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RELATIVE BINDING AFFINITY OF STEROIDS FOR THE CORTICOSTERONE RECEPTOR SYSTEM IN RAT HIPPOCAMPUS

E. R. De Kloet*‡, H. D. Veldhuis*, J. L. Wagenaars† and E. W. Bergink†
*Rudolf Magnus Institute for Pharmacology, Medical Faculty, University of Utrecht, Vondellaan 6, 3521 GD Utrecht, and †Scientific Development Group, Organon International B.V., Oss, The Netherlands

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Summary—In cytosol of the hippocampus corticosterone displays highest affinity for the sites that remain available for binding in the presence of excess RU 26988, which is shown to be a "pure" glucocorticoid. A rather high affinity ($\geq 25\%$) was found for 11β-hydroxyprogesterone, 21-hydroxyprogesterone, 5α-corticosterone, 19-nor-deoxycorticosterone, 11-deoxycorticosterone and cortisol. A moderate affinity ($> 5\%$ and $< 25\%$) was displayed by about 14 steroids among which progesterone, aldosterone, 9α-fluorocortisol and dexamethasone. Corticosterone also shows highest affinity to plasma transcortin and thymus cytosol in the presence of RU 26988. However, the rank-order in affinity by the competing steroids was distinctly different from that observed in the hippocampus; cf. aldosterone and dexamethasone displaced [^{3}H]corticosterone from sites unoccupied by RU 26988 in the hippocampus but not from transcortin or sites in thymus cytosol. In thymus cytosol some potent glucocorticoids have higher affinity for the [^{3}H]dexamethasone labeled sites than dexamethasone. The binding of [^{3}H]dexamethasone in thymus cytosol is completely abolished in the presence of a 100-fold excess of RU 26988.

We conclude that our data support the evidence for RU 26988 as a selective ligand for glucocorticoid receptors. RU 26988 leaves binding sites available with highest affinity for corticosterone in hippocampus cytosol that are distinct from transcortin-like sites as found in thymus cytosol or from plasma transcortin.

INTRODUCTION

The rat brain contains receptor sites for adrenocortical steroid hormones [1]. These receptor sites are heterogeneous and can be distinguished in binding specificity, localization and function [2–5]. *In vivo* administration of tracer amounts of labeled corticosteroids showed pronounced retention of [^{3}H]corticosterone ([^{3}H]B) and [^{3}H]aldosterone ([^{3}H]ALDO) receptor complexes in particular in cell nuclei of the extra-hypothalamic limbic brain regions [6, 7]. Negligible uptake in cell nuclei of hippocampus was observed for [^{3}H]deoxycorticosterone ([^{3}H]DOC), while the uptake of synthetic glucocorticoids such as [^{3}H]dexamethasone ([^{3}H]DEX) was evenly distributed over the various brain structures [8, 9]. Prior treatment with ALDO and DOC blocked cell nuclear retention of [^{3}H]B, while DEX was a poor competitor [6]. Autoradiographic studies showed the principal localization of [^{3}H]ALDO and [^{3}H]B in cell nuclei of limbic nerve cells, particularly in the hippocampal neurons [10–13], while [^{3}H]DEX labeled neurons in the hypothalamus, and in the non-neural brain tissue, e.g. glial cells and vascular endothelial cells [14–16]. These observations on cellular and regional localization of the labeled steroid–receptor complexes were supported by steroid effects on behavior [17–20, 23] and brain biochemistry [21, 22, 24–26] showing that corticoid receptor systems in nerve cells and in non-neural tissue functionally should be distinguished [3–6]. Non-neural tissue (glial cells) contains a glucocorticoid receptor system that, in accordance with that occurring in peripheral glucocoricoid target organs, displays the highest responsiveness to synthetic glucocorticoids such as DEX [3, 4, 6, 26]. The hippocampal neurons contain a receptor system that is responsive exclusively to B, the naturally occurring glucocorticoid of the rat, but that also binds ALDO [4, 5, 6, 27, 28].

In the present study we have investigated the specificity of the remaining corticosterone receptor system after blockade of glucocorticoid receptors with RU 26988 in hippocampus cytosol using a set of 29 reference compounds for competitive binding analysis. A comparison was made between the affinity for these corticosterone receptor sites in hippocampus and thymus cytosol, as well as for transcortin. Also was studied the specificity of the glucocorticoid receptor in thymus using [^{3}H]DEX as ligand. The data show that highest affinity for the receptor system in the hippocampus is displayed by the naturally occurring glucocorticoid of the rat, corticosterone.

MATERIALS AND METHODS

Animals and chemicals

Male Wistar rats (TNO, Zeist, The Netherlands; 160–200 g body weight) were used. All animals were adrenalectomized 3 days before sacrifice. Bilateral

‡To whom correspondence should be addressed.

adrenalectomy was performed in our laboratory through the dorsal approach, and the rats were supplied with saline in drinking bottles. The rats were caged in groups of 5 at 22°C under standard light conditions (14:10 h light–dark period) and allowed food *ad libitum*.

Unlabeled steroids, except RU 26988, were provided by Organon International B.V. (Oss, The Netherlands). RU 26988 was kindly donated by the Roussel UCLAF Research Centre (Romainville, France). 1,2-[^{3}H]Corticosterone (sp. act.: 75 Ci/mmol) and 1,2,4-[^{3}H]dexamethasone (sp. act.: 70 Ci/mmol) were obtained from The Radiochemical Center, Amersham, Great Britain.

Binding studies

For *in vitro* binding experiments in cytosol, rats were anesthetized with Nembutal® and sacrificed by perfusion with saline (25 ml) through the heart. Dissection of hippocampus was performed as described previously [29]. In addition, the thymus was excised.

Cytosol was prepared by homogenizing hippocampal or thymic tissue (w/v = 1:1) in 5 mM Tris, containing 1 mM EDTA disodium salt, 1 mM 2-mercaptoethanol and 5% glycerol, adjusted to pH 7.4 with hydrochloric acid. The homogenate was centrifuged at 2°C for 1 h at 105,000 g_{av} in an International B-60 ultracentrifuge (IEC, Needham Heights, MA).

Scatchard analyses were performed with the results from incubations in microtitration plates of cytosol (0.050 ml) with 0.05 ml Tris buffer, which contained increasing concentrations of [^{3}H]B, [^{3}H]B + 100-fold excess of unlabeled RU 26988, or with [^{3}H]DEX. In addition, blood plasma (0.05 ml) diluted 50-fold with Tris buffer pH = 7.4 was incubated with 0.05 ml Tris buffer containing [^{3}H]B or [^{3}H]B + 100-fold excess RU 26988.

For studies on the relative binding affinity (RBA), a fixed amount (3.9 nM) of tritiated steroid was used together with various concentrations of unlabeled steroids (1.95, 3.9 and 7.8 nM). A 4-h incubation period of the cytosol or plasma was sufficient to obtain binding equilibrium of the labeled steroids to soluble macromolecules. Subsequent separation of bound and unbound steroid was performed as follows: unbound steroid was removed by incubating, with continuous shaking, for 5 min the mixture with 0.1 ml Dextran-coated charcoal suspension (0.25% charcoal, 0.025% Dextran-T-70 in Tris buffer). The microtitration plates were then centrifuged at 1500 g for 15 min. An aliquot of the supernatant (0.1 ml) was added to 9 ml scintillation fluid (Instagel®) and the radioactivity was counted.

In all experiments the specific activity of the radioactive steroids was used to convert radioactivity to femtomoles of steroid. All data were expressed per mg cytosol protein. Protein determinations were performed by the biuret method, adapted for the Centrifichem by A. J. M. Degen[30].

RESULTS AND DISCUSSION

Table 1 shows the apparent binding constants of [^{3}H]B in hippocampus cytosol in the absence or presence of a 100-fold excess of RU 26988. Note that the number of binding sites determined in this study is about four times larger than previously reported, which is probably due to the assay: e.g. in this study the Dextran-charcoal method is used instead of the LH_{20} gelpermeationchromatography. Inclusion of RU 26988 leaves about 75% of the binding sites in the hippocampus available for [^{3}H]B. RU 26988 does not bind to mineralocorticoid receptors nor to transcortin, but has been used as a selective ligand for glucocorticoid receptors in a number of tissues including the brain [see below, 6, 27, 28]. In previous work using the LH_{20} separation technique we found that a 100-fold excess of RU 26988 displaced 25% of the [^{3}H]binding in hippocampus cytosol (unpublished observation). We also have shown that RU 26988 displaces about 80% of the binding sites in the hippocampus labeled with [^{3}H]ALDO. These [^{3}H]ALDO sites have been indicated as presumptive mineralocorticoid receptors [6, 27]. However, these "mineralocorticoid receptors" displayed a higher affinity to B [6]. Therefore, while there is little doubt that RU 26988 occupies selectively glucocorticoid receptors, the nature of the binding sites that remain unoccupied in the presence of RU 26988 is at present not clear; these sites could be mineralocorticoid and/or corticosterone preferring receptor types.

Figure 1 shows the competition of various unlabeled ligands (B, progesterone, DEX and triamcinolone acetonide) for the binding of [^{3}H]B to soluble receptor sites in hippocampal cytosol that remain available after occupation of the glucocorticoid sites with RU 26988. Increasing amounts of unlabeled competing steroids were added and the RBA of the various steroids for these sites was calculated from the displacement of [^{3}H]B (see legend Table 2).

The RBA-values for binding in hippocampal cytosol of 29 steroids related to the corticoid structure are

Table 1. Comparison of equilibrium dissociation constants (K_d) and concentration of binding sites for corticosterone (B_{max}) in the absence or presence of a 100-fold excess of RU 26988 in rat thymus and rat hippocampus

	Thymus			Hippocampus		
Ligand	B_{max} (fmol/mg prot.)	K_d (nM)	$r_{corr.}$	B_{max} (fmol/mg prot.)	K_d (nM)	$r_{corr.}$
[^{3}H]corticosterone	6650	3.1	0.93	2670	2.5	0.89
[^{3}H]corticosterone + 100-fold RU 26988	4700	6.0	0.89	2050	6.5	0.88

Table 2. Relative binding affinities of 29 steroids related to the pregnane structure for binding sites labeled with [^{3}H]DEX or [^{3}H]B in thymus, hippocampus and in blood plasma

No.	Systemic name	Trivial name	[^{3}H]Corticosterone + 100-fold excess RU 26988 (RBA_B = 100%)*			[^{3}H]Dexamethasone (RBA_{DEX} = 100%)
			Hippocampus	Blood plasma	Thymus	Thymus
I	11β,21-Dihydroxy-4-pregnene-3,20-dione	Corticosterone	100	100	100	58 ± 4
II	11β-Hydroxy-4-pregnene-3,20-dione	11β-Hydroxy-progesterone	55 ± 12†	30 (23–39)‡	29 ± 2†	46 ± 18
III	21-Hydroxy-19-nor-4-pregnene-3,20-dione	19-Nor-deoxycorticosterone	43 (31–59)	—	—	43 ± 4
IV	21-Hydroxy-4-pregnene-3,20-dione	Deoxycorticosterone	34 ± 8	6.4 (4.8–8.4)	4.8 (3.7–6.1)	70 ± 14
V	11β,17α,21-Trihydroxy-4-pregnene-3,20-dione	Cortisol	29 ± 7	23 (18–30)	17 (14–21)	48 ± 9
VI	4-Pregnene-3,20-dione	Progesterone	20 (14–27)	—	—	47 ± 7
VII	11β-Hydroxy-4-pregnen-3-one	11β-Hydroxy-pregnenolone	19 (14–37)	—	—	45 (32–62)
VIII	17α,21-Dihydroxy-4-pregnene-3,20-dione	Cortexolone	18 ± 2	2.0 (1.5–2.6)	1.5 (1.1–1.9)	34
IX	11β,21-Dihydroxy-18-al-4-pregnene-3,20-dione	Aldosterone	11	<0.5	<0.5	14 (10–19)
X	9α-Fluoro-11β,21-dihydroxy-16α,17α-dimethyl-21chloro-1,4 prenadiene-3,20-dione	—	11	<0.5	<0.5	370
XI	11β,17α-Dihydroxy-21-methyl-4-pregnene-3,20-dione	21-Deoxy-21-methyl-cortisol	10 (8–12)	16 (13–21)	10 (8–13)	25 (18–35)
XII	11β,21-Dihydroxy-16α,17α-dimethyl-1,4-pregnadiene-3,20-dione	Δ_1-16α,17α-Dimethyl-corticosterone	10	1.2 (0.9–1.6)	0.7 (0.5–0.9)	230 (170–320)
XIII	11β,17α,21-Trihydroxy-1,4-pregnadiene-3,20-dione	Prednisolone	7	—	—	56 (41–76)
XIV	11β,21-Dihydroxy-16α,17α-dimethyl-4-pregnene-3,20-dione	16α,17α-dimethyl-corticosterone	7	<0.5	<0.5	20 (14–29)
XV	9αFluoro-16α-methyl-11β,17α,21-trihydroxy-1,4-pregnadiene -3,20-dione	Dexamethasone	6.6 ± 1.5	<0.5	<0.5	100
XVI	11β,21-Dihydroxy-Δ4-17α-pregnene-3,20-dione	17α-Corticosterone	5.1 (4.2–6.1)	11 (8–14)	9.3 (7.5–11.5)	3.9 (2.8–5.5)
XVII	11-Keto-21-hydroxy-4-pregnene-3,20-dione	11-Keto-corticosterone	4.2 (3.0–5.9)	—	—	1.7 (1.2–2.3)
XVIII	21-Hydroxy-16α,17α-dimethyl-1,4-pregnadiene-3,20-dione	Δ_1-16α,17α-Dimethyl-deoxycorticosterone	2.6 (1.9–3.5)	—	—	140 (100–210)
XIX	9α-Fluoro-11β,16α,17α,21-tetrahydroxy-1,4-pregnadiene-3,20-dione	Triamcinolone acetonide	2.5 (1.8–3.5)	—	—	136 (99–188)
XX	17α,21-Dihydroxy-11α-methyl-4-pregnene-3,20-dione	11α-Methyl-cortisol	1.4 (1.1–1.8)	12 (9–15)	12 (10–19)	<0.5
XXI	11α,17α,21-Trihydroxy-1,4-pregnadiene-3,20-dione	Δ_1-11α-Hydroxy.cortexolone	<0.5	<0.5	<0.5	<0.5
XXII	6αFluoro-11β,17α,21-trihydroxy-4-pregnene-3,20-dione	6αFluoro-16-hydroxy-cortexolone	<0.5	<0.5	<0.5	20 (14–29)
XXIII	11β-Hydroxy-4-pregnene-3,20-dione-17-butanoate	21-Deoxycorticosterone-17-butanoate	<0.5	<0.5	<0.5	230 (170–230)
XXIV	11β,17α-Dihydroxy-21-methyl-1,4-pregnadiene-3,20-dione	21-Deoxy-21-methyl-prednisolone	<0.5	—	—	49 (36–68)
XXV	9α-Fluoro-11β,17α,21-trihydroxy-4-pregnene-3,20-dione	9α-Fluorocortisol	22 (18–28)	—	—	—
XXVI	9α-Fluoro-11β,17α,21,trihydroxy-1,4-pregnadiene-3,20 dione	9α-Fluoroprednisolone	21 (17–26)	—	—	—
XXVII	9α-Fluoro,17α,21-dihydroxy-1,4-pregnadiene-3,11,20 trione	9α-Fluorocortisone	12	—	—	—
XXVIII	11β,21-Dihydroxy-5α-pregnane-3,20 dione	5α-Dihydrocorticosterone	60 (49–74)	—	—	—
XXIX	11β,21-Dihydroxy-5β-pregnane-3,20 dione	5β-Dihydrocorticosterone	<0.5	—	—	—

*The parallel line assay of the plot ln $B/B_t - B$ versus the logarithm of the dose of competitor [44] was chosen to calculate the relative binding affinity RBA. †Mean ± SEM of 3 separate experiments. — is not determined. ‡Result of single experiment with 95% confidential limits as found in the parallel line assay.

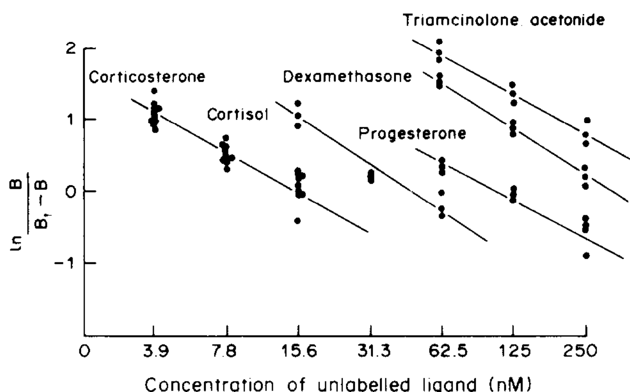

Fig. 1. Competitive binding of [^{3}H]B and various unlabeled ligands (B, cortisol, progesterone, DEX and triamcinolone acetonide) for the receptor system in hippocampal cytosol of the rat. The binding experiment was performed in the presence of a 100-fold excess of unlabeled RU 26988.

given in Table 2 (a similar competition was carried out as with the few examples given in Fig. 1), and compared with the values obtained under identical experimental conditions in thymic cytosol and in diluted blood plasma. The table shows that B has the highest relative affinity for these sites. Consequences of changes in the corticosterone structure for receptor binding in rat hippocampus can be derived from the data.

Some substituents, such as 5α-H, 9α-F, 17α-OH, 18-al reduce the affinity. A similar reduction in affinity occurs with the 1-en, 11-deoxy, 19-nor, 21-deoxy, structural modifications. Addition of 5β-H, 6α-F, 11α-CH_3, 11α-OH, 17-esters or 21-CH_3 are incompatible with the binding and give steroids with very low RBA values.

In contrast, the fluorosubstituents at position 9 and the introduction of a double bond at sites C_{1-2} lead to potent glucocorticoids and increase the affinity for the [^{3}H]DEX labeled sites in thymus. Binding constants for [^{3}H]DEX labeled sites in thymus are B_{max}: 3600 fmol/mg protein; K_d: 3.8 nM. No binding of [^{3}H]DEX occurs in the presence of RU 26988, which supports the evidence for the exclusive glucocorticoid properties of this ligand. Accordingly, these sites are also blocked by RU 26988 in the [^{3}H]B labeled cytosols.

Chemical changes leading to mineralocorticoids do not result in steroids with higher RBA values than B in the hippocampus. Although the mineralocorticoids display some affinity (cf ALDO: 11%; DOC: 33.5%), it is certainly not convincing evidence that the receptor sites labeled with [^{3}H]B in this study are solely mineralocorticoid sites. Rather, the presumptive mineralocorticoid sites may well coexist with the specific B receptor sites as pointed out in a previous study [6]. Proof of receptor heterogeneity could be achieved by isolation of the different receptor types by analytical chemical techniques. Progress in purification has been hampered, however, by instability and proteolysis of the brain corticoid receptors [32–34].

[^{3}H]B has the highest affinity to the receptor sites in the hippocampus, but also in blood plasma and in the thymus, when RU 26988 is included. The sites specifically labeled with [^{3}H]B in the hippocampus are not transcortin molecules. Only trace amounts of transcortin have been detected in brain tissue after extensive perfusion [35]. Furthermore, physicochemical properties and other characteristics, such as association of the corticosterone–receptor complex with cell nuclei allows a clear distinction with transcortin [36]. Moreover, although the order of RBA of some compounds is comparable, at many places there is a distinct difference (cf steroids IV, VIII, IX, X, XII, XIV). In contrast, the RBA values for the B binder in the thymus are quite similar with those observed in blood plasma. These molecules could well be transcortin or transcortin-like molecules that remain associated with the tissue, even after extensive perfusion [37, 38, 39].

In the present study we have attempted to select a series of ligands with a comparable or even more pronounced affinity to the B labeled receptor sites in rat hippocampus. However, B displayed the highest affinity of all steroids tested. This finding is consistent with the unique specificity of B in control of certain behavioral responses [17–20, 22], and serotonin synthesis [21, 40–42]. These indices for neurotransmission and behaviour were not affected by progesterone, DOC, DEX and ALDO. These steroids acted, however, as antagonist when administered prior to B [22, 41]. Since the present study and a previous one [6] have shown that these steroid hormones have some affinity to [^{3}H]B labeled receptor sites, the antagonism probably occurs via competitive binding at the receptor. The selective action of B in the hippocampus and the highest affinity displayed by this steroid to sites excluded by RU 26988, favour the existence of a population of receptor sites preferring the naturally occurring glucocorticoid of the rat. It is tempting to speculate that this neuronal localized population of receptor sites has its origin early in evolution, since B probably served also as sodium and potassium regulating hormone [43]. The glu-

cocorticoid receptor specifically blocked by RU 26988 and a separate population of high affinity mineralocorticoid sites (if present) may be later specializations in corticoid receptor phylogeny.

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