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Glucocorticoid hormones increase the activity of plasma membrane alkaline phosphodiesterase I in rat hepatoma cells

(steroids/enzyme induction/enzyme repression/glucocorticoid domain/hepatoma tissue culture cells)

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ABSTRACT **In rat hepatoma cells the synthetic glucocorticoid dexamethasone causes a 3-fold increase in the activity of the plasma membrane enzyme alkaline phosphodiesterase I (oligonucleate 5′-nucleotidohydrolase, EC 3.1.4.1). The data are consistent with an induction phenomenon mediated by the glucocorticoid receptor involved in tyrosine aminotransferase induction. The effect on alkaline phosphodiesterase I is not a reflection of a general membrane effect of dexamethasone, because the activity of three other enzymes of the plasma membrane is unaffected. On the other hand, nucleoside diphosphatase (nucleoside diphosphate phosphohydrolase acting on ADP) activity is inhibited. Thus, two more enzymes sensitive to glucocorticoids have been identified in a cell line in which these hormones influence only very few gene products. This paper describes enzymatic changes in the plasma membrane of rat hepatoma cells in which glucocorticoids normalize a number of membrane-associated processes that are considered to be characteristic of transformed cells.**

Glucocorticoids modify several properties of cellular plasma membranes. One of the earliest effects of these hormones in lymphoid cells is an inhibition of hexose, nucleoside, and amino acid uptake (1). Glucocorticoid hormones also decrease hexose uptake by fibroblasts (2) and by adipocytes (3). In rat hepatoma tissue culture (HTC) cells, glucocorticoids promote cell adhesiveness and the reappearance of surface antigens present in normal hepatocytes (4). These hormones also inhibit the release of plasminogen activator (5) and the uptake of aminoisobutyric acid (6, 7), and they decrease the number of cell surface microvilli (8). Such modifications of HTC cell membrane functions have been considered as part of a program of glucocorticoid-induced reversal of the transformed phenotype (8). The biochemical reactions underlying these effects have not been elucidated.

Glucocorticoid hormones appear to produce many of their physiological effects through a stimulation of the activity of specific enzymes in target cells, most often as a result of an increase in their rate of synthesis (9). This prompted us to examine whether enzymes located in the plasma membrane could be under glucocorticoid control, an effect that might be related to one or more of the phenomena described above. This possibility was investigated in HTC cells wherein the interaction of steroids with intracellular receptors and the ensuing induction of tyrosine aminotransferase (L-tyrosine:2-oxoglutarate aminotransferase, EC 2.6.1.5) have been extensively studied as a model of glucocorticoid action (10). In this system the hormonal response is very specific in that the number of glucocorticoid-sensitive gene products is quite limited (11). Only seven proteins are reproducibly induced, including two unidentified membrane proteins. We show here that, in HTC cells, glucocorticoid hormones specifically increase the activity of alkaline phosphodiesterase I (oligonucleate 5′-nucleotidohydrolase, EC 3.1.4.1), an enzyme localized in the plasma membrane of HTC (ref. 12; P. Sauvage and P. Tulkens, personal communication) and normal liver cells (13, 14).

MATERIALS AND METHODS

Cell Culture. HTC cells, clone 4 from Gordon Tomkins' laboratory, were grown and maintained as described (4) in Swim's S-77 medium (GIBCO) containing 10% (vol/vol) newborn calf serum. Where indicated (serum-free medium), the latter was replaced by a solution of bovine serum albumin (1 g/liter) in phosphate-buffered saline (0.15 M NaCl/2.5 mM KCl/8 mM Na_2HPO_4/1.5 mM KH_2PO_4, pH 7.45). Steroids in ethanol or ethanol alone were added to cultures. The final concentration of ethanol did not exceed 0.5%. After incubation at 37°C, cells were washed extensively with phosphate-buffered saline before storage at −20°C.

Enzyme Assays. For determination of alkaline phosphodiesterase I, frozen HTC cells were sonicated for 10 sec (Branson Sonifier) in an aqueous solution of Triton X-100 (1 g/liter). Enzyme activity was determined as described by Beaufay *et al.* (15) by incubating the sample for 10 min at 25°C in a mixture containing 0.1 M glycine·NaOH (pH 9.6), 2 mM zinc acetate, and 3 mM *p*-nitrophenylthymidine 5′-phosphate (from Sigma or Boehringer Mannheim). The reaction rate was constant for at least 20 min and the assay was linear with protein concentration up to 120 μg per assay. For determination of tyrosine aminotransferase activity, cells were sonicated as described above in 50 mM potassium phosphate buffer (pH 7.6) containing 5 mM 2-oxoglutarate, 1 mM EDTA, and 0.2 mM pyridoxal phosphate. Enzyme activity was determined according to the method of Diamondstone (16), as modified by Granner *et al.* (17).

Other enzymes were assayed by using cell homogenates (Dounce homogenizer tight pestle) prepared in 0.25 M sucrose/3 mM imidazole·HCl, pH 7.4. Each reaction was conducted in a 1-ml incubation mixture. The alkaline phosphatase (orthophosphoric-monoester phosphohydrolase, EC 3.1.3.1) reaction contained 50 mM glycine·KOH, 5 mM *p*-nitrophenylphosphate, 5 mM $MgCl_2$, 70 mM KCl with incubation for 10 min at 37°C at pH 9.0. *p*-Nitrophenol was measured as described by Beaufay *et al.* (15). The aminopeptidase [α-aminoacyl-peptide hydrolase (microsomal), EC 3.4.11.2] reaction mixture contained 50 mM potassium phosphate and 0.343 mM leucyl-2-naphthylamide with incubation for 30 min at 37°C and pH 7.2. 2-Naphthylamine was assayed by Barrett's method

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Abbreviations: HTC, hepatoma tissue culture; dexamethasone, 1,4-pregnadiene-9α-fluor-16α-methyl-11β,17α,21-triol-3,20-dione.

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(18). Mg^{2+} ATPase (ATP phosphohydrolase, EC 3.6.1.3) assay consisted of 50 mM Tris·HCl, 5 mM ATP, 5 mM $MgCl_2$, 1 mM dithiothreitol, and 2 μg of oligomycin per ml with incubation for 20 min at 37°C and pH 7.4. Nucleoside diphosphatase (nucleoside diphosphate phosphohydrolase acting on ADP) activity was quantitated in a mixture containing 50 mM Tris·HCl, 5 mM ADP, and 5 mM $CaCl_2$ incubated for 20 min at 30°C and pH 7.5. In the last two reactions, 1 ml of 15% trichloroacetic acid was added at the end of the incubation time and phosphate was determined by the method of Fiske and Subbarow (19).

Other Methods. Assays for cell adhesiveness were performed according to Ballard and Tomkins (4). Cells were centrifuged and resuspended in serum-free medium and their concentration was measured by turbidimetry at 650 nm. The cells were allowed to attach to the bottom of the glass vessel for 2 hr and then were agitated for 3 min at 100 rpm on a Buchler SM rotary shaker and the turbidity of the suspension was again determined. The decrease in optical density, expressed as the percentage change in the optical density caused by attachment, was taken as a measure of cell adhesiveness. Protein was determined by the method of Lowry *et al.* (20).

RESULTS

Effect of Dexamethasone on HTC Cell Enzymes. HTC cells were exposed to dexamethasone, a synthetic glucocorticoid, for 48 hr at a concentration (0.1–0.5 μM) that saturates the glucocorticoid receptor and evokes a maximal effect on tyrosine aminotransferase (21). As shown in Table 1, this treatment results in a more than 10-fold increase in the activity of this enzyme. The cell protein content was not significantly modified by dexamethasone. We also confirmed that the adhesiveness to glass of this HTC clone is increased by dexamethasone from 18.8% ± 4.6 to 43.0% ± 7.2 (mean ± SEM of seven separate experiments). The activities of several enzymes were determined after the same glucocorticoid treatment. We consistently observed a 3-fold increase in the activity of the plasma membrane enzyme alkaline phosphodiesterase I (Table 1). This effect appears to be specific because no increase occurred for alkaline phosphatase, Mg^{2+} ATPase, and aminopeptidase (Table 1). Interestingly, there was a decrease in the activity of nucleoside diphosphatase. These four enzymes are considered to be primarily associated with the plasma membrane in hepatocytes (ref. 22; M. Prado-Figueroa, personal communication) and in HTC cells (ref. 12; P. Sauvage and P. Tulkens, personal communication). HTC cells do not contain detectable amounts of the typical 5′-nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5) (12), an enzyme that serves as a marker for the hepatic plasma membrane: 5′-Nucleotidase activity did not appear after dexamethasone treatment of HTC cells. The same is true for $NAD(P)^+$ nucleosidase [$NAD(P)^+$ glycohydrolase, EC 3.2.2.6], another enzyme of rat liver plasma membranes (23) that is absent from HTC cells.

Table 1. Effect of dexamethasone on HTC cell enzymes

Enzyme	Dexamethasone	milliunits/mg of protein	
Tyrosine aminotransferase	−	1.8 ± 0.1	(4)
	+	25.9 ± 7.0	(4)
Alkaline phosphodiesterase I	−	72 ± 16	(6)
	+	229 ± 21	(6)
Alkaline phosphatase	−	8.8 ± 1.2	(5)
	+	8.2 ± 1.9	(5)
Mg^{2+} ATPase	−	43.4 ± 5.8	(3)
	+	49.0 ± 21	(3)
Aminopeptidase	−	23.6 ± 1.9	(2)
	+	22.4	(1)
Nucleoside diphosphatase	−	12.4 ± 0.5	(3)
	+	7.0 ± 0.8	(3)

Cells were exposed for 48 hr to a concentration of dexamethasone (0.1–0.5 μM) that saturates the glucocorticoid receptor (21). The values are means ± SD for the number of experiments indicated in parentheses. Milliunits are nanomoles of product formed per min.

Location and Properties of Alkaline Phosphodiesterase I in Control and Dexamethasone-Treated HTC Cells. The distribution of alkaline phosphodiesterase I is not significantly modified by dexamethasone. First, in two experiments, 93% of the enzyme activity remained associated with the particulate fraction in dexamethasone-treated cells compared to 91% in control cells. Second, upon isopycnic centrifugation of the cell organelles from dexamethasone-treated cells, the gradient subfraction between densities 1.141 and 1.156 had the highest phosphodiesterase I specific activity (enrichment 24-fold over homogenate) with a good yield of 13%. A mean density of ≈1.15 in sucrose/H_2O gradients has been reported for plasma membrane-related structures in rat liver (22) and HTC cells (ref. 12; P. Sauvage and P. Tulkens, personal communication).

In hepatocytes alkaline phosphodiesterase I is thought to be located on the outer surface of the cell (24). Thus, dexamethasone stimulation could cause the release of active enzyme into the culture medium where it would escape detection by our assay procedure. This is not the case in either suspension or monolayer cultures because at least 96% of the activity remains associated with the cells. Evidence suggesting that the enzyme is in the same membrane environment in stimulated as in control cells stems from the Arrhenius plots shown in Fig. 1. These plots exhibit a thermal transition that is typical of some membrane enzymes (25). The energies of activation and the transition temperature are essentially the same with and without stimulation of the cells with dexamethasone. Such is also the case for the dependence on pH and zinc concentration (Table 2).

Finally, we examined whether dexamethasone increased the V_{max} of the enzyme or its affinity for the substrate. The latter

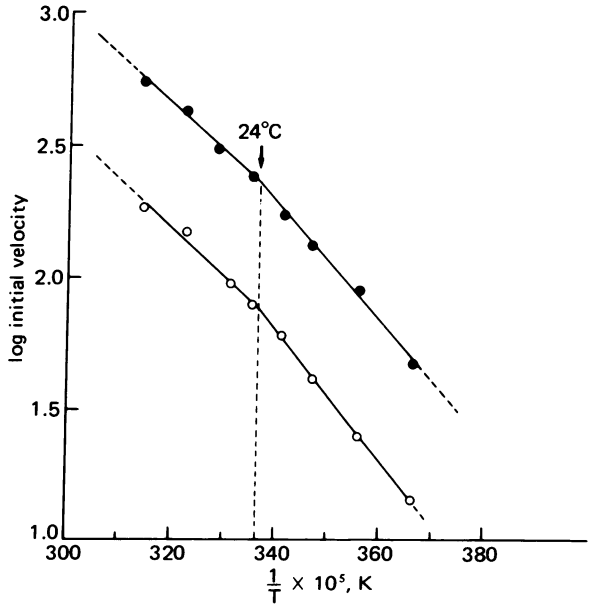

FIG. 1. Arrhenius plots for alkaline phosphodiesterase I activity in extracts from control (○) and dexamethasone-treated (0.5 μM, 48 hr) (●) cells. Initial velocities (milliunits/mg of protein) were calculated by least mean squares fit of time-course determinations (minimum of 12). The reaction rates were constant with time even at the highest temperature studied (45°C).

Table 2. Kinetic parameters of alkaline phosphodiesterase I activity in HTC cells

	Control	Dexamethasone
Apparent K_m, M	6×10^{-4}	6×10^{-4}
V_{max}	70	210
pH for maximal activity	10.0	10.0
$[Zn^{2+}]$ for half-maximal activity	2×10^{-7}	2×10^{-7}
Transition temperature, °C	24	24
Activation energy, kcal		
<24°C	11.4	10.2
>24°C	8.0	8.0
Q_{10} (27–37°C)	1.55	1.55

HTC cells were exposed to 0.5 μM dexamethasone or to the vehicle for either 32 hr in serum-free medium (substrate dependence) or 48 hr in serum-containing medium (other parameters). K_m and V_{max} (milliunits/mg of protein) were calculated by linear regression from 9-points Woolf plots. To establish the pH dependence (16 points), we used 100 mM Tris·HCl (pH 7.2–8.8) and 100 mM glycine·NaOH (pH 7.9–10.2) as buffer systems. The activation energies were calculated by least mean squares fit from the slopes of Arrhenius plots shown in Fig. 1.

possibility was excluded by the observation that the apparent K_m of the enzyme is the same in dexamethasone-treated and control HTC cells (Table 2). Thus, there is an increase in either the concentration of enzyme molecules or in their catalytic activity.

Glucocorticoid Specificity of the Stimulation of Alkaline Phosphodiesterase I. Steroid hormones at high concentration can directly modify certain properties of cellular membranes (8) and purified enzymes (26). It is unlikely that this is the effect of dexamethasone on alkaline phosphodiesterase I. First, the addition of dexamethasone or cortisol (0.1–10 μM) to a cell homogenate *in vitro* (25 hr, 0°C) or to the assay reaction mixture (10 min, 25°C) did not influence the activity of this enzyme. Second, in the intact cell, various steroids increased alkaline phosphodiesterase I activity according to their ability to induce the cytosoluble enzyme tyrosine aminotransferase (Table 3). Of the naturally occurring glucocorticoids, cortisol and corticosterone were the most active. No effect was seen with tetrahydrocorticosterone, an hormonally inactive glucocorticoid metabolite, nor with the sex steroid hormones. Third, a 200-fold excess of the glucocorticoid antagonists estradiol or progesterone (10) or of the partial antagonist deoxycorticosterone (10) inhibited the stimulatory effect of dexamethasone (not shown).

Table 3. Effect of different steroids on the activities of tyrosine aminotransferase and alkaline phosphodiesterase I in HTC cells

Steroid	Tyrosine aminotransferase	Alkaline phosphodiesterase I
Dexamethasone	100	100
Cortisol	81	76
Corticosterone	86	71
Tetrahydrocorticosterone	0	2
Progesterone	4	3
Estradiol	0	4
17α-Methyltestosterone	0	2

HTC cells were resuspended in serum-free medium and incubated at 37°C for 48 hr with or without one of the steroids listed (5 μM). The activities of the two enzymes are expressed as the percentage of the maximum increase over the basal activity measured in the absence of steroid. Mean basal activities were 1.7 and 85 milliunits/mg of protein for tyrosine aminotransferase and alkaline phosphodiesterase I, respectively. The corresponding values in the presence of dexamethasone were 29 and 244 milliunits/mg of protein. Data are means of two experiments.

Fourth, we find that HTC cell alkaline phosphodiesterase I activity is increased by concentrations of dexamethasone as low as 2 nM. The dose–response curve (Fig. 2) shows that this process is as sensitive to dexamethasone as is the induction of tyrosine aminotransferase. The half-maximum effect for both is achieved at 15–20 nM dexamethasone, a concentration similar to the equilibrium dissociation constant for the glucocorticoid receptor (10, 21). This experiment was done in serum-free medium to ensure that the low levels of glucocorticoids present in serum would not shift the dose–response curve. However, the stimulation of alkaline phosphodiesterase I activity also occurs in the presence of serum (Table 1). Taken together, the data presented in this section suggest that this action of glucocorticoid hormones is mediated by the same receptor as that involved in the induction of tyrosine aminotransferase.

Mechanism of the Glucocorticoid Effect. The stimulation of alkaline phosphodiesterase I activity becomes apparent after a lag period of at least 8 hr (Fig. 3) and is maximal after 48–72 hr, depending on the feeding schedule of the culture. The lag period is consistent with an indirect effect of the hormone, perhaps an induction of new enzyme molecules as is the case for tyrosine aminotransferase. It is also possible that glucocorticoids induce a slowly turning-over factor which in turn enhances the catalytic activity of alkaline phosphodiesterase I. Such a mechanism is thought to operate in the stimulation of alkaline phosphatase activity by glucocorticoids in HeLa cells (27). However, alkaline phosphodiesterase I activity in mixtures of extracts from control and dexamethasone-treated cells is strictly additive (Fig. 4). This does not support the idea that dexamethasone promotes the appearance of an activator or inactivates an inhibitor present in control cells.

Upon removal of dexamethasone from HTC cells exposed

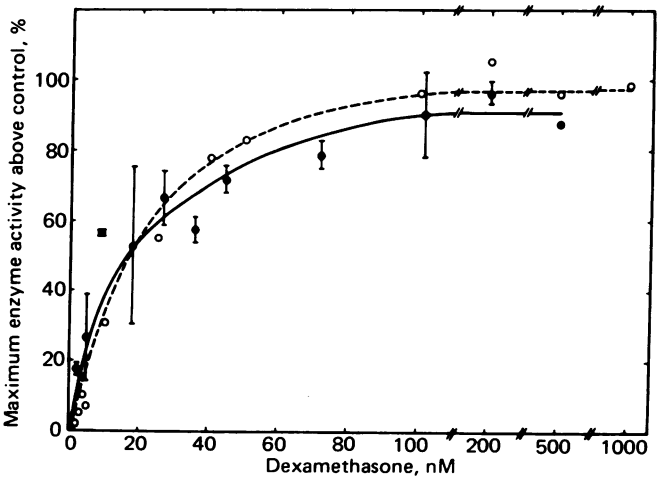

FIG. 2. Effect of various concentrations of dexamethasone on the activity of tyrosine aminotransferase (○) and alkaline phosphodiesterase I (●). HTC cells were incubated in serum-free medium for either 24 hr (○) or 48 hr (●) in the presence of dexamethasone at the indicated concentrations. The activities of the two enzymes are expressed as a percentage of the maximum increase over the basal activity measured in the absence of dexamethasone. Data for tyrosine aminotransferase are single values from three separate experiments. The mean basal and maximum activities were 1.8 and 22 milliunits/mg of protein, respectively. Data for alkaline phosphodiesterase I are means and range from two experiments. The mean basal and maximum activities were 89 and 261 milliunits/mg of protein, respectively.

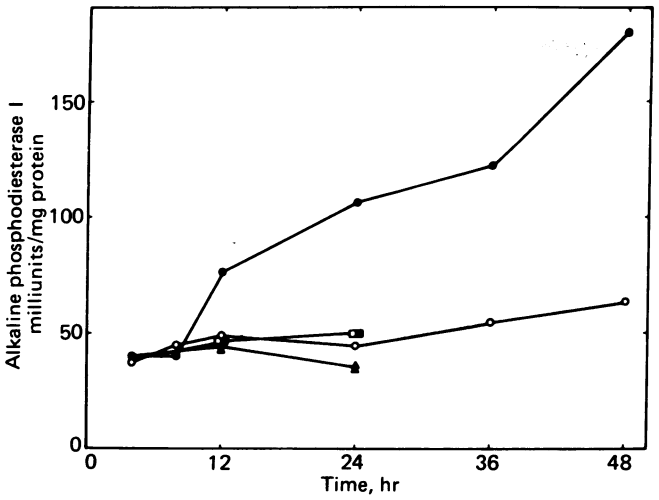

FIG. 3. Time course of stimulation of alkaline phosphodiesterase I by dexamethasone. HTC cells in the exponential phase of growth (0.25×10^6 cells per ml at zero time) were exposed to 0.1 μM dexamethasone (●, ■, ▲) or to the vehicle (○, □, △) and grown without further feeding until the end of the experiment. Aliquots from each of these two cultures received in addition either 0.5 μg of actinomycin D per ml (■, □) or 0.1 mM cycloheximide (▲, △), or were grown without the inhibitors (●, ○). All additions were made at zero time. At the times indicated, samples from the cultures were removed for enzyme assay and protein determination.

to this steroid for 48 hr, alkaline phosphodiesterase I activity returns to control levels with a half-life of at least 10 hr. The longer the half-life of an enzyme, the slower the rate at which its activity will increase in response to an increased rate of synthesis (28). The slow kinetics of appearance and disappearance of the dexamethasone effect are similar to those for

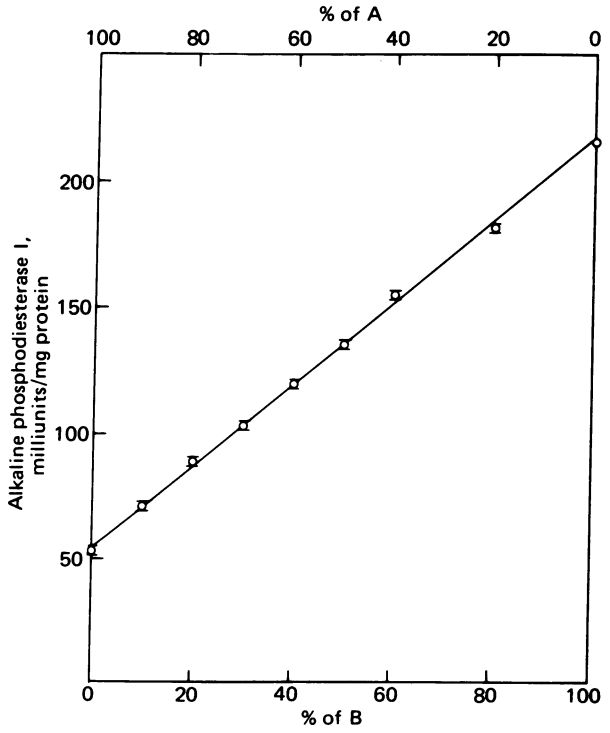

FIG. 4. Alkaline phosphodiesterase I activity in mixtures of extracts from control (A) and dexamethasone-treated (0.5 μM, 48 hr) (B) HTC cells. Both A and B extracts were brought to the same protein concentration. Incubation mixtures contained 100 μg of protein.

the phenomenon of increased cell adhesiveness (4), but differ from the rapid induction and deinduction of tyrosine aminotransferase, an enzyme that has a half-life of 2–3 hr. Such kinetic differences are consistent with the different half-lives of these enzymes if stimulation is the result of an increased rate of synthesis. We investigated whether the stimulation of alkaline phosphodiesterase I by dexamethasone required concomitant RNA and protein synthesis. As shown in Fig. 3, actinomycin D and cycloheximide both prevent the glucocorticoid effect. The induction of tyrosine aminotransferase is also blocked, as expected.

DISCUSSION

We have presented here direct evidence that a plasma membrane enzyme from a cell of hepatic origin is stimulated by glucocorticoid hormones. Based on two-dimensional gel electrophoresis after pulse-labeling with [^{35}S]methionine, Ivarie and O'Farrell (11) have shown that the so-called "domain" of the glucocorticoid response in HTC and other cells is restricted to less than 1% of the detectable proteins. Thus, our observation contributes to a better definition of the glucocorticoid domain in HTC cells. In these cells "Belt I," one of the induced proteins whose functions so far remain unidentified, is reportedly a sialoglycoprotein located on the external side of the plasma membrane (11). Although alkaline phosphodiesterase I is similarly located, it is premature to speculate on the relationship between Belt I and alkaline phosphodiesterase I. First, Belt I is a secreted glycoprotein (H. Baumann, T. D. Gelehrter, and D. Doyle, personal communication). Second, under the denaturing and reducing conditions of these gels, Belt I corresponds to a polypeptide with an apparent molecular weight of about 50,000 (11). In contrast, rat liver alkaline phosphodiesterase I is said to consist of a single polypeptide chain with a molecular weight of about 137,000 (29). The molecular weight of HTC cell alkaline phosphodiesterase I is unknown, but the enzyme is identical to the liver enzyme in K_m, Q_{10}, and pH optimum (unpublished data).

The physiological function of alkaline phosphodiesterase I is unknown. Its external location enables it to interact with the cellular environment and this enzyme responds to glucocorticoids with the same time-course, dose–response, and steroid specificity as does cell adhesiveness (4). It is therefore tempting to speculate that both events are functionally related.

Our observations may also provide insight into the mechanism whereby glucocorticoid hormones regulate the expression of specific genes. This is thought to result from an increase in the concentration of specific mRNAs, presumably by a direct effect on transcription. Indeed, dexamethasone increases the activity of tyrosine aminotransferase mRNA in HTC cells (30). The finding that nucleoside diphosphatase is inhibited by dexamethasone pertains to the question of whether glucocorticoids can repress as well as induce polypeptide synthesis (11). These hormones certainly inhibit several cell functions. However, in the few instances in which the mechanism has been elucidated, the inhibition results from the stimulation of an inhibitory factor. This is the case for the inhibition by dexamethasone of plasminogen activator activity (31) and of amino acid uptake in HTC cells (8). If the inhibition of nucleoside diphosphatase is a direct, receptor-mediated effect of glucocorticoids, then models for steroid action must account for the concomitant stimulation and inhibition of different gene products by the same hormone in the same cell.

A new approach to the study of glucocorticoid action involves the use of HTC cell variants that have apparently normal receptors but have lost the inducibility of several glucocorticoid-responsive functions (32). There is evidence that such losses

are not random but, rather, that certain "clusters" of responses (enzyme induction) are lost, whereas others (membrane-related processes) are retained (33). Work on such variants has already shown that plasminogen activator does not play an important role in the regulation of cell adhesiveness (34). It will be interesting to see whether alkaline phosphodiesterase I behaves as a "membrane function" or an induced protein in these variants. Our preliminary experiments indicate that some variants (generously provided by E. B. Thompson) are "constitutive" for alkaline phosphodiesterase I (i.e., have levels of this enzyme as high as in the glucocorticoid-stimulated wild type), whereas their nucleoside diphosphatase is still inhibited by dexamethasone. These observations might provide the basis for genetic complementation in cell–cell hybridization experiments and should help to correlate membrane enzyme activity with membrane properties.

Finally, the possibility of specifically modifying the concentration of membrane enzymes by glucocorticoids could give clues about the functions and biogenesis of the plasma membrane in HTC (35) and normal cells.

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