ATP-dependent Activation of L Cell Glucocorticoid Receptors to the Steroid Binding Form*

(Received for publication, November 27, 1978)

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The specific glucocorticoid binding capacity in cytosols prepared from L929 mouse fibroblasts (L cells) is inactivated with a half-life of approximately 2 h at 25°C. As previously published, this inactivation can be prevented with 10 mM molybdate and markedly slowed by addition of other phosphatase inhibitors such as glucose 1-phosphate and fluoride. We have now found that ATP (5 to 10 mM) also slows the rate of this inactivation. After extensively inactivating the receptor by preincubating cytosol at 25°C for 4 h and preventing further inactivation by addition of molybdate, addition of ATP results in reactivation of the steroid binding capacity. Maximal reactivation of 40 to 70% is achieved with 5 to 10 mM ATP. The activation is temperature-dependent and specific for ATP. ADP, GTP, CTP, and UTP do not cause activation and preliminary results indicate no effect of cyclic nucleotides in this system. If activation is prevented by addition of 10 mM EDTA to the cytosol, addition of 3 to 10 mM magnesium permits ATP-dependent activation of the binding capacity. The level of reactivation can be enhanced by addition of a heat-stable factor prepared from the same L cell supernatant. These results support the proposal that L cell glucocorticoid receptors can be activated to the glucocorticoid binding state by an ATP-dependent phosphorylation mechanism.

We have recently proposed that unbound glucocorticoid receptors may be inactivated by a mechanism involving dephosphorylation. Two types of studies support this proposal. First, addition of highly purified calf intestinal alkaline phosphatase results in rapid inactivation of the glucocorticoid binding capacity in 100,000 × g supernatants of L929 mouse fibroblasts (L cells) (1). This inactivation shows the zinc dependence and arsenate inhibition characteristic of alkaline phosphatase. The second type of evidence involves inhibition of endogenous receptor inactivation by addition of known phosphatase inhibitors. The endogenous inactivation of the unbound liver receptor is rapid in low speed supernatants but can be slowed by removal of a membrane-bound enzyme which sediments at 100,000 × g (2). The activity of this enzyme can be blocked by the phosphatase inhibitors molybdate, fluoride, and glucose 1-phosphate. Inhibitors of phosphatase action also slow or prevent inactivation of L cell receptors by endogenous enzymes present in cytosol and they block the enhanced L cell receptor inactivation that occurs in the presence of a membrane-bound enzyme from rat thymocytes (3). Evidence presented in the accompanying paper (4) shows that the rapid receptor inactivation which occurs in 100,000 × g supernatants of rat thymocytes can be slowed with fluoride and prevented with molybdate, both at 0°C and 25°C, provided dithiothreitol is present in the preparation. Salomon and Lichti1 have recently shown stabilization of mouse epidermal glucocorticoid receptors by 10 mM fluoride, and Grekin and Sider have reported increased binding of aldosterone to its receptor in the presence of molybdate (5).

Munck and Brink-Johnsen have reported a decrease in glucocorticoid binding capacity and cellular ATP following incubation of whole thymocytes in low glucose and low oxygen (6). The binding capacity is restored following elevation of the ATP levels by returning the cells to a normal oxygen atmosphere. Other studies have also correlated initial glucocorticoid binding capacity with ATP levels and/or oxygenation of the cell suspension medium (7, 8), but no direct evidence has yet been presented for the involvement of phosphorylation in activation of glucocorticoid receptors to the steroid binding form. We have previously reported in vitro activation of unbound thymocyte receptors by addition of a low molecular weight heat-stable factor from L cells (9), but no mechanism for the activation was established in this system. We now report ATP-dependent activation of the glucocorticoid receptor in cytosol preparations from L cells following its inactivation by endogenous enzyme at 25°C.

Part of this work was presented at the Tenth Annual Gregory Pincus Memorial Symposium, October, 1978.

EXPERIMENTAL PROCEDURES

Materials

[1,2,3-3H]Triamcinolone acetonide* (21.6 or 46 Ci/mmol), obtained from New England Nuclear, Boston, Mass., was diluted to 5.3 or 5.6 Ci/mmol prior to use. Nonradioactive dexamethasone, ATP (disodium salt from equine muscle), GTP (sodium salt type III), CTP (type V sodium salt from yeast), UTP, ADP (grade I sodium salt from equine muscle), cyclic AMP (sodium salt, pH 7 in water), cyclic GMP (sodium salt), Firefly Lantern extract, and rabbit muscle protein kinase inhibitor were from Sigma Chemical Co. Joklik-modified minimum essential medium was from Grand Island Biological Co., Grand Island, N. Y. Bovine serum and calf serum were from Kansas City Biologicals, Kansas City, Kansas. All other materials were reagent grade.

* This investigation was supported by Grant CA-16041 awarded by the National Cancer Institute and Grant AM-15740 from the National Institute of Arthritis, Metabolism and Digestive Diseases, Department of Health, Education and Welfare. The project was initiated with funds provided by Institutional Research Grant IN-40N to the University of Michigan from the American Cancer Society. 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.

‡ Supported by a grant from the University of Michigan Cancer Research Institute.

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1 D. S. Salomon, and U. Lichti, personal communication.

2 The trivial names for steroids used are: triamcinolone acetonide, 9α-fluoro-11β,16α,17α,21-tetrahydroxyprogna-1,4-diene-3,20-dione 16,17-acetonide; dexamethasone, 9α-fluoro-16α-methyl-11β,17α,21-trihydroxyprogna-1,4-diene-3,20-dione.
**Methods**

**Cell Culture and Fractionation**—L929 mouse fibroblasts were grown in monolayer culture in Joklik medium supplemented with 10% bovine or calf serum. Cells were harvested in late log phase growth by scraping into Earle’s saline, centrifuged at 800 × g for 10 min in the cold, and washed once by resuspension in several volumes of cold Earle’s saline followed by a second centrifugation at 600 × g. The washed cells were suspended in 1.5 volumes of hypotonic buffer (10 mM Tris, pH 7.35, and 0.1 mM EDTA) and ruptured by Dounce homogenization as described before (3). Ruptured cells were centrifuged for 15 min at 27,000 × g and the resulting supernatant was centrifuged for 45 min at 100,000 × g. All fractionation procedures were carried out at 0-4°C. The 100,000 × g supernatant (referred to here as the cytosol preparation) was quick frozen and stored at -70°C as previously described (9). The cytosol was thawed just prior to use by warming at 20°C, and removing the liquid and transferring it to an ice bath as thawing took place. As described previously (9), cytosol stored at -70°C in hypotonic buffer is equivalent to fresh cytosol in the level of glucocorticoid binding capacity and in the inactivation rate for decay of that binding capacity. Stored cytosol also supports the same amount of ATP-dependent activation as does fresh cytosol.

In the experiments presented in Fig. 6 a heat-treated L cell fraction was added to cytosols as a source of a heat-stable factor that promotes more extensive receptor reactivation. The fraction was prepared by heating a sample of the L cell 100,000 × g supernatant at 90°C for 15 min and removing the precipitate by centrifuging at 27,000 × g for 10 min. This preparation was concentrated severalfold by flash evaporation at 40°C.

**Incubation Conditions and Steroid Binding Assay**—Incubations containing L cell cytosol (50 to 80%) and other additions were prepared as noted in the figure legends. In the experiments of Fig. 6, the concentrated heat-treated preparation was added in a volume such that its final concentration in the incubation was diluted back to that of cytosol (i.e. if it was concentrated 4-fold originally it was added in one-fourth of the final incubation volume). All incubations were carried out at 25°C except where noted. After varying times of incubation, duplicate 0.36-ml aliquots were removed and incubated for 2 h at 0°C in a total volume of 0.4 ml with 5 × 10⁻⁵ M [³H]-triamcinolone acetonide in the presence of either a 1000-fold excess of nonradioactive dexamethasone or vehicle as previously described. This concentration of [³H]-triamcinolone acetonide is an order of magnitude higher than that required to saturate specific binding sites and the binding reaction is complete in 2 h (10). Bound steroid was separated from free material by chromatography on small columns of Sephadex G-25 and bound radioactivity was assayed as described before (11). The specific binding capacity represents the bound counts per min measured in the absence of dexamethasone minus that measured in the presence of the competing steroid. All binding values presented in this paper represent the average of duplicate determinations.

**ATP Assays**—ATP concentrations were measured by a modification of the luciferin-luciferase assay as described by Karl and Holm-Hansen (12). The 5-mg vials of Firefly Lantern extract were dissolved in 5 ml of distilled water and the undissolved material was removed by centrifugation. Scintillation vials containing 0.3 ml of this material plus 0.05 ml of buffer (20 mM MgSO₄, 10 mM Tris, pH 7.4) were counted for 1 min in a Packard Tri-Carb scintillation counter set at 4% gain, window 40-1000 to obtain a background counts per min value. Immediately upon obtaining each background value, 5 to 20 μl of ATP solution were pipetted into the same vial and it was recounted. The difference between these values was converted to ATP concentration by calculating from a standard curve prepared each time assays were done using freshly made ATP standards at nanomolar concentrations. The standard curves are not linear so a wide range of standard ATP concentrations was used to define the curves. Due to the sensitivity of this assay, cytosols usually required 10- to 1000-fold dilution. Assays were carried out at least in duplicate and values for identical conditions from different experiments agreed well from day to day.

**Protein Determinations**—Protein concentrations for undiluted supernatant used in each experiment were determined by the method of Oyama and Eagle (13), using bovine serum albumin (Sigma, Fraction V) as a reference standard. L cell cytosols contained 12 to 17 mg of cytosol protein/ml. After heating for 15 min at 90°C, the cytosol preparations contained 1.5 to 2.2 mg of protein/ml.

**RESULTS**

Since we have previously shown inhibition of inactivation of unoccupied L cell glucocorticoid receptors by the phosphatase inhibitors molybdate, fluoride, and glucose 1-phosphate, we examined the effect of ATP on this inactivation process. Fig. 1 shows the effect of 5 μM ATP as compared to 10 mM molybdate on the inactivation of the glucocorticoid binding capacity in cytosol at 25°C. The binding capacity of control L cell cytosol prepared with hypotonic buffer is inactivated with a half-life of about 2 h at this temperature and molybdate almost totally prevents this inactivation. Addition of 5 mM ATP at time zero slows the inactivation rate significantly (t½ now about 8 h) but is not as effective as molybdate. Higher concentrations of ATP are less effective, and 20 mM ATP inhibits initial binding capacity.

In order to test whether the addition of ATP can activate receptors to the steroid binding state, a 100,000 × g supernatant of L cells was first incubated at 25°C for 4 h to permit extensive inactivation of the binding capacity (Fig. 2). By this time, the inactivation rate was completely saturated. Incubations were then performed at 0°C with either ATP or molybdate. The results are shown in Fig. 2. It is obvious that ATP inactivates the capacity much more slowly than does molybdate. The ATP concentration is 100× greater than that required to inactivate the unoccupied receptor at 25°C. However, the ATP concentration is 1000× less than that of the molybdate.

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**Figure 1**

Stabilization of unbound L cell glucocorticoid binding capacity at 25°C by molybdate and ATP. L cell cytosol was incubated at 25°C with buffer (O), 5 mM ATP (A), or 10 mM molybdate (I). Aliquots were removed at the indicated times and assayed for steroid binding capacity as described under "Methods." The specific binding capacity in 0.40-ml aliquots of each incubation mixture is plotted versus the time in hours.

**Figure 2**

Reactivation of L cell glucocorticoid receptor with ATP and molybdate. L cell cytosol was incubated at 25°C with no addition (C) or with 10 mM molybdate (D), and aliquots were removed at the indicated times and assayed for glucocorticoid binding capacity as described under "Methods." After 4 h of incubation of the control, 10 mM molybdate (I), 10 mM ATP (A), or 10 mM molybdate plus 10 mM ATP (O) were added to portions of the supernatant. Again, aliquots were removed at the indicated times and assayed for glucocorticoid binding capacity.
time, only about 25% of the glucocorticoid binding capacity remained. Addition of 10 mM molybdate at 4 h stabilized this remaining receptor for another 4 h just as it stabilized the receptor when added at time zero. Addition of 10 mM ATP at 4 h resulted in a slight increase in binding capacity over the next 4 h, and addition of 10 mM molybdate plus 10 mM ATP resulted in approximately 50% reactivation of the inactivated binding capacity within 30 min. The resultant increased binding capacity was stabilized for the remainder of the 25°C incubation. Fig. 3 shows a concentration dependence for this ATP-dependent activation of the receptor. The control binding capacity is inactivated to about 36% by preincubation for 4 h, and as above, addition of 10 mM molybdate at 4 h prevents continued inactivation. The solid bars representing addition of varying ATP concentrations with the molybdate show an optimum reactivation at 5 to 10 mM ATP.

To understand how these added ATP concentrations giving maximal activation compare to those normally present in our L cell supernatants, we measured both ATP and glucocorticoid binding activity during a 6-h incubation of the supernatant at 25°C. Fig. 4 shows that the initial ATP concentration of the freshly thawed supernatant is approximately 1.7 mM. The ATP levels drop at a much faster rate than the steroid binding capacity. The kinetics of the decay of glucocorticoid binding capacity are first order, whereas the loss of ATP from the cytosol is not. Assay of the ATP levels during incubation with molybdate (data not presented) showed a decrease of approximately 2 orders of magnitude in measurable ATP at time zero. This decrease is probably due to the formation of phosphomolybdate complexes which lower the concentration of free ATP that can be assayed. Very little additional decrease occurred during incubation under these conditions.

The nucleotide specificity for reactivation of the L cell glucocorticoid receptor is presented in Table I. In this particular experiment, the binding capacity of L cell cytosol was rapidly inactivated with a loss of more than 75% of the binding capacity occurring in 2 h of preincubation. At this time, 10 mM molybdate and 10 mM concentrations of the indicated nucleotides were added to replicate portions of the supernatant, and the incubation was continued for an additional 1 h. Only ATP resulted in significant reactivation (36%) of the glucocorticoid binding capacity.

![Fig. 3. Dependence of reactivation on ATP concentration. L cell cytosol was preincubated at 25°C for 4 h. Control A represents binding capacity assayed at time zero. Control B represents binding capacity after 4 h of inactivation at which time 10 mM molybdate was added with or without varying concentrations of ATP. Control C represents the binding capacity assayed 30 min after addition of molybdate alone. The solid bars represent binding capacity 30 min after addition of molybdate with the indicated concentrations of ATP.](image-url)

![Fig. 4. Loss of ATP and receptor levels during 25°C incubation. L cell cytosol was incubated for 4 h at 25°C. At the indicated times, aliquots were removed and assayed for glucocorticoid binding capacity and a separate aliquot was quick frozen and stored at -70°C for subsequent ATP assay. The assays were carried out as described under "Methods." The left axis indicates ATP concentrations (mM) represented by ▲. ● represents glucocorticoid binding capacity and corresponds to the units given on the right axis.](image-url)

### Table I

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>Specific binding capacity (cpm/0.4 ml incubation)</th>
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<tbody>
<tr>
<td>At 2 h</td>
<td>At 3 h</td>
</tr>
<tr>
<td>L&lt;sub&gt;100&lt;/sub&gt; alone</td>
<td>4,800</td>
</tr>
<tr>
<td>Plus MoO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>4,600</td>
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<td>Plus MoO&lt;sub&gt;4&lt;/sub&gt; and ATP</td>
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<td>Plus MoO&lt;sub&gt;4&lt;/sub&gt; and CTP</td>
<td>4,990</td>
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<tr>
<td>Plus MoO&lt;sub&gt;4&lt;/sub&gt; and UTP</td>
<td>6,100</td>
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We attempted to ascertain if there was a magnesium requirement for the activation by adding magnesium chloride and looking for enhanced activation with ATP. Magnesium (0.2 to 2.0 mM) had no effect and higher concentrations inhibited the initial binding capacity. Therefore, EDTA (10 mM) was added to chelate cellular cation and, as shown in Table II, the addition of 10 mM EDTA with the ATP and molybdate prevents almost all of the activation. Addition of increasing magnesium concentrations under these conditions overcomes the EDTA inhibition. The experiments presented in Fig. 5 show that the activation process is temperature-dependent. After inactivating 80 to 90% of the glucocorticoid binding capacity by preincubating the supernatant at 30°C or 37°C, varying amounts of reactivation are obtained by adding 10 mM molybdate and ATP and incubating portions of the supernatant at different temperatures. Part A of this figure shows that activation is more rapid
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at 30°C than at 20°C. From Parts A and B of Fig. 5 it can be seen that, while activation is rapid at 30°C and 37°C, the increased binding capacity is not stabilized and receptor activity soon decays again. The use of a 25°C incubation temperature allows an optimal combination of rapid activation rate and subsequent stabilization of the activated receptor. It is not possible to accurately determine activation rates for these experiments because the 2-h incubation required for binding assay at each time point may allow some continued activation at a slower rate.

We have not been able to demonstrate any effect of cAMP or cGMP at concentrations from 0.1 µM to 1 mM on the ATP-dependent receptor activation (data not shown). These experiments were done in the presence of 1 mM isobutylmethylxanthine which itself has no effect on the initial binding capacity or on the activation process. At a concentration of 100 µg/ml, the rabbit muscle inhibitor of cAMP-dependent protein kinase (14) also had no effect on the ATP-dependent reactivation of L cell glucocorticoid receptors.

In all of the experiments presented, a limit of approximately 50% reactivation was obtained even when optimal concentrations of molybdate and ATP were used. Since Rees and Bell (15), and, more recently, Granberg and Ballard (16) have reported partial activation of unbound glucocorticoid receptors with dithiothreitol, we tested its ability to enhance activation in the L cell system. Dithiothreitol had no effect on the control or molybdate-stabilized inactivation of L cell cytoplasmic receptors nor did it affect activation of these recep-

![Figure 5](http://www.jbc.org/)

**Fig. 5.** Temperature dependence of activation. In A, L cell cytosol was preincubated at 30°C for 3 h to allow endogenous inactivation of the receptor. At this time, 10 mM molybdate plus 10 mM ATP was added to three portions. These portions were then incubated at 0°C (●), 20°C (▲), or 30°C (▲) and aliquots were removed at the indicated times and assayed for glucocorticoid binding capacity. The experiment of B was similar except that the preincubation was carried out at 37°C for 20 min and the reactivation with molybdate and ATP was done at 25°C (▲) and 37°C (▲). ▲, Specific binding at the end of the preincubation, zero time for reactivation. Binding expressed as percentage of control at zero time.

![Figure 6](http://www.jbc.org/)

**Fig. 6.** Stimulation of ATP-dependent reactivation of L cell glucocorticoid receptor by addition of a heat-treated preparation of the L cell supernatant. In A, L cell cytosol was incubated at 25°C (▲), and at time zero, 45 min, 2 h, and 4 h, 10 mM molybdate and 10 mM ATP were added to replicate aliquots of the incubation either with a heat-treated preparation of L cell supernatant (▲), or with an equal volume of buffer (▲). The heat-treated L cell supernatant was prepared as described under "Methods" and it had no binding capacity of its own even when assayed with molybdate and ATP. Glucocorticoid binding capacity was assayed 30 min after each addition was made. B shows the results of a similar experiment in which 10 mM ATP, 10 mM molybdate, and the heat-treated preparation were added (▲) after various times of incubation of the control (▲). In this figure, a time course for effect of these activating conditions is presented.
ators by itself or when added with molybdate, ATP, or both (data not shown).

Since we have reported activation of thymocyte receptors by addition of a low molecular weight, heat-stable factor from L cells (9), we examined the possibility that the binding capacity could not be completely reactivated because this factor was being lost from the system. The experiment of Fig. 6 shows the effect that time of preincubation at 25°C had on the maximum level of receptor activation and the effect of adding more heat-stable activator. From the open triangles of Part A it can be seen that the addition of ATP yields complete reactivation when it is added at 45 min but the maximum level of reactivation decreases greatly with increasing time of incubation of the unbound supernatant. The solid triangles show that addition of heat-treated L cell preparation, at a concentration equivalent to that of the untreated supernatant, provides no advantage at time zero or 45 min, but improves activation after 2 and 4 h of incubation. No increased stimulation is observed if higher concentrations of the heat-treated preparation are used. The heat-treated preparation has no activating effect in the absence of ATP and molybdate. Part B of this figure shows a time course for the activation with ATP, molybdate, and the heat-treated preparation added after the same times of incubation of the control. It is unlikely that the heat-treated preparation is adding any glucocorticoid receptor because it contains no binding capacity of its own even after incubation with molybdate and ATP.

The increasing activation obtained on addition of the heat-treated cytosol preparation at later time points suggests that the active material in the receptor-containing cytosol may be enzymatically inactivated during the 25°C incubation. To test this possibility, whole 100,000 x g L cell supernatant was incubated at 25°C and aliquots removed and heat-treated (90°C, 10 min) at various times up to 8 h. These heat-treated preparations were then added, with ATP and molybdate, to a fresh preparation of inactivated L cell cytosol and no significant difference in the stimulation of activation by the different preparations was observed (data not shown).

We have previously suggested (9) that changes in activation or inactivation of glucocorticoid binding capacity may explain the low apparent receptor levels present in some cells that are resistant to glucocorticoids. Several years ago we obtained a glucocorticoid-resistant subline of L cells by a stepwise selection procedure (17). The resistant line was subsequently cloned and shown to have only 10 to 15% of the specific binding capacity of the sensitive parent cells (18). The receptors in both lines bind glucocorticoids with the same affinity (18), and we have therefore examined the rates of inactivation and activation of the binding capacity in the resistant cell line in order to see if there are differences that might explain the low steroid binding capacity. The rate of inactivation of the binding capacity of resistant cell cytosol as shown in Fig. 7A was measured on several occasions. The $t_{1/2}$ for inactivation of the resistant cytosol ranged from 0.5 to 2 h at 25°C. As shown in Fig. 7B, the binding capacity of resistant cell cytosol can be reactivated by ATP in the presence of molybdate but increase in binding capacity to greater than time zero resistant cell values does not occur. To see whether any components present in sensitive cell cytosol, but absent or deficient in the preparation from resistant cells, could stimulate activation of the resistant cell receptor to a level near that in the sensitive line, we mixed cytosols of the two lines in the presence of 10 mM ATP and 10 mM molybdate. A time course for binding capacity in the mixture incubated for 6 h at 25°C showed no more than a summation of the binding capacity measured in each preparation alone (not shown).

**DISCUSSION**

It is quite surprising that the addition of 5 mM ATP at time zero slows the rate of inactivation of the binding capacity as shown in Fig. 1. This could represent ATP-dependent activation taking place without a change in inactivation rate or it may reflect considerable inhibition of the inactivation rate. We are unable to assess the relative contribution of these two possible mechanisms to the observed stabilization. Given the rate of degradation of ATP in the L cell cytosol preparation at 25°C (Fig. 4), it is very surprising that the ATP effect is sustained over a long period.

A clear demonstration of activation of glucocorticoid binding capacity is obtained after allowing endogenous inactivation of the receptor by incubation of the 100,000 x g supernatant at 25°C, blocking further inactivation by addition of molybdate, and simultaneously adding ATP (Fig. 2). The activation is specific for ATP: ADP, GTP, CTP, and UTP having no significant effect (Table I). It is maximal at ATP concentrations of 5 to 10 mM (Fig. 3). These levels are somewhat higher than the 1.7 mM ATP measured in the supernatant at the beginning of the incubation period. It should be remembered, however, that the inactivation process must be inhibited with molybdate in order to visualize ATP-dependent activation in this system. The free concentration of ATP in the presence of...
molybdate is probably much lower than the 5 to 10 mM because of the ability of molybdate to form phosphomolybdate complexes (19). The demonstration that molybdate greatly lowers the ATP measurable in L cell cytosol by the luciferin-luciferase assay supports this possibility.

The temperature dependence for receptor activation (Fig. 5) supports an enzymatic mechanism for the process. Activation is more rapid at 30°C than at 20°C and is negligible at 0°C. The activated receptor is not stable at 30°C or 37°C, however, and binding capacity quickly returns to the lower levels. Molybdate does not effectively inhibit the increased phosphatase activity occurring at these higher temperatures. In addition, it is possible that increased protease activity may contribute to the loss of binding activity observed at 30°C and 37°C. The use of an ATP generating system in the presence of another type of phosphatase inhibitor (i.e., one that does not form phosphomolybdate complexes) may eventually permit more extensive, sustained activation at higher temperatures.

If the activation reflects the activity of a kinase enzyme, one would expect to observe a magnesium requirement. There is apparently sufficient magnesium present in these rather concentrated cytosol preparations to permit activation. To prevent activation 10 mM EDTA was required and the addition of magnesium clearly reverses this inhibition (Table II). We have not chosen to test the relative efficacy of various added cations in this crude system. The addition of other cations chelated by EDTA may simply compete for the chelation of magnesium and raise the amount of the uncomplexed form of that ion. The unoccupied receptor is completely inactivated via a cyclic nucleotide-dependent kinase. It is still quite possible that a cyclic nucleotide-independent kinase does mediate the effect.

In most of the experiments a maximum of only about 50% activation was obtained after 4 h of inactivation at 25°C. The experiment of Fig. 6A shows that the maximum reactivation obtainable decreases with increasing time of inactivation of the control at 25°C. In that figure it is also shown that the addition of an equivalent concentration of a heat-treated preparation of the same L cell supernatant will enhance any reactivation after separation of receptor from endogenous ions in this manner. Preliminary results have demonstrated no effect of cyclic nucleotides (0.1 mM to 1 mM cyclic AMP or cyclic GMP) on the ATP-dependent receptor activation. Nor did 100 µg/ml of the protein inhibitor of cyclic AMP-dependent kinases (14) have any effect in a single pilot experiment. These results argue strongly against activation via a cyclic nucleotide-dependent kinase. It is still quite possible that a cyclic nucleotide-independent kinase does mediate the effect.

Although we have generally found the rate of inactivation of unbound receptor to be somewhat faster in the resistant cell cytosol than in that from sensitive cells, the range of inactivation observed with the resistant cells is broad (t/2 from 0.5 to 2 h) and clearly overlaps with that of the sensitive cell line. We have previously demonstrated that a great deal of receptor-inactivating enzyme activity is present in the material sedimented by centrifugation of rat thymus or liver homogenates at 100,000 X g (2, 3). In the experiments of Fig. 7, we have utilized only the supernatant preparation and we have not yet examined the possibility that resistant cells have high levels of membrane-bound phosphatase activity. The resistant cell receptor can be reactivated after partial inactivation at 25°C. Unfortunately, because of the low binding values (and consequently the small changes observed) in this system and the difficulty of obtaining an accurate assessment of reactivation rates with the methods we have used, we cannot say that the rates of ATP-dependent activation are different in sensitive and resistant cells. Attempts to increase the receptor activity in resistant cell cytosol to nearer the level in the sensitive preparation by mixing the two supernatants in the presence of ATP and molybdate at zero time also revealed no stimulation of binding capacity in the resistant cells. These results suggest there is not a pool of soluble inactive receptor present in resistant cells that can be activated by enzyme or other activating factors present in excess in sensitive cell cytosol. Examination of the mechanism for decreased steroid binding capacity in other lines resistant to glucocorticoids may indicate that some forms of resistance are due to changes in receptor activating or inactivating capacity. The identification of such a resistant line would help clarify the mechanisms for these processes.

Other laboratories have reported activation of glucocorticoid receptors in thymocyte and lung cytosols by the addition of reducing agents (15, 16). As reported previously for liver cytosol (16), the glucocorticoid binding capacity of L cell cytosol is not affected by the addition of reducing agents like dithiothreitol. Also, the activation of L cell glucocorticoid binding capacity is not affected by dithiothreitol. The relationship between the activation of thymocyte receptors by reducing agents and ATP will be discussed in detail in the accompanying paper (4). The object of the phosphorylation process proposed in this paper has not been established. The simplest explanation, still, is that phosphorylation of the receptor itself is required for steroid binding, but it is quite possible that another component required for steroid binding activity is activated and inactivated by a phosphorylation-denphosphorylation mechanism.

To our knowledge this is the first report showing that the ability of a receptor to bind a hormone or drug can be altered by what is likely to be a phosphorylation mechanism. The observation that specific binding capacity can be altered in subcellular systems suggests that the process may be operative in the intact cell as proposed by Munck and Brinck-Johnsen in 1968 (6). It would not be surprising to find that receptors for some other steroid hormones and perhaps those for some neurotransmitters may also be activated and inactivated by similar processes. It is also possible that hormones may be able to alter the activation and/or inactivation rates of their own receptors or even of receptors for other hormones. In that case control over rapid and reversible alteration in the capacity of receptors to bind hormones may exist as an important regulatory mechanism.

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