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Renal mineralocorticoid receptors and hippocampal corticosterone-binding species have identical intrinsic steroid specificity

(transcortin/steroid sequestration/glucocorticoid receptors)

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ABSTRACT **There is current evidence for two classes of hippocampal glucocorticoid receptors (GR)—one classical, [^{3}H]dexamethasone ([^{3}H]Dex)-binding sites in glial cells, and the other [^{3}H]corticosterone-preferring sites in neuronal cells. In the presence of 1 μM of the synthetic glucocorticoid RU26988 (11β,17β-dihydroxy-17α-propynylandrost-1,4,6,-trien-3-one) to exclude tracer from [^{3}H]Dex sites, hippocampal cytosol from adrenalectomized/ovariectomized Sprague–Dawley rats binds [^{3}H]Dex to sites (K_d at 4°C, 0.77 × 10^{-9} M; 65 fmol/mg of protein) with the following order of specificity: aldosterone (Aldo) = 9 α-fluorocortisol (9αF-cortisol) = deoxycorticosterone (DOC) = corticosterone > cortisol ≫ Dex; [^{3}H]Aldo, [^{3}H]DOC, and [^{3}H]corticosterone binding show identical specificity in the presence of RU26988. Addition of 1% adrenalectomized/ovariectomized rat plasma (but not plasma heated at 56°C for 30 min) alters the specificity to: 9αF-cortisol ≥ Aldo ≥ DOC ≫ Dex ≥ corticosterone ≥ cortisol, consistent with sequestration of DOC, corticosterone, and cortisol by transcortin and similar to classical mineralocorticoid receptor (MR) binding of [^{3}H]Aldo in renal cytosol (9αF-cortisol ≥ Aldo ≥ DOC ≫ corticosterone ≥ cortisol ≥ Dex). Separation of other renal binders from transcortin by hydroxylapatite adsorption established the intrinsic specificity of [^{3}H]Aldo binding to MR as: DOC ≥ Aldo ≥ 9αF-cortisol ≥ corticosterone > cortisol ≫ Dex, parallel to that of the [^{3}H]corticosterone-binding sites in hippocampus. These studies suggest (*i*) that hippocampal [^{3}H]corticosterone-binding sites and renal MR may have identical intrinsic specificity for steroids, with apparent specificity differences the result of tissue-specific sequestration of naturally occurring steroids other than Aldo and (*ii*) that an identical steroid-binding species may thus be occupied under physiological conditions by a mineralocorticoid in one tissue (kidney) and a glucocorticoid in another (hippocampus).**

Though many adrenal steroids show both glucocorticoid and mineralocorticoid activity, studies on the control of adrenal steroidogenesis strongly suggest aldosterone (Aldo) to be the principal mammalian mineralocorticoid and cortisol or corticosterone to be the principal glucocorticoid. A decade ago, the concept of distinct mineralocorticoid and glucocorticoid effects was strengthened by the demonstration of separate mineralocorticoid and glucocorticoid receptors (MR and GR) in tissues—e.g., kidney—that were recognized as targets for both classes of hormones. [^{3}H]Aldo bound to two classes of saturable binding sites (1, 2); from those of higher affinity (type I), [^{3}H]Aldo was displaced by steroids with a hierarchy (Aldo ≥ deoxycorticosterone (DOC) > corticosterone), consistent with a physiological MR role (2). On similar criteria, the lower affinity (type II) [^{3}H]-Aldo sites were identified as classical, dexamethasone (Dex)-binding GR, previously described in a variety of tissues (3).

In parallel, there is increasingly compelling evidence for GR distinct from and in addition to the type II Dex-binding GR. The intranuclear localization of [^{3}H]Dex and [^{3}H]corticosterone, administered *in vivo* to adrenalectomized rats, has shown two separate types of putative GR in the central nervous system, most particularly in the hippocampus (4–7). In this tissue, binding of Dex appears confined to glial cells and corticosterone-preferring sites appear confined to neurons. More recently, a specific receptor role for such sites has been postulated on the basis of the induction of protein 1 in hippocampal neurons of adrenalectomized rats by corticosterone but not by equal doses of Dex (8).

Simultaneously, there have been a series of reports of "MR-like" binding in rat brain (9–11), mouse pituitary tumor cell lines (12), and cultured rat aortae (13). The salient difference between classical renal MR and these sites is that corticosterone, DOC, and Aldo appear to have equivalent affinity for such sites. Given the much higher plasma-free levels of corticosterone than Aldo in the rat, substantial mineralocorticoid occupancy of such sites appears unlikely.

In the present study, we present data suggesting that renal MR (2), hippocampal corticosterone-preferring GR (4–7), and, by inference, the nonclassical mineralocorticoid-like receptors (9–13) share an identical intrinsic hierarchy of affinity for a range of natural and synthetic steroids. Secondly, these studies suggest that the differences in specificity found in previous studies reflect different tissue levels of transcortin and, thus, differential sequestration of transcortin-bound steroids. Finally, these studies suggest that an identical species in terms of steroid specificity may operate in one tissue (e.g., kidney) as a MR and in another (hippocampus) as a GR.

METHODS

[^{3}H]Dex (50 Ci/mmol; 1 Ci = 37 GBq), [^{3}H]corticosterone (105 Ci/mmol), [^{3}H]DOC (55 Ci/mmol), and [^{3}H]Aldo (77 Ci/mmol) were purchased from New England Nuclear. Dex was a gift from Merck Sharp & Dohme (Sydney, Australia), Spirolactone was from Searle (Skokie, IL), and the synthetic glucocorticoid 11β,17β-dihydroxy-17α-propynylandrost-1,4,6-trien-3-one (designated RU26988) was from Roussel-Uclaf (Romainville, France); other steroids were from Ikapharm (Ramat-Gan, Israel) and Steraloids (Wilton, NH).

In all studies, female Sprague–Dawley rats, body weight 150–200 g, were used 4 days after adrenalectomy and ovariectomy. Rats were killed by decapitation, and the thymus, kidneys, and hippocampus were removed into ice-cold saline. Tissues were

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Abbreviations: Aldo, aldosterone; MR, mineralocorticoid receptor; GR, glucocorticoid receptor; Dex, dexamethasone; DOC, deoxycorticosterone; 9αF-cortisol, 9α-fluorocortisol.

* Deceased.

*Correction - Proc. Natl. Acad. Sci. USA, Jan. 1984

minced and thoroughly washed in ice-cold saline before homogenization. Blood was collected into heparin-treated tubes and centrifuged at 1,000 × g for 5 min, and plasma was decanted. All procedures were carried out at 4°C.

Tissues were homogenized (glass/glass) in either TEMGD buffer (10 mM Tris/1.5 mM EDTA/20 mM Na_2MoO_4/10% (vol/vol) glycerol/2 mM dithiothreitol, pH 7.4) or TSMD buffer (100 mM Tris/250 mM sucrose/100 mM Na_2MoO_4/2 mM dithiothreitol, pH 7.4). Homogenates were centrifuged at 200,000 × g for 40 min at 4°C to yield a cytosol. Cytosols (100 μl) or hydroxylapatite eluates (100 μl) were added to 50 μl of buffer containing tritiated ligand and 50 μl of buffer either steroid-free or with various concentrations of competitor. Incubation was either for 16 hr at 4°C or for 40 min at 22°C. Bound and free steroids were separated by use of hydroxylapatite or dextran-coated charcoal as indicated in figure legends.

In the studies using hydroxylapatite, bound and free steroids were separated by the addition of 300 μl of an ice-cold suspension of hydroxylapatite (15%, wt/vol) in 50 mM Tris/10 mM KH_2PO_4, pH 7.2. After incubation for 20 min at 4°C with intermittent shaking, the tubes were centrifuged (1,000 × g for 5 min), the supernatant was aspirated, and the pellet was washed three times with 1 ml of 10 mM Tris/5 mM NaH_2PO_4/1.5 mM EDTA, pH 7.2/1% Tween 80. Washed hydroxylapatite pellets were resuspended in 2 ml of ethanol at room temperature for 15 min and centrifuged (1,000 × g for 5 min), and the supernatant was taken for liquid scintillation spectrometry.

In other studies, 300 μl of ice-cold 0.5% charcoal/0.05% dextran in TSMD was added to the incubate and incubated for 20 min at 4°C before centrifugation (1,000 × g for 10 min). Aliquots of the supernatant were taken for liquid scintillation spectrometry.

Preliminary experiments verified that a batch hydroxylapatite technique separated steroid receptors from transcortin (14, 15). Accordingly, ice-cold hydroxylapatite (50% wt/vol) in TSMD containing 10 mM KH_2PO_4 was added to an equal volume of cytosol and incubated for 20 min at 4°C with intermittent shaking. The suspension was then centrifuged (1,000 × g for 5 min), the supernatant was aspirated, and the pellet was washed free of transcortin with TSMD buffer containing 5 mM KH_2PO_4 (3-ml washes three times). Cytoplasmic steroid-binding sites were eluted from the hydroxylapatite with three 2-ml portions of TSMD buffer containing 0.4 M KH_2PO_4, and the eluate was centrifuged at 2,000 × g for 15 min to remove fines. Protein determinations in all studies were performed by the Bradford method (16).

RESULTS

Hippocampal cytosols incubated with [^{3}H]Dex or [^{3}H]corticosterone yielded rectilinear Scatchard plots (Fig. 1). The inclusion of 1 μM RU26988 reduced [^{3}H]Dex binding to ≈15%; however, the affinity of the remaining [^{3}H]Dex binding sites could not be determined with accuracy. When [^{3}H]corticosterone binding in the presence and absence of RU26988 was compared, the apparent affinity was also similar; in contrast with Dex, however, about half the [^{3}H]corticosterone binding sites were not blocked by RU26988.

That this difference reflects binding of both ligands to more than one binding site in hippocampal cytosol despite the rectilinear Scatchard plots is suggested by Fig. 2 *A* and *B*. The binding of [^{3}H]Dex consistently was displaced better by Dex than by corticosterone, and vice versa. The simplest interpretation of the data in Figs. 1 and 2 is that there exist in hippocampal cytosol two classes of sites, one with higher affinity for corticosterone than Dex (corticosterone-preferring sites) and the other with higher affinity for Dex than for corticosterone, consistent with earlier studies from other laboratories (4–8).

This interpretation is further strengthened by the clear differences in the ability of RU26988 to compete (Fig. 2 *A* and *B*). RU26988 had negligible affinity for ≈60% of the sites labeled with [^{3}H]corticosterone; at the concentration of [^{3}H]Dex used (2.6 nM), about 15% appeared to be bound to sites with negligible affinity for RU26988, consistent with the findings of Fig. 1. In contrast, RU26988 competed for all [^{3}H]Dex and [^{3}H]corticosterone binding sites in thymus cytosol derived from the same rats and run as parallel, internal controls (Fig. 2 *C* and *D*).

To establish the steroid specificity of the RU26988-insensitive [^{3}H]corticosterone binding sites, hippocampal cytosol was incubated with [^{3}H]corticosterone and a >200-fold excess of RU26988 in the presence and absence of a range of concentrations of natural and synthetic steroids. The results of such a specificity study are shown in Fig. 3*A*; identical patterns were seen when [^{3}H]Aldo and [^{3}H]DOC were used as probes (Fig. 4). This hierarchy of affinity is clearly different from that found for [^{3}H]Aldo in renal cytosol (Fig. 3*B*). In both cytosols, Aldo and 9αF-cortisol appear to have equivalent affinity for the steroid-binding sites, in each case ≥ 10-fold higher than Dex; the most salient differences in Fig. 3 *A* and *B* are the lower apparent affinities of DOC and cortisol, and the very much lower affinity of corticosterone in kidney compared with hippocampus.

The data shown in Fig. 3 *C* and *D* suggest that these dif-

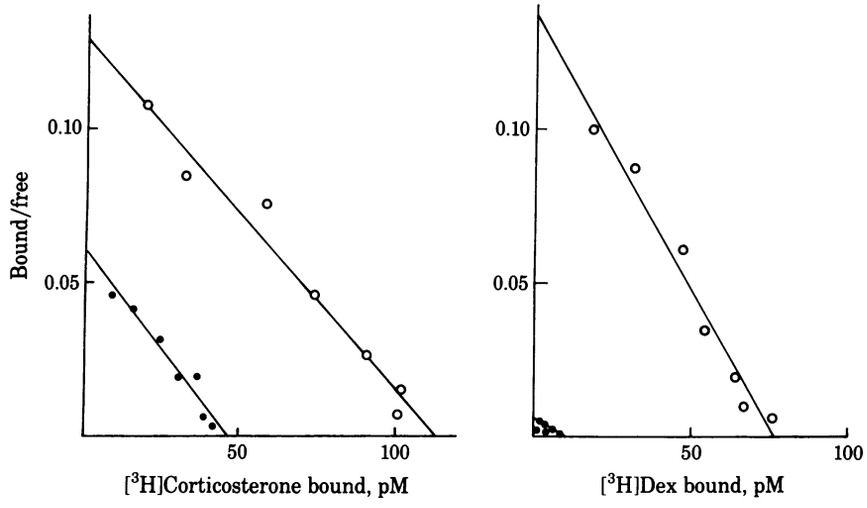

FIG. 1. Scatchard plot analyses of the specific binding of [^{3}H]Dex (*Left*) and [^{3}H]corticosterone (*Right*) in hippocampal cytosol in the presence (●) and absence (○) of 1 μM RU26988. Cytosol was prepared in TEGMD buffer. Incubation with [^{3}H]Dex (0.2–12.9 nM) and [^{3}H]corticosterone (0.2–13.8 nM) was overnight at 4°C. Separation of bound and free was by the use of hydroxylapatite. Nonspecific binding was estimated by the inclusion of 1 μM Dex or 1 μM corticosterone at each concentration of radioligand. For [^{3}H]Dex: without RU26988, n = 105 fmol/mg of protein and $K_d = 0.56 \times 10^{-9}$ M; with RU26988, n = 13.7 fmol/mg of protein and $K_d = 2.6 \times 10^{-9}$ M. For [^{3}H]corticosterone: without RU26988, n = 157 fmol/mg of protein and $K_d = 0.88 \times 10^{-9}$ M; with RU26988, n = 65 fmol/mg of protein and $K_d = 0.77 \times 10^{-9}$ M.

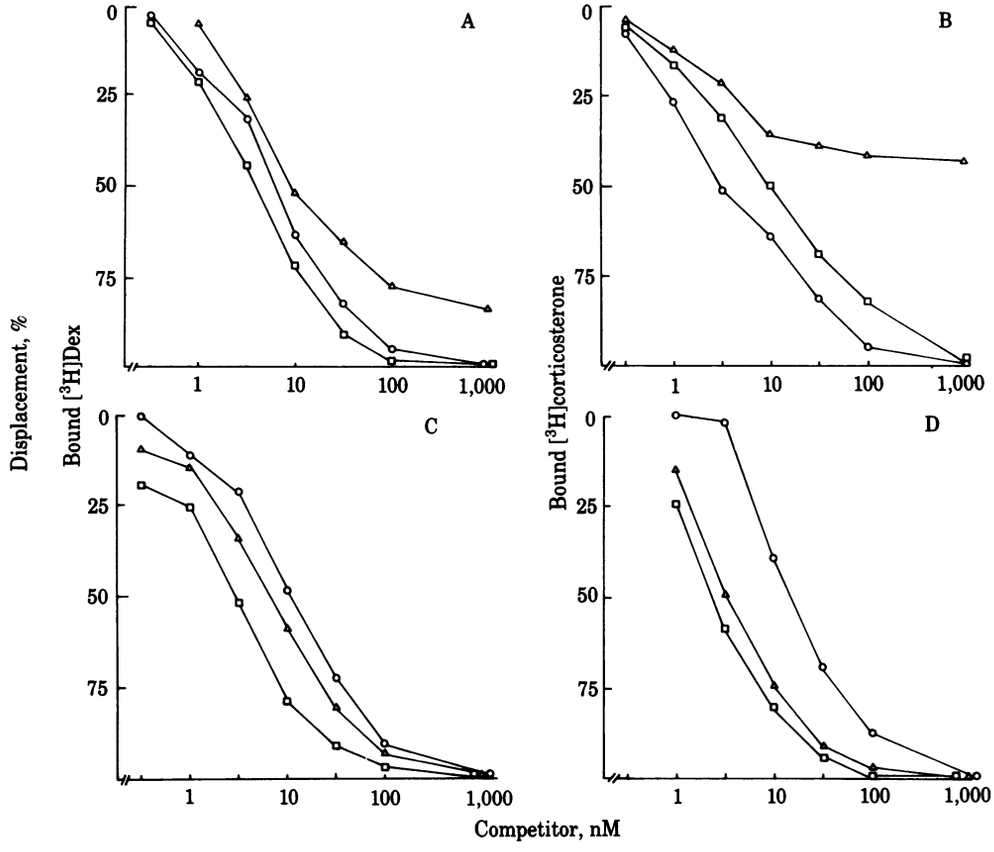

FIG. 2. Steroid specificity of [^{3}H]corticosterone and [^{3}H]Dex binding in hippocampal (*A* and *B*) and thymic (*C* and *D*) cytosols prepared in TSMD buffer and incubated with 2.6 nM [^{3}H]Dex (*A*), 1.8 nM [^{3}H]corticosterone (*B*), 2.6 nM [^{3}H]Dex (*C*), and 2.1 nM [^{3}H]corticosterone (*D*) for 40 min at 22°C with various concentrations of Dex (□), corticosterone (○), or RU26988 (△). Specific binding (fmol/mg of protein) was 58 (*A*), 99 (*B*), 163 (*C*), and 77 (*D*). Bound and free steroid were separated on hydroxylapatite; each point is the mean of duplicates.

ferences are not intrinsic to the binding sites in the two tissues but are a reflection of a different cytosol milieu. When hydroxylapatite was used to separate renal steroid receptors from transcortin before binding, the specificity of renal [^{3}H]Aldo-binding sites (Fig. 3*C*) approximated closely that seen in untreated hippocampal cytosol, with DOC > Aldo = 9αF-cortisol = corticosterone. Similar pretreatment of hippocampal cytosol with hydroxylapatite did not affect the apparent specificity (data not shown), suggesting that transcortin was not present in any significant amounts. When hippocampal cytosol was made 1% (vol/vol) with adrenalectomized rat plasma (Fig. 3*D*), the pattern of specificity of [^{3}H]corticosterone binding was markedly altered to one that was equivalent to a "classical" renal MR (9αF-cortisol $\geq$ Aldo > DOC $\gg$ Dex $\geq$ corticosterone > cortisol); heated (56°C for 30 min) plasma from adrenalectomized rats did not mimic this effect.

DISCUSSION

On the background of the numerous studies over the past decade on mineralocorticoid and glucocorticoid binding, the data reported above strongly suggest that at least the steroid-binding moiety of such receptors can be divided into two classes. One class, which we shall term type I, may represent MR in the kidney and GR in the hippocampus; the other class, which we shall term type II, is the classic [^{3}H]Dex-binding GR. If type I receptors have identical intrinsic steroid specificity in kidney and hippocampus, there must exist extrinsic specificity-conferring mechanisms determining occupancy *in vivo*. Finally, if both type I in some tissues and type II sites are GR, the implications of two such distinct classes of glucocorticoid receptor merit discussion.

Until details of protein sequence and structure are available, the identity of renal [^{3}H]Aldo-binding sites and hippocampal [^{3}H]corticosterone-preferring sites remains a working hypothesis rather than established fact. However, such a hypothesis is not only consistent with the numerous studies on binding of various ligands ([^{3}H]Aldo, [^{3}H]DOC, [^{3}H]corticosterone, and [^{3}H]Dex) over the past decade but allows interpretation of the sometimes disparate and often puzzling findings of such studies.

In the present studies, we have discriminated between steroid bound to putative receptors and to transcortin by the selective adsorption of the former onto hydroxylapatite; we have discriminated between steroid binding to [^{3}H]Dex-binding type II GR and other steroid receptors by using unlabeled RU26988, which has a high specificity for type II sites (11). On this basis there appear to be two classes of binding site for adrenal steroids in rat hippocampal cytosol; one with high affinity ($<1 \times 10^{-9}$ M) for corticosterone, the putative physiological ligand, and an affinity for Dex of $\approx 1 \times 10^{-8}$ M; the other, with not dissimilar affinity for [^{3}H]Dex ($\approx 3 \times 10^{-9}$ M) and corticosterone ($\approx$10 nM). The concentrations of both classes of sites are similar; because both steroids have affinities in the range 10^{-8}–10^{-9} M for both classes of site, it is not surprising that, for both ligands, rectilinear Scatchard plots were found, as has been described on theoretical grounds (17). Finally, we found no evidence for a distinct MR as has been suggested by Anderson and

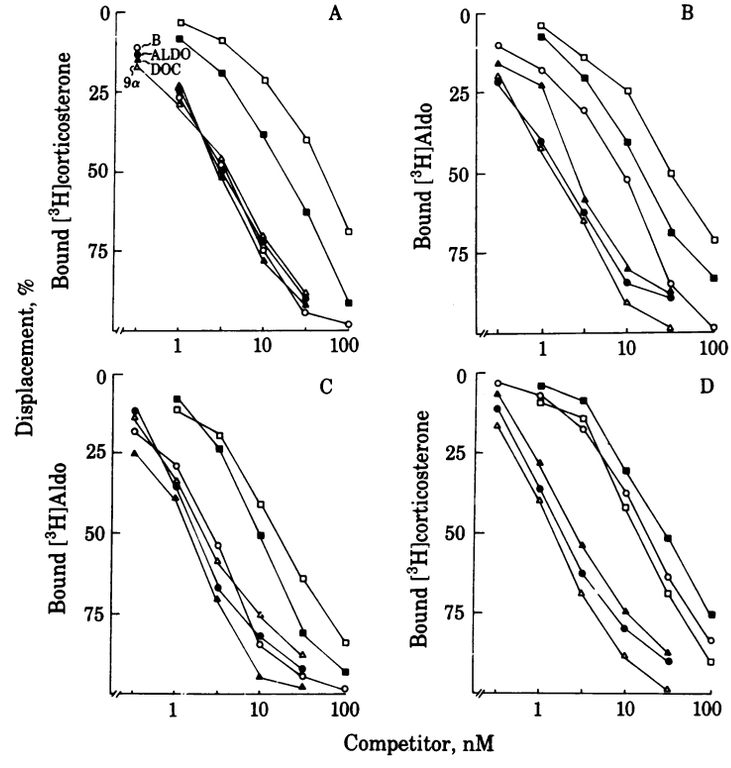

FIG. 3. Steroid specificity of hippocampal [^{3}H]corticosterone (*A* and *D*) and renal [^{3}H]Aldo (*B* and *C*) binding in the presence and absence of plasma. Hippocampal (*A*) and renal (*C*) cytosols in TSMD were adsorbed onto hydroxylapatite and washed free of transcortin, and the receptors were eluted with TSMD containing 0.4 M KH_2PO_4. Alternatively, untreated renal cytosol (*B*) or hippocampal cytosol made 1% in plasma (*D*) were used; in preliminary studies, addition of 0.4 M K_2HPO_4 was shown not to affect the affinity or specificity of binding. Cytosols were incubated at 22°C for 40 min with 2.1 nM [^{3}H]corticosterone (*A*), 1.5 nM [^{3}H]Aldo (*B*), 1.5 nM [^{3}H]Aldo (*C*), and 4.7 nM [^{3}H]corticosterone (*D*), all in the presence of 1 μM RU26988 and various concentrations of Aldo (●), corticosterone (○), DOC (▲), 9α-F-cortisol (△), cortisol (■), or Dex (□). Specific binding (fmol/mg of protein) was 129 (*A*), 39 (*B*), 26 (*C*), and 110 (*D*). Bound and free steroid were separated by dextran-coated charcoal (*A* and *C*) or hydroxylapatite (*B* and *D*); each point is the mean of duplicates.

Fanestil (10), Veldhuis *et al.* (†), and Moguilewsky and Raynaud (11); in our studies, whether [^{3}H]corticosterone, [^{3}H]Aldo, or [^{3}H]DOC was used as a probe, the binding sites so labeled showed absolutely equivalent affinity for corticosterone, DOC, and Aldo.

That the renal [^{3}H]Aldo-binding type I receptor has an equivalent intrinsic specificity is strongly suggested by the competition studies on cytosol preadsorbed with hydroxylapatite; that the extrinsic, modifying factor may be transcortin is suggested on a number of grounds. First, high levels of transcortin or a transcortin-like binder have been demonstrated in renal cytosol; that negligible levels are found in hippocampal cytosol is suggested by the identical potency of corticosterone for [^{3}H]corticosterone-binding sites in hydroxylapatite-pretreated and nonpretreated cytosol. Second, the change in relative affinity after hydroxylapatite adsorption is consistent with the known higher affinity of transcortin for the naturally occurring steroids corticosterone, DOC, and cortisol; the relative potencies of Aldo, 9α-F-cortisol, and Dex, which bind poorly to transcortin, remain unaffected. Third, addition of 1% plasma from adrenalectomized rats to hippocampal cytosol causes a marked change in specificity of the corticosterone-preferring sites, to a hierarchy indistinguishable from that of a classical renal MR; this change does not occur when the transcortin is destroyed by heating to 56°C for 30 min. Taken together, these findings strongly support a crucial role for transcortin or a related protein in determining the specificity of type I receptors in certain target tissues.

Intravascular binding of corticosterone and DOC by transcortin has been postulated as a crucial specificity-conferring mechanism, allowing Aldo access to renal MR (2). From the studies described in this paper, it appears likely to have an additional crucial role at the tissue level; the exact operation of such a specificity-conferring mechanism remains to be established. Various studies have shown that the levels of transcortin-like binding in a range of tissues are higher than could be accounted for by plasma contamination (18). Evidence that such sites may be intracellular in the kidney is disputed (19, 20), although the weight of more recent evidence (21) is against such a location. The relatively low affinity of DOC for [^{3}H]Aldo-binding sites in isolated rabbit collecting tubules (≈5% of that of Aldo and approximately that of spirolactone) might be interpreted as evidence for a site in close apposition to the cells (22); similarly, an extravascular, extracellular location of transcortin would explain the observed differences in specificity of Aldo binding between cultured AtT 20 cells (12) and whole pituitary glands (23).

Whatever the localization of the tissue transcortin, if it is simply a sequestration site, the problem remains of its eventual saturation under equilibrium conditions and, thus, of its becoming ineffective. For example, progressive dilution of renal cytosol *in vitro* results in a progressive increase in the apparent affinity of corticosterone for type I binding sites (unpublished data). One *in vivo* mechanism, whereby extravascular transcortin might act as a renewable sink for corticosterone binding, is in tissues where the vascular architecture is recurrent and blood

† Veldhuis, H. D., Van Kopper, C., Van Ittersum, M. & De Kloet, E. R., Proceedings of the Twenty-Second Dutch Federation Meeting, April, 1981, Utrecht, Netherlands, p. 448 (abstr.).

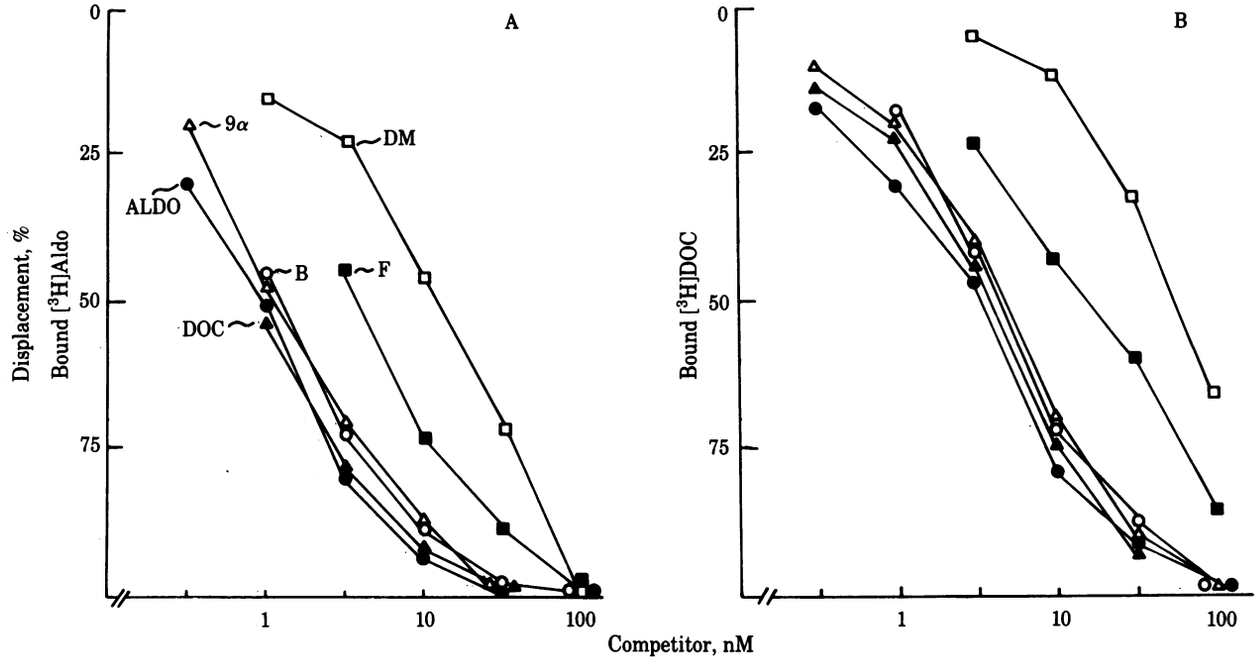

FIG. 4. Steroid specificity of [^{3}H]Aldo and [^{3}H]DOC binding in hippocampal cytosol. Cytosol in TSMD buffer was incubated with 1.6 nM [^{3}H]-Aldo (*A*) or 3.6 nM [^{3}H]DOC (*B*) in the presence of 1 μM RU26988. Experimental details were as for Fig. 2 except that the following additional steroid competitors were used: Aldo, DOC, 9α-F-cortisol, and cortisol. Specific binding (fmol/mg of protein) was 74 (*A*) and 57 (*B*). Each point is the mean of duplicates. B, corticosterone; F, cortisol; 9α, 9αF-cortisol; DM, dexamethasone.

flow is rapid—for example, in the renal inner medulla/papilla.

The final area for discussion is that of the existence and relative affinity for corticosterone of two distinct classes of GR. There is general consensus that type II, Dex-preferring, RU26988-blockable sites are physiological GR; the demonstration of various behavioral effects elicited by corticosterone but not Dex (24, 25) and the recent report of the induction of protein I in rat hippocampus by corticosterone but not Dex (8) constitute strong evidence for a receptor role for these corticosterone-preferring type I sites. What has become clear from the present studies is that there is a clear difference in the affinity of corticosterone, the endogenous glucocorticoid in the rat, for the two types of sites. In the present *in vitro* studies, this difference is at least an order of magnitude; from previous *in vivo* studies, the much higher affinity of hippocampal than pituitary [^{3}H]corticosterone binding could confidently be expected (6).

As a corollary of this difference in affinity, it might reasonably be predicted that type I GR are at least potentially capable of responding to fluctuations in corticosterone levels within the normal, baseline range of diurnal variation; in contrast, the much lower affinity of type II GR for corticosterone suggests that they are significantly occupied only when free corticosteroid levels are increased. In terms, then, of potential physiological roles, the type I receptors may be involved in the modulation of circadian responses, whereas the type II receptors may be involved in responses to increased glucocorticoid levels. The implications of this distinction—particularly given the effects of steroid administration and withdrawal upon the central nervous system (26) and the loss of diurnal rhythmicity of cortisol secretion in a large proportion of patients with endogenous depressions (27)—await exploration. Similarly awaiting exploration is the evolutionary significance of the emergence of two distinct GR, with a plasma steroid-binding globulin that selectively confers MR selectivity upon one of them in a tissue specific fashion.

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