Inactivation of Glucocorticoid Receptors in Cell-free Preparations of Rat Liver

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SUMMARY

We have examined the rates of inactivation of glucocorticoid receptors in cell-free preparations from several rat tissues. The $t_{1/2}$ of inactivation of the glucocorticoid-binding ability of thymus, heart, and kidney cytosols (37,000 × g supernatants) ranges from 2 to 4 hr at 0°C, whereas that of liver is much slower (15 to 25 hr). The rate of inactivation of the glucocorticoid-binding capacity of soluble preparations from liver varies roughly according to the g force at which they have been centrifuged. The 100,000 × g particulate material from liver contains a heat-labile component that inactivates the steroid-binding capacity of the 100,000 × g supernatant. The ability of the particulate enzyme to inactivate glucocorticoid receptors at 0°C is not affected by protease inhibitors but is inhibited by fluoride and molybdate. The rapid inactivation of unbound glucocorticoid receptors that occurs in a high-speed (100,000 × g) supernatant preparation from rat liver at 25°C can be completely inhibited by molybdate. These observations suggest that the inactivation of glucocorticoid receptors observed in cell-free liver preparations in vitro is due to a nonproteolytic enzymatic function.

INTRODUCTION

Cell-free preparations from different tissues of the same animal vary widely in their capacity to bind specifically glucocorticoids (1, 3). Binding capacity can also vary within a single cell type. In lymphocytes (14, 22) and fibroblasts (8), decreased ability of cytosol to bind glucocorticoids has in some cases been found to be associated with resistance to the hormone effect. Marked changes in specific glucocorticoid binding can occur in some organs during the course of development (7). The basis for the differences in binding capacity between tissues and within a single cell type is not well understood. These differences could reflect variations in rates of receptor synthesis and/or degradation. However, it has been proposed that glucocorticoid receptors in intact cells are subject to activation and inactivation by a process that requires energy and is independent of protein synthesis (10, 15, 16). Such an activation-inactivation system could also play a role in regulating specific binding capacity.

Glucocorticoid receptors in cell-free preparations are very labile unless they are bound to steroids. The instability of the unoccupied receptor and stabilization by glucocorticoids have been observed in thymic lymphocytes (4), murine lymphosarcoma (12), fibroblasts (19), and hepatocytes (2). It is not yet clear whether the lability of the receptor in cell-free preparations reflects a degradation or inactivation mechanism of importance in the intact cell. Efforts to prevent inactivation of the unbound receptor have not been particularly successful. Glycerol (at low temperature), EDTA, and sulfhydryl-protecting reagents provide some slowing of the inactivation rate of the unbound thymocyte receptor (20, 24), and both glycerol and some phosphorylated sugars have been reported to retard the decay in the binding capacity of mouse fibroblast cytosol (9). Several investigators have suggested that the inactivation of the binding capacity observed in vitro is a physical rather than an enzymatic process (9, 12, 20). The purpose of the work presented in this paper is to examine the rate of inactivation of glucocorticoid-binding capacity in cell-free preparations from several rat tissues and to demonstrate that the inactivation in cell-free preparations of rat liver is due to an enzymatic process that can be inhibited by both molybdate and fluoride.

MATERIALS AND METHODS

Chemicals. [1,2,4-3H]Dexamethasone (9α-fluoro-16α-methyl-11β,17α,21-trihydroxypregna-1,4-diene-3,20-dione) (10 Ci/m mole) and [1,2,3-3H]triamcinolone acetonide (9α-fluoro-11β,16α,17α,21-tetrahydroxypregna-1,4-diene-3,20-dione 16,17-acetonide) were purchased from Schwarz BioResearch, Inc., Orangeburg, N. Y. The specific activity of triamcinolone acetonide was diluted to 5.3 Ci/m mole prior to use. Nonradioactive dexamethasone, PMSF, TLCK, TPCK, and α-chymotrypsin (EC 3.4.2.11) from bovine pancreas were obtained from Sigma Chemical Co., St. Louis, Mo. SBTI was purchased from Worthington Biochemical Corp., Freehold, N. J. 125I-Labeled bovine serum albumin came from Amersham/Searle Corp., Arlington.
Glucocorticoid Receptor Inactivation

RESULTS

Specific Binding Capacity in Soluble Preparations of Tissues from Different Strains of Rats. The specific binding capacities of 37,000 × g supernatants prepared from 4 tissues of 3 strains of rats are presented in Table 1. The amount of specific glucocorticoid binding observed under these conditions of cell rupture and assay is markedly different in the various tissue preparations. However, the binding capacity of each tissue is quite consistent from 1 strain of rat to another. The differences in specific activity of binding from 1 tissue homogenate to another do not simply reflect differences in protein content. Ballard et al. (1) examined the specific binding in cell-free preparations of various organs of the Buffalo rat, and similar differences were also observed when binding was expressed on the basis of DNA.

We had hoped to find 1 or more differences in the amount of specific binding obtained from the tissues of the various strains of rats. This would have provided a genetic model with which to examine those factors that control receptor levels. As this was not the case, we focused on the possibility that the rates of inactivation of the binding capacity might vary among the 4 tissues.

Decay of Specific Binding Capacity. The rates of decay in the binding capacity of 37,000 × g supernatants prepared from liver, thymus, heart, and kidney of Buffalo and Sprague-Dawley rats are presented in Chart 1. In these experiments the specific binding capacity was determined by 2-hr assays on samples removed at various times from soluble preparations maintained in an ice bath. Thus, the rates described by the individual data points represent the rates of inactivation of the unbound receptor at 0°. Again, no difference is observed between Buffalo (solid symbols) and Sprague-Dawley (open symbols) rats. However, it is clear that under these conditions there is a substantial difference in the rate of decay of the liver-binding capacity as compared to the rates for thymus, kidney, and heart preparations. Whereas the latter 3 tissues produce a supernatant in which the specific binding capacity disappears with a half-life of 2 to 4 hr, the liver preparation has a half-life of 15 to 25 hr. The inset in Chart 1 presents the extended decay curve for liver.

Variation of Inactivation Rate with Speed of Centrifugation and Ionic Strength. The rate of inactivation of specific binding capacity in the 37,000 × g supernatant from thymocytes (Chart 1) is the same as that observed by Bell and Munck (4) in low-speed (1500 × g) thymus cytosol prepared at a lower salt concentration. The rate of inactivation in the liver supernatant is considerably faster than the 50-hr half-life reported by Beato and Feigelson (3) for preparations of rat (Sprague-Dawley) liver that had been centrifuged at 10,000 × g.

Table 1

<table>
<thead>
<tr>
<th>Type of rat</th>
<th>Liver</th>
<th>Thymus</th>
<th>Kidney</th>
<th>Heart</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sprague-Dawley</td>
<td>0.80 ± 0.03</td>
<td>0.35 ± 0.02</td>
<td>0.18 ± 0.03</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td>Buffalo</td>
<td>0.62 ± 0.04</td>
<td>0.28 ± 0.01</td>
<td>0.19 ± 0.02</td>
<td>0.15 ± 0.02</td>
</tr>
<tr>
<td>Wistar</td>
<td>0.86 ± 0.12</td>
<td>0.30 ± 0.05</td>
<td>0.24 ± 0.02</td>
<td>0.11 ± 0.03</td>
</tr>
</tbody>
</table>

Glucocorticoid-binding capacity of cytosol preparations from organs of various strains of rats

Cytosols (37,000 × g supernatants) were prepared from liver, thymus, kidney, and heart obtained from 3 strains of rats, and the ability of each preparation to bind dexamethasone in a specific manner was measured as described in "Materials and Methods." The values represent the means of 2 experiments (2 determinations/experiment) expressed as pmoles specifically bound per mg of supernatant protein ± the range. The assay values in raw counts specifically bound varied from 5,000 to 70,000 according to the tissue examined.

Specific glucocorticoid-binding capacity (pmoles/mg of protein)
are the same as those for the main chart).

various times and incubating them for 2 hr with radiolabeled dexamethasone. The specific binding capacity at each assay time is presented as a percentage of the value determined at zero time versus the incubation time in hr. Each value is an average of duplicate determinations. Solid symbols, Buffalo rat; open symbols, Sprague-Dawley rat; △, liver; ○, thymus; ○, heart; ▽, kidney. Inset, the liver data over a longer time course (the units of the inset are the same as those for the main chart).

The rate of inactivation of the unbound receptor in cell-free preparations from several tissues of 2 strains of rats. Cytosols (37,000 × g supernatants) were prepared from liver, thymus, kidney, and heart of Sprague-Dawley and Buffalo rats. Each cytosol was incubated at 0°, and the specific binding capacity was determined by removing aliquots of the supernatant at various times and incubating them for 2 hr with radiolabeled dexamethasone. The specific binding capacity at each assay time is presented as a percentage of the value determined at zero time versus the incubation time in hr. Each value is an average of duplicate determinations. Solid symbols, Buffalo rat; open symbols, Sprague-Dawley rat; △, liver; ○, thymus; ○, heart; ▽, kidney. Inset, the liver data over a longer time course (the units of the inset are the same as those for the main chart).

240,000 × g. Accordingly, the rate of inactivation of the specific binding capacity in 27,000 × g supernatants from liver and thymus of Sprague-Dawley rats was compared to that in 100,000 × g supernatants from the same cell preparations. As can be seen in the inset of Chart 2, the specific binding capacity decays at approximately the same rate in low- and high-speed supernatants from thymocytes. However, the specific binding capacity in the 100,000 × g supernatant from liver decayed at a much slower rate than did the low-speed preparation from the same broken cells (Chart 2). This rate of decay in the 100,000 × g preparation is the same as that observed in the 240,000 × g supernatant by Beato and Feigelson (3).

Other investigators have noted that salt can affect the rate of inactivation of the binding capacity in cell-free preparations from rat thymocytes (24) and mouse fibroblasts (13). Accordingly, the rate of inactivation of the receptor was measured in a portion of a 100,000 × g liver supernatant prepared in only the hypotonic buffer (data marked as low salt in Chart 2). The rate of inactivation of the unbound receptor in the hypotonic supernatant from liver is slower than in the salt-containing sample. The effect of salt was examined in greater detail in the experiment shown in Chart 3. The inactivation rate is not affected by sodium chloride at 0.1 M, but at 0.3 M salt it is very rapid. The inactivation rate of the hypotonic liver preparation is slowest when only the top half of the 100,000 × g supernatant is carefully removed and used for specific binding assay.

Inactivation Rate in Mixed Preparations. The high-speed supernatant of liver could have a slower rate of decay than low-speed fractions because particulate-bound inactivating or degrading enzymes have been centrifuged out of the preparation. For testing of this possibility, the experiments shown in Chart 4 were carried out. In Chart 4A, a 27,000 × g supernatant was prepared from rat liver in hypotonic buffer only. This supernatant was then centrifuged at 100,000 × g, and the pellet portion was resuspended in hypotonic buffer. The resuspended liver100 pellet has no glucocorticoid-binding capacity. The liver100 pellet was then mixed with the 100,000 × g supernatant, and the specific binding capacity was determined at various times while the mixture was maintained in ice. As presented in the chart, the binding capacity of the high-speed liver supernatant decays slowly, whereas that of the mixture declines with a half-life of approximately 4 hr. As shown by the triangles, the 100,000 × g pellet preparation has no effect on specific binding capacity if it is first heated for 10 min at 90°. From Chart 4B,
it is clear that the specific binding capacity of the liver supernatant can also be inactivated by the 100,000 × g particulate fraction prepared from thymic lymphocytes.

As shown in Table 2, the ability of the 100,000 × g liver pellet to inactivate the binding capacity of the high-speed liver supernatant is not affected by the protease inhibitors PMSF, TLCK, TPCK, and SBTI. That the protease inhibitors can operate effectively under the conditions of the experiment is demonstrated by the fact that the loss of binding capacity due to the effect of α-chymotrypsin can be completely blocked by TPCK and PMSF. The protease inhibitors also have no effect on the ability of the thymocyte particulate enzyme to inactivate glucocorticoid-binding capacity (data not shown). We have also examined the ability of the 27,000 × g liver supernatant to digest ¹²⁵I-labeled bovine serum albumin at 25°. As can be seen from the data in Table 3, the specific binding capacity of the liver preparation is reduced by 76% within 1 hr at 25°, whereas there is almost no digestion of radiolabeled albumin added to the same preparation, even after a much longer incubation time.

Inhibition of the Inactivating Effect. In view of the fact that the binding capacity of the high-speed supernatant from liver can be inactivated by purified calf intestine alkaline phosphatase (16), we examined the effect of some
Comparison of the rates of receptor inactivation and \textsuperscript{14}C-labeled albumin digestion in the 27,000 x g liver supernatant

A 27,000 x g supernatant was prepared from Sprague-Dawley rat liver. A portion of the supernatant was incubated with 16 \mu g of \textsuperscript{14}C-labeled bovine serum albumin in a total volume of 0.5 ml at 25°. Samples (5 \mu l) were removed at the beginning and after 4 or 8 hr of incubation, and the radioactivity accompanying the albumin peak was determined after electrophoresis on 10% polyacrylamide gels under denaturing conditions, as previously described (25). The specific steroid-binding capacity was determined with \textsuperscript{3}H triamcinolone acetonide at zero time and after 1 hr in 0.4-ml aliquots of the same 27,000 x g supernatant without radioactive albumin.

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Radioactivity recovered in the albumin band (cpm x 10^-4)</th>
<th>Specific binding capacity (cpm/0.5 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>570 100</td>
<td>13,940 100</td>
</tr>
<tr>
<td>1</td>
<td>568 100</td>
<td>3,390 24</td>
</tr>
<tr>
<td>4</td>
<td>551 97</td>
<td>908 27</td>
</tr>
</tbody>
</table>

The effect of fluoride on the inactivation of the binding capacity of liver particulate enzyme activity of liver and thymocytes is presented in Table 4. A 100,000 x g supernatant from liver was incubated at 0° with 0.14-ml portions of resuspended thymus or liver pellet in the presence or absence of 0.1 M sodium fluoride. The liver pellet had been resuspended in an amount of hypotonic buffer equivalent to one-third the volume of the original 27,000 x g supernatant, and the thymus pellet had been resuspended in a volume equivalent to its supernatant. Specific binding assays were performed at zero time and after 1 hr of incubation. The values represent the averages of 2 determinations expressed as cpm of \textsuperscript{3}H triamcinolone acetonide specifically bound per 0.6-ml incubation. The control binding at the beginning of the incubation was 31,652 cpm/0.6 ml.

Table 4

The effect of fluoride on the inactivation of the binding capacity of liver\textsubscript{100} supernatant by liver\textsubscript{100} and thymus\textsubscript{100} pellets

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>Without fluoride</th>
<th>With fluoride</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver\textsubscript{100} alone</td>
<td>30,086 31,693</td>
<td></td>
</tr>
<tr>
<td>Plus liver\textsubscript{100} pellet</td>
<td>11,782 31,858</td>
<td></td>
</tr>
<tr>
<td>Plus thymus\textsubscript{100} pellet</td>
<td>11,519 9,543</td>
<td></td>
</tr>
</tbody>
</table>

Phosphatase inhibitors on the inactivating activity in the 100,000 x g pellet fraction. The effect of fluoride on the particulate enzyme activity of liver and thymocytes is presented in Table 4. A 100,000 x g supernatant from liver was incubated at 0° with an amount of particulate material sufficient to inactivate two-thirds of the binding capacity in 14 hr. Although fluoride has no effect on the activity of the thymocyte particulate enzyme, there is complete inhibition of the effect of the liver preparation. At higher concentrations of the liver particulate fraction, the amount of inhibition produced by fluoride decreases.

Chart 5 presents the effects of 3 phosphatase inhibitors on the inactivating activity of the high-speed liver supernatant at 25°. Levamisole, an inhibitor of alkaline phosphatase, has no effect. Fluoride produces a partial inhibition, and molybdate ion can nearly block the inactivating activity at this temperature. In this experiment both inhibitors were present at a concentration of 100 mM, a maximally effective concentration for fluoride in this system. As shown in Chart 6, however, molybdate at 1 mM is able to prevent inactivation of the binding capacity in the high-speed liver supernatant at 25°.

DISCUSSION

This work was undertaken to find a way to study in vitro some of the mechanisms controlling glucocorticoid receptor levels in various tissues. Cell-free preparations from liver, thymus, heart, and kidney of Buffalo rats had all been reported to have substantial binding activity (1). Beato and Feigelson (3), however, did not find specific glucocorticoid binding in cytosol prepared from hearts of Sprague-Dawley rats. In contrast, Funder et al. (6) reported the receptor level in cytosol from Wistar rat hearts to be almost twice that found in the Buffalo rat preparation. The amount of specific binding found in cytosol of Wistar rat kidney (6) was nearly 4 times that found in kidney for the Buffalo strain. Thus, from the data available in the literature, it seemed possible that there could be rather large differences between rat strains.
with respect to the levels of specific binding capacity in certain tissues [as has been described for A/J and C57BL/6J mice (23)]. We chose to compare the amount of specific binding in tissues from Buffalo, Sprague-Dawley, and Wis
tar rats in the hope that, in defining the differences, we might determine that the receptor is degraded or inactivated at a different rate in 1 strain than in others. It is clear from the data of Table 1 that, when the 3 rat strains are compared with the same techniques, no significant strain variations in the amount of binding are observed.

There is a large difference between rates of inactivation of the binding capacity in 37,000 x g supernatants from thymus, heart, and kidney and the rate observed in the analogous liver preparation (Chart 1). These tissues were all homogenized under the same conditions in the same buffer, and it seemed unlikely that the difference in rates would be attributable to differences in molecular properties of receptors produced in various organs of the same animals. The rate of inactivation of the unoccupied receptor in liver cytosol varies very roughly according to the force at which it is centrifuged (Chart 2). This is not true for the thymus (Chart 2, inset). As shown in Chart 4, the high-speed particulate material from liver contains a heat-labile activity that can inactivate the hepatic glucocorticoid receptor. It is reasonable to propose that there is an inactivating enzyme that is, at least in part, associated with the particulate material. We have not attempted to define the subcellular location of the enzyme further because our assay (measuring the rate of receptor inactivation) does not yield readily defined units and because our method of cell rupture under hypotonic conditions does not leave liver cell nuclei intact.

The mechanism by which the enzyme inactivates the binding capacity is not yet defined. From the experiments presented in Tables 2 and 3, we would suggest that it does not represent a general proteolytic effect. The observation that the effect of the liver particulate enzyme at 0° can be inhibited by fluoride suggests the possibility of a phosphatase activity. The action of the liver particulate enzyme can also be blocked by molybdate. This anion has been shown to be a potent inhibitor of phosphatase activity in a number of systems (18, 21). Since the action of the thymus particulate-inactivating enzyme is not affected by either of these inhibitors, there is clearly a difference between the nature of the activity in the thymocyte and liver particulate preparations.

Since we have recently shown that alkaline phosphatase from calf intestine can inactivate glucocorticoid-binding capacity in soluble preparations from mouse L-cells or rat liver (16), it is important to ask whether the inactivation observed in this work could result from alkaline phosphatase, an enzyme that is located largely in the microsomal fraction of eukaryotic cells. For this purpose we examined the effect of levamisole on the inactivation process. Although the mechanism of the action of levamisole is not known, it has been demonstrated to be a potent inhibitor of alkaline phosphatase activity in liver as well as in many other tissues (except intestine) from several animal species, including the rat (5, 26). This inhibitor has no effect on the particulate receptor-inactivating activity or on the loss of binding capacity that takes place in the high-speed supernatant alone at 25° (Chart 5).

Although a phosphatase mechanism can be tentatively proposed on the basis of fluoride and molybdate inhibition, the target of such a dephosphorylation process has not been defined. There are several possibilities: (a) the receptor itself may be inactivated by dephosphorylation; (b) an as yet unidentified phosphorylated cofactor may be required to permit binding; or (c) a rather specific protease may be activated by a dephosphorylation process. Regardless of the mechanism, an examination of the basis for the enzymatic inactivation of the binding capacity in subcellular preparations should provide some interesting insight into the nature of the glucocorticoid receptor.

From experiments with intact cells, both Munck et al. (15) and Ishii et al. (10) have obtained evidence that glucocorticoid receptors are reversibly activated and inactivated by an energy-requiring mechanism that is independent of protein synthesis. It is possible that the inactivation process reported here is related to the inactivation that occurs in the intact cell. The fact that inactivation in the high-speed supernatant can be inhibited could prove useful. The rapid rate of glucocorticoid receptor inactivation occurring in cell-free preparations at temperatures above 0° has proven to be an impediment to the definitive study of binding kinetics. The ability of molybdate to block inactivation at 25° (Charts 5 and 6) should permit additional thermodynamic experiments to define better the nature of the hormone-receptor interaction.

**REFERENCES**

C. J. Nielsen et al.


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