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INTERACTION OF ANTIGLUCOCORTICOID RU 486 WITH RAT KIDNEY GLUCOCORTICOID RECEPTOR

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SUMMARY: [^{3}H]RU 486 competes with dexamethasone for rat kidney glucocorticoid receptor (GR) occupancy in vitro, exhibiting a higher association constant for binding to GR than [^{3}H]dexamethasone. Unlike [^{3}H]dexamethasone-receptor complexes which dissociate rapidly at 37°C even in the presence of molybdate, [^{3}H]RU 486-receptor complexes remained more stable both in the presence and in the absence of molybdate. Interestingly, sulfhydryl reagents such as N-ethylmaleimide, iodoacetamide and tosyllysyl chloromethane at 5mM concentration almost completely inhibited binding of [^{3}H]dexamethasone to GR, whereas 20-30% binding to [^{3}H]RU 486 was inhibited by these reagents. [^{3}H]RU 486-receptor complexes readily undergo temperature-dependent activation in vitro as judged by their binding to DNA-cellulose. We propose that changes in binding affinity, stability and sulfhydryl reagent sensitivity between glucocorticoid agonist and antagonist may be due to subtle differences in the binding of the agonist and antagonist to the steroid binding domain of the receptor. This may have a direct relevance to the antiglucocorticoid properties of RU 486.

INTRODUCTION: RU 486 exhibits potent antiglucocorticoid activity both in vivo and in vitro without any observable agonist activity even at concentrations almost ten times those of dexamethasone (1). Besides antiglucocorticoid action, RU 486 also possesses potent antiprogestinic properties (2,3). Currently, considerable work is being carried out to understand the molecular mechanism(s) by which RU 486 exerts its potent antagonistic effects on various glucocorticoid responsive target tissues (4). It appears from these studies that [^{3}H]RU 486 binds with high affinity to glucocorticoid receptors in various target tissues such as liver, thymus and various regions of the brain (5,6,7). However, the precise molecular mechanism by which this compound antagonizes glucocorticoid action is yet to be delineated. Kidney is a well known

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glucocorticoid responsive tissue. In the present study, we have characterized the binding, activation and some physicochemical properties of the potent glucocorticoid antagonist RU 486 in rat kidney and compare it with the potent glucocorticoid agonist dexamethasone in order to understand the molecular mechanism by which RU 486 antagonizes glucocorticoid action.

MATERIALS AND METHODS: [6,7-^{3}H]Dexamethasone (44.1 Ci/mmol) was obtained from New England Nuclear. [^{3}H]RU 486 (Ref. 20063-194A), specific activity 50.6 Ci/mmol and unlabeled RU 486 were kindly supplied free of charge by Roussel-Uclaf, France. Unlabeled steroids were purchased from Sigma. All other reagents were of analytical grade.

Male Sprague-Dawley rats, 150-250g, were used. The rats were adrenalectomized bilaterally 3 to 6 days before use and were maintained on standard Purina rat chow, 0.9% NaCl solution (saline) and water ad libitum. According to institutional animal care and use committee guidelines, proper care was taken to minimize the pain during the surgical procedure and while killing the animals.

Animals were sacrificed by exposure to carbon dioxide. Kidney tissues were removed and homogenized (1:3 w/v) in 10mM Tris-HCl, 0.25M sucrose, pH 7.5 at 4°C. The cytosol was obtained after centrifugation at 105,000g for 60 min at 4°C. The cytosol was adjusted to about 10mg of protein per ml, and a final pH of 7.5 at 4°C, and used. Protein content was determined by the method of Lowry et al. (8). The cytosolic samples were incubated with 1-50nM of [^{3}H]dexamethasone or [^{3}H]RU 486 in the presence or absence of 1000-fold excess of unlabeled steroids. After incubation for 4 h at 0°C, the specific macromolecular binding was determined using the charcoal-dextran technique (9).

Denaturation studies were carried out by incubating renal cytosol for 4 h at 0°C with 10nM [^{3}H]RU 486 or [^{3}H]dexamethasone with or without 10mM sodium molybdate plus 5mM dithiothreitol. The triplicate samples were incubated at 35°C for various times, treated with charcoal-dextran and counted using Beckman Ready Solve - EP.

Activation studies were carried out by preincubating the cytosol with either [^{3}H]RU 486 or [^{3}H]dexamethasone for 4 h at 0°C with or without sodium molybdate. Portions of cytosol were then further incubated for 45 min at 25°C. 200μl samples were mixed with 100μl DNA-cellulose (1.0 mg native calf thymus DNA/ml cellulose). The samples were incubated for 60 min at 4°C with occasional stirring. The samples were washed thrice with 1 ml of 10mM Tris-0.25M sucrose buffer, pH 7.5 at 4°C, and the pellet was suspended overnight in the Beckman Ready Solve - EP solvent and counted as described before (9).

Tosyllysyl, iodoacetamide and N-ethylmaleimide were prepared in concentrated stock solution (100mM, pH 7.5) before use. The cytosol containing chemical reagents was incubated for 30 min at 0°C in the dark before addition of [^{3}H]steroids and specific binding was determined as described before.

DNA was measured according to the diphenylamine assay of Burton (10).

Sucrose density gradient analysis was performed using linear 5-20% sucrose gradients prepared in 10mM Tris buffer, pH 7.5 containing 30mM KCl (low salt gradients) or 300mM KCl (high salt gradients). Cytosol was equilibrated with 10nM of either [^{3}H]RU 486 or [^{3}H]dexamethasone for 4 h at 0°C and 400μl was layered on the top of the gradients after charcoal-dextran treatment. Samples were centrifuged for 18 h at 4°C in a Spinco SW rotor at 35,000 rpm. 8 drop

samples were collected with a Gilford Densiflow apparatus, starting from the top of the gradients. Bovine Serum albumin (4.6 S) and human gamma globulin (7.1 S) were used as references to estimate sedimentation coefficients (11).

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RESULTS: As determined from Scatchard plot analysis of data (not presented) [^{3}H]dexamethasone bound to kidney cytosolic GR with an apparent association constant of 2.3 x 10^8 M^{-1} at 0°C. [^{3}H]RU 486 binding to GR was about 2 times higher than that of dexamethasone (Ka = 4.8 x 10^8 M^{-1} at 0°C).

The rate of dissociation of [^{3}H]dexamethasone from the rat kidney cytosolic GR at 0°C was rather rapid with T ½ of 12 hours (Fig. 1) when compared to [^{3}H]RU 486 which dissociated with a T ½ of about 70 hours.

Data presented in Fig. 2 show that prebound kidney cytosolic [^{3}H]dexamethasone-receptor complex completely dissociated within 30 min at 35°C. Addition of 10mM molybdate plus 5mM dithiothreitol stabilized the

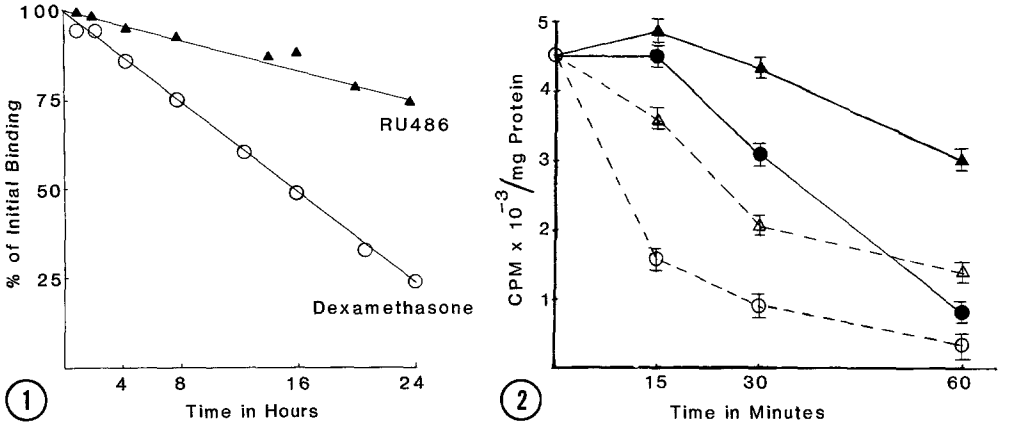

Fig. 1. DISSOCIATION RATE OF GLUCOCORTICOID AGONIST AND ANTAGONIST FROM RAT KIDNEY CYTOSOLIC GR.

Samples were incubated with 10nM concentration of [^{3}H]dexamethasone or [^{3}H]RU 486 at 0°C for 4 hours. A 1000-fold excess of the corresponding nonlabeled steroid was added at the end of the incubation. At different time intervals, triplicate aliquots were withdrawn and specifically bound radioactivity was determined. Results are averages of three determinations.

Fig. 2. STABILITY OF PREFORMED [^{3}H]DEXAMETHASONE OR [^{3}H]RU 486-RECEPTOR COMPLEX.

Samples were incubated in triplicate with 10nM of either [^{3}H]dexamethasone (O) or [^{3}H]RU 486 (Δ) alone or in the presence of 10mM sodium molybdate plus 5mM dithiothreitol (●,Dex) (▲,RU 486) for 4 h at 0°C. Aliquots were removed, incubated at 35°C and, after incubation for the times indicated, bound radioactivity was determined as described under Methods. Results are mean ± S.E. of three experiments done in triplicate.

[^{3}H]dexamethasone-receptor complex significantly. [^{3}H]RU 486-receptor complex, on the other hand, was considerably more stable under similar conditions. The stability of [^{3}H]RU 486-receptor complex was further enhanced by addition of molybdate and dithiothreitol; at up to 60 min of incubation at 35°C, only 50% degradation of the [^{3}H]RU 486-receptor complex was observed.

Steroid specificity of binding (Fig. 3) shows that [^{3}H]RU 486 was displaced in the order of RU 486 > corticosterone > dexamethasone > progesterone. 17β Estradiol and aldosterone displaced poorly with [^{3}H]RU 486.

Pretreatment of kidney cytosol with sulfhydryl blocking reagents such as iodoacetamide, tosyllysyl chloromethane or N-ethylmaleimide resulted in complete loss in subsequent [^{3}H]dexamethasone binding. Interestingly, only 20-30% loss in [^{3}H]RU 486 binding was observed under similar conditions (Fig. 4).

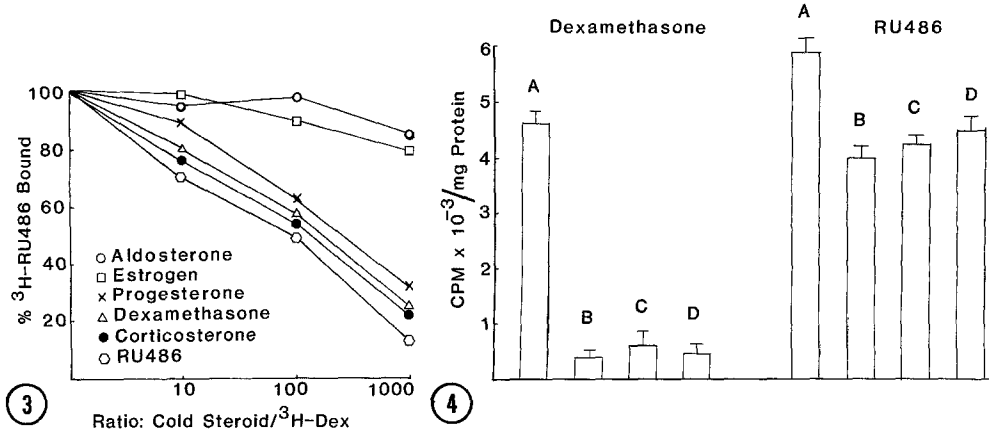

Fig. 3. STEROID SPECIFICITY OF ANTAGONIST BINDING.

Samples in triplicate were incubated with 10nM [^{3}H]RU 486 alone or in the presence of various concentrations of mentioned unlabeled steroids and specific bound radioactivity determined as described under Methods. Each point is the mean value of three experiments.

Fig. 4. EFFECT OF SULFHYDRYL BLOCKING REAGENTS ON RAT KIDNEY GR BINDING OF GLUCOCORTICOID AGONIST AND ANTAGONIST.

Samples were treated with 5mM concentration of iodoacetamide, N-ethylmaleimide or tosyllysyl chloromethane for 30 min at 0°C before 10nM of either [^{3}H]dexamethasone or [^{3}H]RU 486 was added. The samples were then incubated for 4 hours at 0°C and specific binding was determined as described in Methods. Results are mean ± S.E. of four determinations done in triplicate. Control (A), Iodoacetamide treated (B), Tosyllysyl Chloromethane treated (C) and N-ethylmaleimide treated (D).

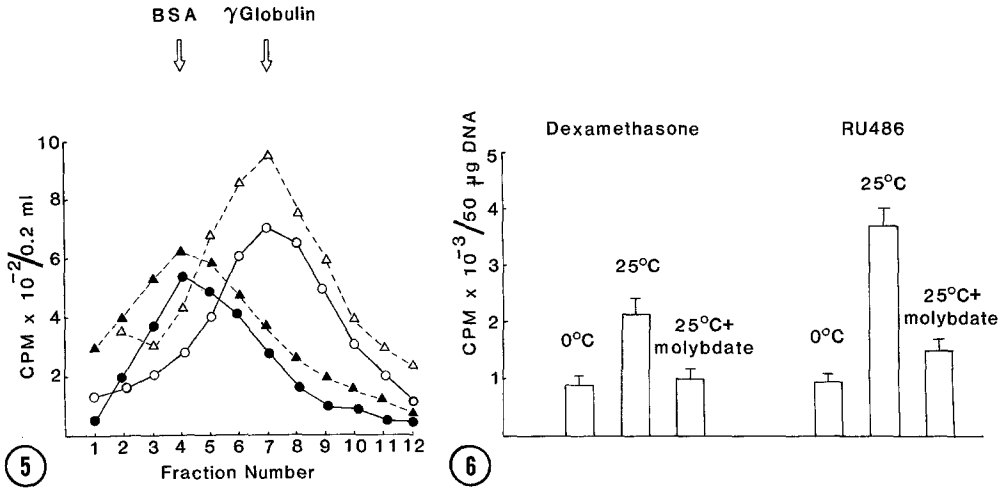

Fig. 5. SUCROSE DENSITY GRADIENT ANALYSIS OF THE RAT KIDNEY [^{3}H]RU 486-RECEPTOR COMPLEX.

Cytosol pretreated with 10nM of either [^{3}H]dexamethasone or [^{3}H]RU 486 at 0°C for 4 hours was charcoal-dextran treated and 400µl of supernatant sample was applied to low salt (30mM KCl) or high salt (300mM KCl) gradients. All other conditions are described in Methods. BSA = bovine serum albumin. Dex high salt (●), Dex low salt (○), RU 486 high salt (▲), RU 486 low salt (△).

Fig. 6. DNA-CELLULOSE BINDING OF GLUCOCORTICOID AGONIST OR ANTAGONIST-RECEPTOR COMPLEX.

Samples were preincubated at 0°C in presence of 10nM [^{3}H]dexamethasone or [^{3}H]RU 486. Samples were then incubated for 45 min at 25°C. DNA-cellulose bound radioactivity was determined as described in Methods. Each point is mean ± S.E. of four experiments done in triplicate.

Both the [^{3}H]RU 486-receptor complex and [^{3}H]dexamethasone-receptor complex sedimented at about 7 S in low ionic strength sucrose density gradients and at about 4 S in high ionic strength sucrose density gradients (Fig. 5).

Data presented in Fig. 6 show that kidney cytosolic [^{3}H]dexamethasone-receptor complex binding to DNA-cellulose was enhanced 2-fold after *in vitro* thermal activation at 25°C for 45 min, compared to control untreated sample. [^{3}H]RU 486-receptor complex was more readily activated as judged from a 3-4-fold increase in the binding to DNA-cellulose when compared to [^{3}H]dexamethasone. Addition of 10mM molybdate completely inhibited DNA-cellulose binding of the [^{3}H]dexamethasone-receptor complex. Interestingly, [^{3}H]RU 486-receptor complex binding to DNA-cellulose was only inhibited up to 60-70%.

DISCUSSION: The data obtained from rat kidney represent some similarities and differences to those reported from other rat tissues. For example, the high affinity binding of antiglucocorticoid RU 486 to rat kidney GR and its slower dissociation rate are similar to those reported for rat thymus (6) and in hepatoma cells (12) but differ from rat liver (5) where lower affinity as well as higher dissociation rate of [^{3}H]RU 486-receptor complex versus [^{3}H]dexamethasone-receptor complex is reported. Agarwal et al. (5) reported thermal activation of [^{3}H]RU 486-receptor complex in rat liver, while Philibert and Moguilewsky (6) observed impaired in vitro thermal activation of antagonist-receptor complex in rat thymus cytosol. Philibert and Moguilewsky (6) also noted lower affinity of [^{3}H]RU 486-receptor complex for DNA-cellulose and low level of specific in vitro retention of antagonist-receptor complex into purified nuclei of thymus when compared to agonist GR complex. These obvious inconsistencies in data reported from rat liver, kidney and thymus suggest that no generalization can be made from one tissue to the other in terms of either binding or activation properties of the glucocorticoid antagonist RU 486 and, even though overall mechanisms of the antagonism may remain the same from tissue to tissue, subtle differences may exist depending on the tissue to account for the observed tissue dependent changes in physicochemical properties of [^{3}H]RU 486 binding.

It is likely that the glucocorticoid antagonist properties of RU 486 may reside in its initial binding to the GR, activation, nuclear binding of the activated steroid-receptor complex or somewhere distal to nuclear binding. Overall, results obtained indicate that binding of RU 486 to the steroid binding domain of GR may be crucial to its antagonist properties. The presence of a bulky residue, in terms of the 11β-4-dimethyl-amino phenyl derivative in RU 486 nucleus, may modify the interaction of RU 486 with some of the amino acids that represent the steroid binding domain of the receptor in such a fashion that resulting conformational changes may lead to higher affinity and stability of the antagonist-receptor complex. However, unlike the agonist-receptor complex, the antagonist-receptor complex (even though capable of activation in vitro) may lack the ability to properly interact with the

regulatory region of the genes whose expression is regulated by glucocorticoids. The above hypothesis of differences in ligand structure between agonist and antagonist which may result in changes in its interaction with steroid binding domain of GR is supported by our observation that the glucocorticoid agonist dexamethasone and antagonist RU 486 display differences in their sensitivity toward sulfhydryl blocking reagents. This suggests that at least one -SH group required for agonist binding to the receptor may be different from the antagonist binding to the GR. Recent advances in cloning of GR and the availability of the newer probes to explore the steroid binding domain of the GR may precisely localize these changes at the molecular level.

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