Communication

Specific Glucocorticoid Receptors in Thymus Cells

LOCALIZATION IN THE NUCLEUS AND EXTRACTION OF THE CORTISOL-RECEPTOR COMPLEX*

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SUMMARY

Specific glucocorticoid receptors previously identified in rat thymus cells by measurements of steroid binding to whole cells are found to be located largely, if not entirely, in the nucleus. Localization is accomplished by showing that cortisol-3H, which is specifically bound to whole cells, remains with the nuclei (identified as such by electron microscopy) after cell membranes and cytoplasmic materials are removed by hypotonic shock in 1.5 mM MgCl2 at 3°.

The cortisol-receptor complex has been extracted from nuclei and separated from free cortisol by gel chromatography. It is rapidly degraded by Pronase. The rate at which cortisol dissociates at 37° from the isolated complex, and from nuclei, is indistinguishable from the characteristic slow rate at which specifically bound cortisol dissociates from whole cells, showing that this latter rate is an intrinsic property of the cortisol-receptor complex. The rate at which cortisol associates specifically with whole cells at 37° is indistinguishable from the rate at which it appears in the nuclear receptor complex.

We have recently found that rat thymus cells contain binding sites specific for cortisol and related glucocorticoids (1, 2). These sites, of which there are about 5000 per cell, have been identified by using cell suspensions in which added glucocorticoids produce metabolic effects comparable to their effects in vivo. An important additional property of such suspensions is that they metabolize glucocorticoids very slowly if at all, so that even after prolonged incubation radioactivity introduced as cortisol-3H can be recovered unaltered (2). The specific binding sites can be distinguished from the much more numerous nonspecific sites by the fact that cortisol (and all other glucocorticoids tested so far) dissociates slowly from the former (time constant of 3 min at 37°) and rapidly from the latter (time constant of less than 15 sec).

Several observations (1, 2) support our hypothesis that the specific sites are the glucocorticoid "receptors" of thymus cells, i.e., the molecular entities through which glucocorticoids initiate their specific physiological effects. In contrast to the nonspecific sites, the specific sites become saturated at physiological glucocorticoid concentrations and are competted for by a variety of steroids roughly in proportion to glucocorticoid activity. They are occupied fast enough (7 to 10 min) by cortisol to account for the earliest cortisol effects (15 to 20 min (3)) observed so far with thymus cells. These effects are abolished if cortisol is prevented from binding to the specific sites by the metabolically inactive competing steroid, corticosterone (2). Somewhat similar binding experiments have been described by Schaumburg and Bojesen (4), but they did not find good correlation between binding and glucocorticoid activity, perhaps because most of their measurements were at 4° rather than 37°.

We wish to report here results that show that most, if not all, of these glucocorticoid receptors are in the nucleus, and to describe a method by which the cortisol-receptor complex may be extracted. Some of these results have been presented (5).

Our evidence for nuclear sites was obtained using a procedure described by Cooper, Baliga, and Munro (6) for disrupting lymphoid cells by hypotonic shock with dilute MgCl2. This procedure breaks the cell membranes and releases cytoplasmic materials, but leaves nuclei relatively intact.

The curves in Fig. 1, obtained by methods given in Reference 2, illustrate the time course of steroid binding to thymus cells in suspensions initially equilibrated for 20 min with cortisol-3H or cortisone-3H in Krebs-Ringer bicarbonate buffer at 37°, then diluted 50-fold into Krebs-Ringer bicarbonate buffer or 1.5 mM MgCl2 in water at 3°, and finally rewarmed to 37°. In the ratio of (SC) to (S)o, which is proportional to the amount of steroid bound to cells, (S), is a constant (6 nm for all curves) equal to the concentration of steroid in the buffer during the initial equilibrium period; (SC) is the concentration of steroid bound to the cells (or to nuclei from disrupted cells), in nanomoles per liter of packed cells. The packed cell volume used after dilution is calculated from the value measured before dilution, multiplied by the dilution factor (see Reference 2). With cells that are disrupted by dilution, the packed cell volume is the volume the cells would have occupied had they remained intact, and serves merely as a useful basis for comparing data. (SC) after dilution is found from the radioactivity in pellets obtained by centrifuging aliquots of the suspension for 105 sec in a Beckman microfuge (2); nuclei from disrupted cells are sedimented as effectively by this procedure as whole cells.

Only the parts of the curves after dilution in Fig. 1 are important for the present discussion. The top curve, obtained previously (2) but included here because it provides the basis for interpreting the other curves, shows that when cells equilibrated with cortisol-3H are diluted into Krebs-Ringer bicarbonate buffer at 3°, the nonspecifically bound steroid dissociates in about 40 min. The fraction of cortisol that remains with the cells can be clearly identified as that bound to specific sites, since (a) it coincides closely in amount with the cortisol bound to specific sites before the dilution at 3°; (b) at 37° (after the suspension is rewarmed) it dissociates at the rate characteristic of specifically bound cortisol; (c) no such fraction is found with cortisone (data not shown), a steroid that has nonspecific properties similar to cortisol.

1 The trivial name used is: cortexolone, 17,21-dihydroxyprogesterone-3,20-dione.
membranes and contents remain relatively intact and are free of cytoplasmic materials. The fraction of cortisol that in this case is also similar to the 3° section of the top curve in Fig. 1, displaced downward by about 0.7 unit.

If cells equilibrated with cortisol are diluted into 1.5 mM MgCl₂ (Fig. 1, middle curve), the nonspecifically bound steroid dissociates within 3 min. Electron micrographs of these cells fixed with glutaraldehyde at various times after dilution show that by 5 min cell membranes are completely disrupted, whereas nuclear membranes and contents remain relatively intact and are free of cytoplasmic materials. The fraction of cortisol that in this case remain with the nuclei can again be identified as that bound to specific sites, for reasons similar to those that apply to the top curve. (a) The fraction is of the right magnitude, even though recoveries vary somewhat erratically from 50 to 90% of the specifically bound cortisol present in the intact cells before dilution. (b) Despite cellular disruption, this nuclear-bound fraction dissociates at 37° at a rate very similar to that in whole cells. (c) No comparable fraction is obtained with cortisone-3H (inner curve in Fig. 1). (d) With cortisol-3H no nuclear fraction is retained at 3° if cortisol (5 μM) is present in the initial incubation at 37°. The curve obtained is in fact practically superimposable on the cortisone curve of Fig. 1. Thus, we conclude that at 37° most, if not all, of the specific cortisol-receptor complex in thymus cells is found in the nucleus.

We have done a number of control experiments that strengthen this conclusion. No nuclear-bound fraction is formed if cortisol-3H is added to the MgCl₂ solution rather than to the medium in which the initial equilibration at 3° is carried out, showing that the nuclear-bound fraction is unlikely to be an artifact formed during cell disruption, but rather that it results from a process that takes place in the intact cells. Fragments of cell membranes that remain attached to nuclei after cell disruption in MgCl₂ carry a negligible amount of the nuclear-bound fraction; these fragments can be removed (by squirting the MgCl₂ suspension through a 22-gauge Teflon needle and then separating nuclei and membranes on a sucrose gradient) without loss of cortisol from the nuclei.

Extraction of the cortisol-receptor complex from thymus nuclei has been accomplished as follows. Cells are incubated with cortisol-3H and disrupted by dilution into 1.5 mM MgCl₂ at 3°, as described above, to yield nuclei with only specifically bound cortisol. All subsequent steps are at 3°. The MgCl₂ solution for this procedure contains 0.5 mg of deoxyribonuclease (Sigma) per ml, which prevents formation of a DNA gel in later steps. After 30 min of gentle shaking, the suspension is centrifuged at 106,000 X g for 2 min to sediment the nuclei. The supernatant is drawn off, and the pellet, which contains nuclei that are still relatively intact and retain most of the specifically bound cortisol, is homogenized gently by hand in a solution containing 0.6 M KCl, 0.1 M tris(hydroxymethyl)aminomethane and 0.0015 M ethylenediaminetetraacetic acid, at pH 8.0 (0.2 ml per ml of MgCl₂ suspension). The homogenate is then centrifuged at 106,000 X g for 2 min to sediment particulate matter that now retains almost none of the cortisol. A 0.4-ml aliquot of the supernatant is chromatographed using the KCl extraction medium for elution on a column (8 mm diameter, 140 mm length) of Sephadex G-25 Fine (Pharmacia) to separate free cortisol from cortisol bound to macromolecules, and successive 5-drop fractions are analyzed for radioactivity.

Results of such an experiment are given by the solid line in Fig. 2. The peak on the right (B) is free cortisol. Peak A, which emerges with the void volume of the column and usually accounts for 30 to 50% of the cortisol in the nuclei from which the extract is prepared, can be identified with some confidence as the cortisol-nuclear receptor complex. It does not appear to be an artifact formed in the course of the extraction procedure, since if cortisol-3H is added at any stage after the initial incubation

![Fig. 1. Dissociation from thymus cells of bound cortisol and cortisone after 20 min of incubation at 37°, followed by 50-fold dilution at 3°, and then rewarmed to 37°. Thymus cell suspensions (0.37 ml of packed cells per ml of cell suspension) were incubated at 37° with cortisol-3H (2.5 nCi per ml) or corticosterone-3H (2.5 nCi per ml) at concentrations of (S) and (S°) in Krebs-Ringer bicarbonate buffer (KRB) containing 2.8 mM glucose. After 20 min, they were diluted 50-fold into either Krebs-Ringer bicarbonate buffer or 1.5 mM MgCl₂ at 3°. After further incubation periods, the diluted cortisol suspensions were rewarmed within 25 sec to 37°. At various times before and after incubation, samples were taken for determination of (Se), the nanomoles of bound steroid per liter of packed cells. For further details see text and Reference 2.](http://www.jbc.org/)

![Fig. 2. Separation on a Sephadex column of the cortisol-nuclear receptor complex (Peak A) from free cortisol (Peak B), and progressive dissociation of the complex at 37°. Nuclear extracts, prepared as described in the text from thymus cells incubated with cortisol-3H (8 nCi) for 30 min at 37°, were chromatographed on Sephadex G-25 columns. The solid line gives the elution curve of an aliquot of extract that was kept at 3° throughout the procedure. The dashed lines give the elution curves of aliquots that were warmed at 37° for 2 min (.), 5 min (O), and 15 min (■), then recooled at 3° and chromatographed.](http://www.jbc.org/)
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with whole cells, no such peak is found. No comparable peak is formed with cortisone-\(^{3}H\), added at any stage of the procedure.

Furthermore, the rate at which cortisol dissociates at 37° from the complex in Peak A is remarkably similar to the rate at which specifically bound cortisol dissociates from whole cells. Dissociation of Peak A is measured by warming aliquots of the KCl extract to 37° for various intervals, cooling them to 3° again, and then chromatographing them. The series of elution curves thus obtained are shown with dashed lines in Fig. 2, where the numbers are the minutes of exposure to 37°. From the amount of cortisol under Peak A at successive times the time constant for dissociation can be estimated to be around 2 min. Given the error in these estimates, this value is indistinguishable from the 3-min time constant characteristic of specifically bound cortisol in whole cells, suggesting strongly that this latter constant reflects an intrinsic property of the cortisol-nuclear receptor complex.

We have also measured the rate of association of cortisol to the nuclear receptor in whole cells, by incubating cell suspensions at 37° with cortisol-\(^{3}H\) for timed intervals, diluting them into cold 1.5 M MgCl\(_2\), and then measuring the amount of cortisol-receptor complex by the procedure described above. These experiments have shown that the previously measured rate (2) at which cortisol reaches its specific sites at 37° in whole cells is indistinguishable from the rate at which it is found attached to the nuclear receptors, again underlining the predominant role of nuclear receptors in accounting for specific binding to whole cells. An immediate conclusion from these results is that the time lag observed between specific binding and the metabolic effects of cortisol must be due to steps lying beyond the association with nuclear receptors.

Preliminary experiments with enzymatic digestion indicate that the cortisol receptor is insensitive to deoxyribonuclease and ribonuclease, but is rapidly degraded by Pronase, and thus can tentatively be considered to be at least in part a protein.

So far we have been unable to obtain any significant degree of direct binding of cortisol either to the receptor (isolated first as the cortisol-receptor complex which is then allowed to dissociate) or to nuclei. This failure may be due simply to technical difficulties in preserving the integrity of the receptor, or it may reflect a more fundamental problem of the type encountered with estrogen receptors in the uterus, where formation of a nuclear estradiol-receptor complex must apparently be preceded by formation of a cytoplasmic complex (7).

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