretation is correct—i.e., that CM is an antiglucocorticoid. Furthermore, CM apparently is a potent antiglucocorticoid.

If CM inhibits TyrATase induction by interaction with glucocorticoid receptors (which has not yet been ascertained), then the K_i for CM would reflect the strength of its association with receptors. Analysis of Fig. 3 allowed a rough estimation of the IC_{50}, or apparent K_i, for CM ($\approx 10^{-7}$M) that was an order of magnitude lower than that expected from the apparent affinity of CM for HTC-cell receptors (see Fig. 1). This fact suggested that the kinetic parameters of CM binding to receptors would be completely different from those of the parent steroid cortisol—i.e., a very slow rate of association but an extremely slow rate of dissociation (27)—or that CM is acting as an irreversible inhibitor of glucocorticoid action. One approach to establishing CM as an irreversible inhibitor of TyrATase induction by glucocorticoids is to see if the effects of CM can be reversed by washing the cells. Most glucocorticoids are readily removed from whole cells by washing (9). Thus HTC cells were preincubated overnight with vehicle, progesterone [a known reversible antiglucocorticoid (9, 26)], or CM. The cells were then washed once and resuspended in fresh medium containing 20 nM dexamethasone with or without the steroid of preincubation. A plot of the ensuing time course of TyrATase induction (Fig. 4) shows the normal induction of TyrATase for vehicle- or progesterone-pretreated cells, confirming the reversible nature of progesterone action (9, 26) and the efficacy of our procedures in removing noncovalently bound steroid. In marked contrast, however, these procedures did not reverse the effects of the CM preincubation. There was no induction of TyrATase in the CM-pretreated, dexamethasone-posttreated cells until ≈8 hr after the wash procedure, a time when the vehicle- and progesterone-pretreated cells showed considerable induction of TyrATase (Fig. 4). Even 24 hr after the wash

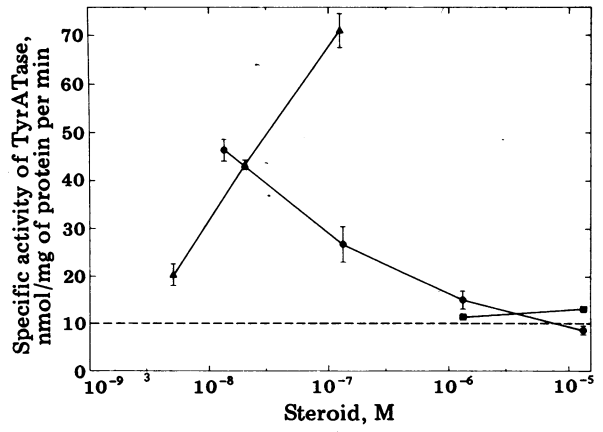

FIG. 3. Inhibition of dexamethasone induction of TyrATase activity in whole HTC cells by CM. Duplicate monolayer plates of HTC cells were treated with medium containing 1% EtOH with or without dexamethasone (▲) or CM (■) as controls and 20 nM dexamethasone with varying concentrations of CM (●). After incubation for 18 hr at 37°C, the specific enzyme activity of TyrATase in cell extracts (21) was plotted against the concentration of that steroid present in varying amounts. The range of each duplicate determination is shown by error bars when it exceeds the area of the data points. The basal level of TyrATase activity is indicated by the dashed line.

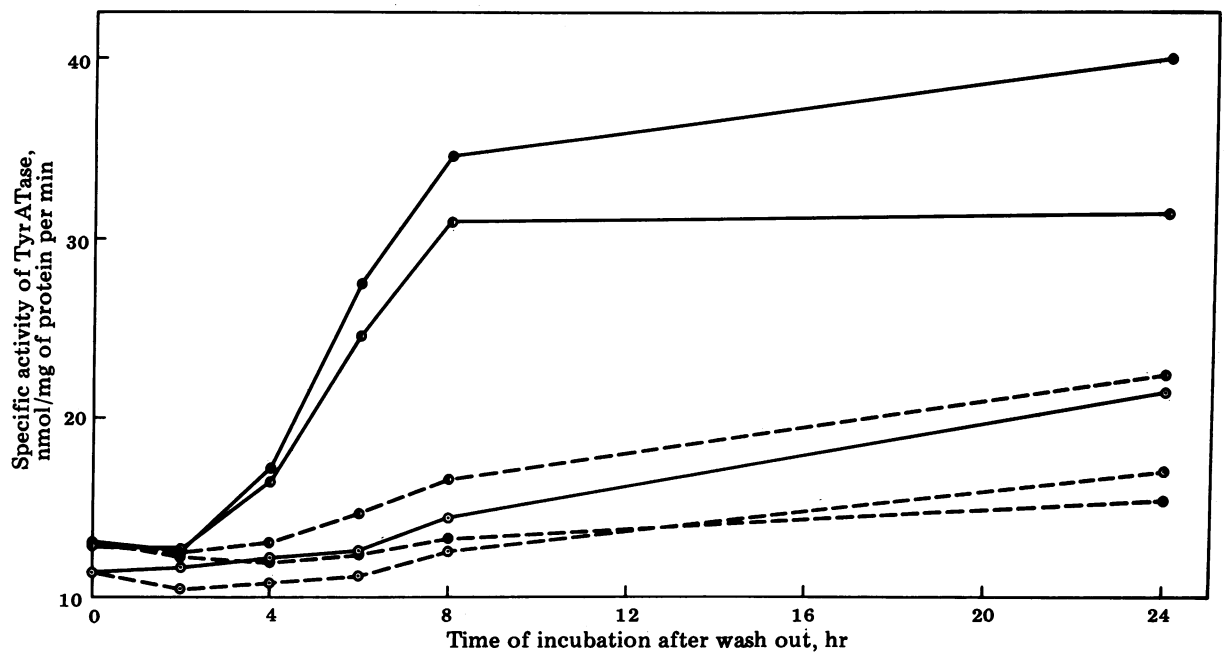

FIG. 4. TyrATase induction after EtOH, progesterone, or CM pretreatment of HTC cells. Spinner cultures containing 200 ml of HTC cells at 2×10^5 cells per ml were treated with 0.938 ml of EtOH with or without 0.469 mM progesterone or 1.28 mM CM. After incubation at 37°C for 15.5 hr, $\approx 6 \times 10^7$ cells were centrifuged at $600 \times g$ for 10 min at 22°C and resuspended in 200 ml of "conditioned" medium that also had been "incubated" at 37°C for 15.5 hr. After a second centrifugation, each cell pellet was again resuspended in 210 ml of "conditioned" medium. Duplicate aliquots (3 ml) were removed for zero-time protein and TyrATase determinations, and duplicate 50-ml aliquots were placed in 100-ml Wheaton bottles containing 300 μl of EtOH with or without 3.67 μM dexamethasone for the EtOH preincubated cells and 300 μl of EtOH solutions of 3.67 μM dexamethasone without or without 0.367 mM progesterone (for the progesterone pretreated cells) or 1.0 mM CM (for the CM pretreated cells). The cells in Wheaton bottles were incubated in a rotating water bath (37°C at 175 rpm) with 3-ml aliquots being removed at various time points for protein and TyrATase determinations in the usual manner (21). The specific enzyme activity of TyrATase was then plotted against the length of time of incubation after the addition of steroid to washed cells for (*i*) EtOH pretreated cells treated after wash out with dexamethasone (●—●) or EtOH (●---●); (*ii*) progesterone pretreated cells treated after wash out with dexamethasone (◐—◐) or dexamethasone with progesterone (◐---◐); and (*iii*) CM pretreated cells treated after wash out with dexamethasone (○—○) or dexamethasone + CM (○---○). The average variation among duplicates was ±2.4%. Similar results have been obtained when the final concentrations of CM and progesterone were equal (i.e., 4.0 μM; data not shown).

procedure, the magnitude of TyrATase induction in the CM-pretreated, dexamethasone-posttreated cells was significantly reduced; this limited induction could be blocked if CM is added with dexamethasone after the wash procedure. Finally, it is noteworthy that CM, 24 hr after being "removed" from the cells by the washing procedure, was just as efficient in reducing the magnitude of TyrATase induction as was progesterone when it was continuously present in the cells (Fig. 4).

The observed inhibition of dexamethasone induction of TyrATase by CM does not seem to be due to general toxicity of the reactive CM for several reasons. (*i*) In two experiments, comparison after 16–24 hr of cells treated with 13 μM CM and 0.1 μM cortisol or with 6 μM CM and 0.04 μM dexamethasone to vehicle-, cortisol-, or dexamethasone-treated controls revealed no effect on cell number and cell viability (93–94% compared to 93–97% for controls, as determined by trypan blue exclusion) and only minor effects on total cell protein ($\approx$1% increase to 6% decrease). (*ii*) In six experiments, including one of the above, the effect of the maximum dose of 13 μM CM for 16–18 hr on total HTC-cell protein content was minor (average of 12% lower than vehicle-, cortisol- or dexamethasone-treated controls). In three other experiments involving longer exposure to CM (i.e., 16-hr preincubation with vehicle or 3–6 μM CM followed by another 24 hr with added dexamethasone), the proteins in the cells chronically treated with CM were an average of 20% lower than those of the vehicle, with or without dexamethasone, controls. (*iii*) After $\approx$24 hr, CM was no longer completely inhibitory, and some inducibility of TyrATase returned to HTC cells (data not shown).

We next looked to see if the cell-free action of CM—i.e., prevention of dexamethasone binding to HTC-cell receptors—was reversible. By using our described exchange conditions (18), crude HTC-cell receptors were incubated with enough cortisol or CM to occupy $\approx$75% of the receptor (based on data of Fig. 1). After $\approx$3 hr, activated charcoal was added to remove free steroid and to inactivate steroid-free receptors (18). An excess of [^{3}H]dexamethasone was then introduced to examine the ability of receptors previously exposed to cortisol or CM to bind the added ^{3}H-labeled steroid. The preincubation of HTC-cell receptors with CM reduced by $\approx$75% the ability of these receptors to subsequently bind [^{3}H]dexamethasone (Fig. 5). Thus CM is either a long-acting or an irreversible inhibitor of one aspect of glucocorticoid action in broken cells—i.e., the ability of glucocorticoids to bind to glucocorticoid pretreated receptors.

DISCUSSION

We have investigated some of the biological properties of CM, a chemically reactive derivative of cortisol, in cell-free and whole-cell assays utilizing the well-studied line of HTC cells (9, 17, 18, 21, 23, 25, 26, 28). CM contains the novel α-keto mesylate group that specifically and rapidly reacts with thiols at 0°C (18, 19). Thus CM is a new alkylating steroid that would be predicted to readily form covalent steroid complexes with thiol-containing molecules, peptides, and proteins. This steroid is an inhibitor of glucocorticoid action in a whole-cell biological assay (Fig. 3). As such, CM is one of a small number of anti-glucocorticoids containing glucocorticoid-specific structures

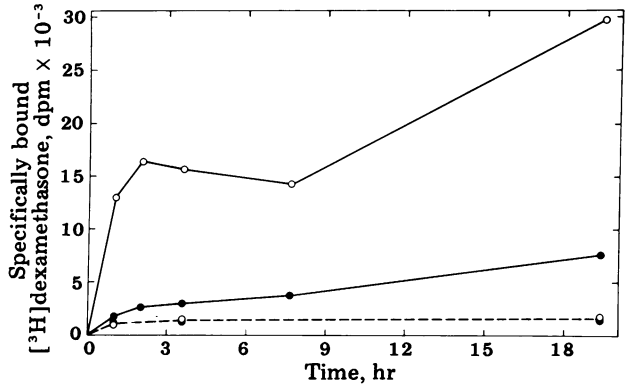

FIG. 5. Inhibition of [^{3}H]dexamethasone exchange binding to preformed complexes by CM. By using the described exchange assay (18), crude HTC-cell receptors were preincubated with 60 nM cortisol or 1.35 μM CM for 2.8 hr at 0°C (final protein concentration, 7.0 mg/ml). After addition of activated charcoal to remove free steroid and to inactivate steroid-free receptors, followed by centrifugation to pellet the activated charcoal, the preincubated cytosols were adjusted to 19 nM [^{3}H]dexamethasone with or without 11 μM dexamethasone. After subsequent incubation for the indicated time, activated charcoal was again added to remove free steroid, and the amount of specifically bound [^{3}H]dexamethasone (equals total minus nonspecific binding) that was formed by exchange binding in cortisol (○—○) or CM (●—●) preincubated cytosols was determined. For comparison, the amount of nonspecific binding in cortisol (○) or CM (●) preincubated receptor solutions is shown by the dashed line.

(29, 30). Furthermore, the data of Figs. 4 and 5 indicate that the effects of CM are not readily reversible under conditions that do reverse the actions of other known reversible steroids such as progesterone (Fig. 4) or cortisol (Fig. 5). Thus, CM is a unique, long-acting, and possibly irreversible antiglucocorticoid in whole cell and broken cell systems.

At this point it must be emphasized that while CM does compete with [^{3}H]dexamethasone for binding to glucocorticoid receptors (Fig. 1), we do not know if the long-acting, apparently irreversible effects of CM (Figs. 4 and 5) are due to reaction with receptors. We cannot rule out other noncompetitive interactions of CM with receptors or reactions of CM with unknown factors that might be needed for receptor activity. Radioactively-labeled CM is required to answer these questions and to begin to elucidate the mechanism of CM action.

Although there are a few apparent exceptions (21), the concentration of a given steroid required to give half-maximal biological response is greater than or equal to the K_d of the binding of the same steroid to its receptor (23, 26). In contrast, the IC_{50} (or apparent K_i) of CM is $\approx$0.1 μM (Fig. 3), which is equal to the K_d of cortisol induction of TyrATase in HTC cells (data not shown). This IC_{50} (apparent K_i) is an order of magnitude *less* than the apparent K_d of CM for HTC-cell glucocorticoid receptors (Fig. 1). This disagreement is most easily explained by nonequilibrium conditions with the chemically reactive CM, especially in the cell-free competition assay of Fig. 1. While short-time competition assays with the tritiated forms of potent glucocorticoids such as dexamethasone are routinely used to determine the relative K_ds of unlabeled steroids for receptors (17, 18, 21, 26, 30–32), a half-time of 3–10 hr‡ for the dissociation of dexamethasone from receptors (34) requires that competition assays of 15–50 hr be used to obtain true equilibrium measurements. Thus, for steroids with slow dissociation rates, these competition assays largely reflect the varying association rates of steroid and receptor (21). Because 3 hr at 0°C is enough to inhibit $\approx$75% of the expected exchange-binding of [^{3}H]dexamethasone with CM–receptor§ complexes (Fig. 5), the above inequality of IC_{50} or $K_i \neq K_d$ can be explained by Eq. 1,

$$\text{CM} + \text{receptor} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \underset{\text{noncovalent}}{\overset{(\text{Dex})}{\text{CM·receptor}}} \overset{k_2}{\rightleftharpoons} \underset{\text{covalent}}{\overset{(\text{Dex})}{\text{CM-receptor}}}, \quad [1]$$

in which $k_{-1} \ll k_1$ for dexamethasone and CM¶ or, if CM gives a covalent complex, $k_2 \gg k_{-1}$ for CM. Thus k_1 for CM and dexamethasone would be rate limiting in the cell-free competition assay. Only in the much longer whole cell experiment at 37°C (Fig. 3) would the binding of dexamethasone be close to true equilibrium (21), in which case the tight CM·receptor complex or the irreversible formation of covalent CM-receptor would significantly displace the equilibrium with dexamethasone to cause the low IC_{50} of CM. In this respect, it is also of interest to compare CM with progesterone, a known, rapidly reversible antiglucocorticoid (26). The apparent K_ds of these steroids with HTC-cell glucocorticoid receptors are 15 and 5 times greater, respectively, than the K_d of cortisol (31). Thus, all other things being equal, a concentration of CM 3 times that of progesterone would be required to cause equivalent effects. However, due to the long-acting or irreversible nature of CM, Fig. 4 shows that, over a 24-hr period, a preincubation with CM, followed by washing the cells to remove the free CM, causes just as much antiglucocorticoid activity as does progesterone that is *continuously* present at one-third the CM concentration.

The observation that CM is a long-acting or irreversible antiglucocorticoid in whole cells would not have been possible if both of the following two conditions did not exist. First, CM has to be reactive and stable, in order to give some reaction with those components that are mandatory for steroid action, but not be consumed by reacting at 37°C with the large excess of other compounds that are present in HTC cells and the tissue culture medium. The β-hydroxy-α-keto mesylate functional group of CM rapidly reacts with thiolate anions ($R\text{-}S^-$) at 0°C but is essentially inert toward other nucleophiles such as thiols, carboxylates, imidazoles, amines, and alcohols (19). Even so, we were pleasantly surprised when the kinetics of TyrATase inhibition in dexamethasone/CM solutions (data not shown) and of experiments of the type in Fig. 4 indicated that CM was fully active as an antiglucocorticoid in tissue culture for 17–18 hr. Thus, the reactive CM is a long-lasting antiglucocorticoid.

The second necessary condition is that replenishment of inactivated receptors or other cell factors needed for steroid activity be slow. If inactivated receptors or factors were rapidly resynthesized as soon as they were inactivated by CM, it would be very difficult to observe any prolonged activity or irreversibility on the part of CM. However, this is obviously not the case, because removing free CM from pretreated cells and then adding dexamethasone cause no induction of any TyrATase for 6–8 hr and only partial induction of TyrATase after 24 hr (Fig. 4). In three experiments, TyrATase induced by dexamethasone in CM-pretreated cells appeared an average of 2.7 hr after the

‡ With activation of the receptor–steroid complex, the half-time for dissociation increases to about 30 hr (33; W. B. Pratt, personal communication).

§ The terms CM-receptor and CM·receptor (see also Eq. 1) do not imply that CM occupies the steroid-binding cavity of the receptor but rather that CM interacts with receptors or factors required for receptor activity in a manner that prevents the binding of dexamethasone.

¶ For cortisol, k_{-1} is not $\ll k_1$ and the half-time for dissociation is $\approx$1 hr (27).

onset of TyrATase induction in EtOH or progesterone-pretreated cells. Thus, it would appear that the rate of synthesis of new receptors or factors is relatively slow. Further work is required to answer this question, but experiments with CM of the type of Fig. 4 should enable a determination of the rate of synthesis of new, functionally active receptors or factors required for steroid activity.

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1. Clark, J. H., Peck, E. J., Jr., Hardin, J. W. & Eriksson, H. (1978) in *Receptors and Hormone Action*, eds. O'Malley, B. W. & Birnbaumer, L. (Academic, New York), Vol. 2, pp. 1–31.
2. Clark, J. H., Peck, E. J., Jr. & Anderson, J. N. (1974) *Nature (London)* **251**, 446–448.
3. Katzenellenbogen, B. S. & Ferguson, E. R. (1975) *Endocrinology* **97**, 1–12.
4. Katzenellenbogen, B. S., Ferguson, E. R. & Lan, N. C. (1977) *Endocrinology* **100**, 1252–1259.
5. Rochefort, H. & Capony, F. (1977) *Biochem. Biophys. Res. Commun.* **75**, 227–285.
6. Horwitz, K. B. & McGuire, W. L. (1978) *J. Biol. Chem.* **253**, 8185–8191.
7. Legha, S. S. & Carter, S. K. (1976) *Cancer Treatment Rev.* **3**, 205–216.
8. Kiang, D. T. & Kennedy, B. J. (1977) *Ann. Intern. Med.* **87**, 687–690.
9. Samuels, H. H. & Tomkins, G. M. (1970) *J. Mol. Biol.* **52**, 57–74.
10. Addison, G. M., Asmussen, N. W., Corvol, P., Kloppenborg, P. W. C., Norman, N., Schröder, R. & Robertson, J. I. S., eds. (1978) *Aldosterone Antagonists in Clinical Medicine* (Excerpta Medica, Amsterdam).
11. Bartter, F. C., ed. (1960) *The Clinical Use of Aldosterone Antagonists* (Thomas, Springfield, IL).
12. Harper, M. J. K. & Walpole, A. L. (1966) *Nature (London)* **212**, 87.
13. Munck, A. & Brink-Johnsen, T. (1968) *J. Biol. Chem.* **243**, 5556–5565.
14. Hormones and Antagonists—Proceedings of the Fourth International Seminar on Reproductive Physiology and Sexual Endocrinology in *Gynecologic Investigation* **2**, (1971/1972) 150–323 and **3**, (1972) 2–164.
15. Neumann, F. & Steinbeck, H. (1974) in *Androgens II and Antiandrogens* eds. Eichler, O., Farah, A., Herken, H. & Welch, A. D. (Springer, New York), pp. 235–484.
16. King, R. J. B. & Mainwaring, W. I. P. (1974) *Steroid-Cell Interactions* (University Park Press, Baltimore, MD).
17. Simons, S. S., Jr., Thompson, E. B. & Johnson, D. F. (1979) *Biochemistry* **18**, 4915–4922.
18. Simons, S. S., Jr., Thompson, E. B., Merchlinsky, M. J. & Johnson, D. F. (1980) *J. Steroid Biochem.* **13**, 311–322.
19. Simons, S. S., Jr., Pons, M. & Johnson, D. F. (1980) *J. Org. Chem.* **45**, 3084–3088.
20. Gopalakrishnan, T. V. & Thompson, E. B. (1977) *J. Biol. Chem.* **252**, 2717–2725.
21. Simons, S. S., Jr., Thompson, E. B. & Johnson, D. F. (1979) *Biochem. Biophys. Res. Commun.* **86**, 793–800.
22. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
23. Baxter, J. D., Rousseau, G. G., Higgins, S. J. & Tompkins, G. M. (1973) in *Biochemistry of Gene Expression in Higher Organisms*, eds. Pollack, J. K. & Lee, J. W. (Australia and New Zealand Book Co., Sydney, Australia), pp. 206–224.
24. Failla, D., Tomkins, G. M. & Santi, D. V. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 3849–3852.
25. Ivarie, R. D. & O'Farrell, P. H. (1978) *Cell* **13**, 41–55.
26. Rousseau, G. G., Baxter, J. D. & Tompkins, G. M. (1972) *J. Mol. Biol.* **67**, 99–115.
27. Pratt, W. B., Kaine, J. L. & Pratt, D. V. (1975) *J. Biol. Chem.* **250**, 4584–4591.
28. Simons, S. S., Jr. (1979) in *Glucocorticoid Hormone Action*, eds. Baxter, J. D. & Rousseau, G. G. (Springer, Berlin), pp. 161–187.
29. Cutler, G. B., Jr., Barnes, K. M., Sauer, M. A. & Loriaux, D. L. (1979) *Endocrinology* **104**, 1839–1844.
30. Rousseau, G. G., Kirchhoff, J., Formstecher, P. & Lustenberger, P. (1979) *Nature (London)* **279**, 158–160.
31. Rousseau, G. G. & Schmit, J. P. (1977) *J. Steroid Bhiochem.* **8**, 911–919.
32. Turnell, R. W., Kaiser, N., Milholland, R. J. & Rosen, F. (1974) *J. Biol. Chem.* **249**, 1133–1138.
33. Kaine, J. L., Nielsen, C. J. & Pratt, W. B. (1975) *Mol. Pharmacol.* **11**, 578–587.
34. Rousseau, G. G. & Baxter, J. D. (1979) in *Glucocorticoid Hormone Action*, eds. Baxter, J. D. & Rousseau, G. G. (Springer, Berlin), pp. 49–78.