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PHYSIOLOGICAL ACTION AND RECEPTOR BINDING OF A NEWLY SYNTHESIZED AND NOVEL ANTIGLUCOCORTICOID

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SUMMARY: RU 38486, a newly synthesized molecule, reversed glucocorticoid mediated enzyme induction and gluconeogenesis in the liver, and RNA synthesis in rat thymocytes. The transfer of radiolabelled dexamethasone from the cytoplasm to the nucleus was also opposed by RU 38486 in intact thymocytes. Although RU 38486 saturated the same molecular species of the receptor as the hormone in the liver, differences seemed to appear when thymus was taken into account. Along with the ongoing clinical trials, an important new tool thus appears at hand to understand and harness the molecular action of glucocorticoid hormones in mammalian systems.

INTRODUCTION: Substances that oppose the action of steroid hormones are important not only to understand the organization and expression of the complex mammalian genome but are also endowed with much clinical promise. Although antagonists for other classes of steroid hormones have been known and employed in medicine for some time now, specific anti-glucocorticoids have hitherto been wanting (reviews in 1-3). RU 38486 (11β-(4-dimethylaminophenyl)-17β-hydroxy,-17α-(prop-1-ynyl)-estra-4,9-dien-3-one) is a newly synthesized substance exhibiting potent anti-gestational and anti-glucocorticoid action (3,4). The tritiated derivative of this material has only recently been synthesized (3) and permits molecular analysis of the glucocorticoid hormone action in relation to receptor conformations in various target organs. The results are embodied in this report.

MATERIALS AND METHODS: Male, Wistar rats (150-250 g) were bilaterally adrenalectomized 5-7 days prior to use and maintained on laboratory pellet food and water ad libitum. Liver tryptophan pyrrolase (TP), tyrosine transaminase (TT) and glycogen levels were determined by well established procedures (5) used in our laboratory for nearly two decades (1,2,6,7). These are expressed

Abbreviations: Glucocorticoid Receptor = GR; Transcortin = T = Corticosteroid Binding Globulin = CBG; Tryptophan pyrrolase = TP; Tyrosine transminase = TT.

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as μM kynurenine/g liver/h, μg parahydroxyphenylpyruvic acid/mg liver/10 min, and mg %, respectively. The student's t test was used to assess significance.

RNA synthesis and nuclear transfer in thymocytes were quantitated by the technique described in detail earlier (8). Briefly, thymocytes from adrenalectomized rats (10^8 per assay tube), exhibiting >90% viability by the trypan blue test, were incubated in duplicate (37° C 2h) under CO_2 in R_7 medium containing either 5 x 10^{-8} M ^{3}H-dexamethasone alone or with 10^{-8} or 10^{-7} M RU 38486; vehicle and antagonist control tubes were also included. Aliquots of 2 x 10^{-7} cells were thereafter incubated with 2 μCi ^{3}H-uridine (60 min 37°C).

To determine nuclear transfer, the remainder of the original incubation mixture (8 x 10^7 cells) was homogenized in 15 mM Tris-HCl pH 7.8, 5 mM $MgCl_2$, 5 mM 6-mercaptoethanol,and centrifuged at 105,000 g at 4°C to obtain a clear cytosol. The pellet was washed twice with the above buffer containing 50 mM NaCl, 250 mM sucrose and 20% glycerin. Thereafter, the pellet was suspended in the Tris buffer containing 50 mM NaCl and 2.2 M sucrose and centrifuged at 25,000 rpm for 90 min at 4°C to obtain the nuclear pellet.

Both the nuclear and the cytosol fractions were incubated (10 min 4°C) with an equal volume of 0.5% charcoal dextran which was removed by centrifugation at 3000 rpm 20 min 4°C. The supernates were counted in Bray solution in a Packard Tricarb Scintillation spectrometer. Protein was quantitated by the Bradford method (8). The results are expressed as CPM/mg protein.

For determination of RNA, after incubation with tritiated uridine, the assay mixtures were treated with 10% TCA to remove protein and the pellets washed repeatedly with ethanol and ether to remove steroids. The RNA was then hydrolyzed by overnight incubation in presence of 0.3 M NaOH. The radioactivity was quantitated in Bray solution. DNA was quantitated by the well known GABA method (8) and the synthesis of RNA expressed as CPM/mg of cellular DNA.

For kinetics of association, 0.5 ml liver cytosol (in 0.01 M Tris-HCl pH 7.4) was incubated (60 min 4°C) with the desired tritiated material alone or in presence of an excess of cold steroid of choice. Free steroids were removed by further incubation (10 min 4°C) in presence of 0.5 ml activated charcoal (50 mg/ml) which was removed by centrifugation (3000 rpm 20 min 4°C). Aliquots of 0.5 ml were mixed with 10 ml ACS fluid (Amersham) and counted in the Packard spectrometer. Results are expressed as CPM/mg protein (Bradford method).

For chromatography, liver cytosol in the initial buffer was equilibrated (60 min 4°C) with the tritiated steroid of choice, charcoal treated, and then passed through glass wool to remover traces of charcoal. Rat blood serum was similarly equilibrated with ^{14}C-corticosterone as a marker in double labelled chromatography that we established (1,2,6,7); see also figure legends.

^{3}H-RU 38486 (Ref. 20063-194 A), specific activity 50.6 Ci/mM, was synthesized by Roussel-Uclaf,France and kindly supplied to us free of charge. 1,2,^{3}H-dexamethasone (Batch 19; 25 Ci/mM), 5,6,^{3}H-uridine (Batch 123, 43.6 Ci/mM) and 4-^{14}C-corticosterone (52 mCi/mM; batch 12) were purchased from Amersham, G.B. Corresponding radioinert steroids were provided by Roussel or Amersham, or purchased from Sigma, U.S.A. All reagents were high purity analytical grade, mostly from Merck.

RESULTS AND DISCUSSION: The physiological action of RU 38486 is presented in table 1. Dexamethasone induced ($p < 0.01$ vs control in all cases) liver TP, TT and glycogen within 4 h, as previously well established (3,5). Although by itself RU 38486 did not influence the endogenous levels of any of these parameters, this substance reversed the inductive effect of dexamethasone in a dose dependent manner.

Table 1. ANTI-INDUCIVE EFFECT OF RU 38486 ON SEVERAL LIVER FUNCTIONS

Treatment	Tryptophan Pyrrolase	Tyrosine Transaminase	Glycogen
1. Vehicle	1.23 ± 0.14	0.59 ± 0.13	4.87 ± 1.56
2. Dexamethasone	4.96 ± 0.42	3.72 ± 0.52	11.32 ± 3.71
3. RU 38486	1.07 ± 0.06	0.70 ± 0.04	4.90 ± 1.92
4. DEX + RU	3.75 ± 0.38	3.73 ± 0.49	12.53 ± 3.84
5. DEX + RU	1.76 ± 0.79	1.98 ± 0.27	4.49 ± 1.63

Dexamethasone (5 μg) and RU 38486 (5 mg for lines 3 & 5 and 0.5 mg for line 4) were given intraperitoneally 4 h prior to assay. Detailed account of the techniques has been published repeatedly (5-7).

Glucocorticoids exert a catabolic action in thymocytes, contrary to the anabolic action described above in the liver (3,5). Diminished macromolecular synthesis and thymus involution are commonly observed effects of glucocorticoid therapy (1,2,3,5). Since steroid hormone action in general is believed to require a prior transfer of bound hormone from the cytoplasm to the nucleus (9), we wished to analyze the influence of RU 38486 on the relationship between RNA synthesis and nuclear transfer of dexamethasone in thymocytes.

This was accomplished by studying simultaneously, in the same assay, the incorporation of ^{3}H-uridine into RNA and of the amount of ^{3}H-dexamethasone in the cytoplasm relative to that in the nucleus in presence of various concentrations of RU 38486. Data in table 2 show that just 5×10^{-8} dexamethasone abolished ^{3}H-uridine incorporation by >90% over that obtained with 10^{-7} M RU 38486 alone which did not differ from the vehicle control (not shown; see also (3)). However, just 10^{-8} M RU 38486 antagonized the effect of 5×10^{-8} dexamethasone, and the incorporation of ^{3}H-uridine increased to control level with 10^{-7} M RU 38486.

Data in table 2 also show that RU 38486 reversed, in a dose dependent manner, the quantity of ^{3}H-dexamethasone eluted from the nuclear pellet in these very thymocytes. In addition, the formation of cytoplasmic ^{3}H-dexamethasone receptor complexes, too, was essentially totally reversed when just 10^{-8} M RU 38486 competed with five times higher concentration of dexamethasone.

Table 2. EFFECT OF RU 38486 ON RNA SYNTHESIS AND THE GLUCOCRTICOID RECEPTOR IN RAT THYMOCYTES

Treatment	CPM/mg DNA		CPM/mg Protein
	^{3}H-Uridine	^{3}H-Dex (Nuclear)	^{3}H-Dex (Cytoplasmic)
RU 38486 (10^{-7}M)	1112	-	-
DEXAMETHASONE (5×10^{-7}M)	80	87710	1200
DEX + RU (10^{-8}M)	172	35489	461
DEX + RU (10^{-7}M)	1085	26351	452

Vehicle control did not differ from RU 38486 alone for RNA synthesis. No similar control could be tested for the cellular receptor since even a trace of hormone, required for receptor quantitation, leads to some nuclear transfer; lines 3 & 4 should therefore be compared to line 2. For details of techniques see Methods and (5).

Thus, RU 38486 seems to possess higher affinity for GR than dexamethasone but nuclear events require an excess of antagonist for still unclear reasons.

Since an antagonist for glucocorticoid action seems indeed to have been synthesized, it was of obvious interest therefore to analyze the nature of the cytoplasmic GR that is said to mediate steroid hormone action and that is known to exhibit molecular heterogeneity (10).

Data in figure 1 show that the binding of both ^{3}H-dexamethasone and ^{3}H-RU 38486 was biphasic, albeit the affinity of the latter was somewhat less than that of the hormone at lower dexamethasone concentrations. Thus, despite higher specific activity of the antagonist, the binding of the agonist was just as good between 10^{-9} and 10^{-8} M of either material. This conclusion, although different from that with thymocytes (table 2), must take into account the differences in the techniques used to quantitate cytoplasmic GR.

Data in figure 2 show that, taking into account their respective specific activities, both the agonist and the antagonist saturated liver GR to a similar extent. Furthermore, one thousandfold excess of the homologous, radioinert ligand reduced binding by >50% in each case. In cross competition studies, RU 38486 appeared as potent in displacing ^{3}H-dexamethasone from GR as dexamethasone in displacing ^{3}H-RU 38486. These suggest that both the agonist and the antagonist appear to be active via the intervention of a common receptor

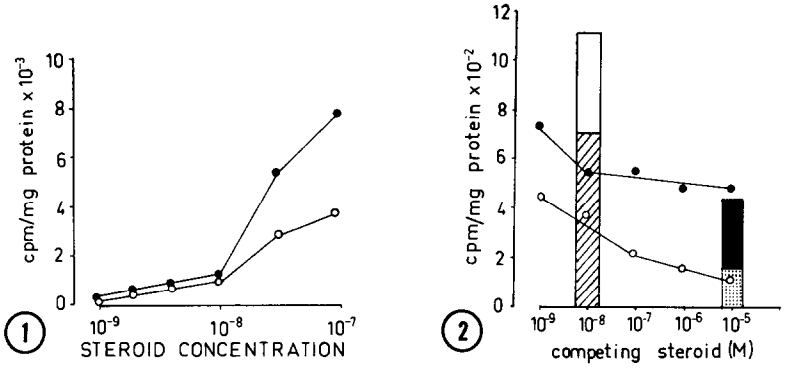

Fig. 1. KINETICS OF AGONIST AND ANTAGONIST BINDING TO THE LIVER GLUCOCORTICOID RECEPTOR.

Liver cytosol was incubated, in triplicate, in presencce of either ^{3}H-RU 38486 (●) or ^{3}H-dexamethasone (○), alone or with one thousandfold excess of the homologous, unlabelled molecule to account for nonspecific binding which was subtracted from the receptor bound steroid.

Fig. 2. CROSS COMPETITION BETWEEN GLUCOCORTICOID AGONIST AND ANTAGONIST FOR BINDING TO THE LIVER RECEPTOR.

Assay mixtures were incubated with 10^{-8} M of either ^{3}H-RU 38486 (□), ^{3}H-dexamethasone (▨), alone, or in presence of 10^{-5} M non-radioactive RU 38486 (■) or dexamethasone (▩). For cross competition, the desired amount of dexamethasone (●) or RU 38486 (○) was incubated with 10^{-8} M of the tritiated, heterologous steroid.

system. This is important since receptor heterogeneity is widely recognized (10) and an antagonist specific receptor is said to be present in selected targets of estradiol (11).

Data in figure 3 show that ^{3}H-RU 38486 saturated the GR_1 and GR_3 species of the GR complex, eluted in 0.001 M and 0.04 M phosphate (0.18 and 5.8 mili-Siemens), respectively, from the DEAE-cellulose-52 columns. These were clearly distinct from blood serum transcortin (T) eluted in 0.06 M phosphate (9.2 mS), in the double labelled chromatography according to our original method (6,7). Similar results were obtained with dexamethasone in place of RU 38486.

Molecular filtration on Ultrogel-ACA-44 columns, too, revealed a single peak of bound ^{3}H-RU 38486, excluded before transcortin, in the double labelled chromatography as per our original technique (6,7). Again, this was similar to dexamthasone labelled profiles (7,10,12) (Fig. 4).

In conclusion, RU 38486 seems to be an excellent tool to elucidate the molecular nature of both the anabolic and catabolic action of glucocorticoid hormones. Since both the agonist and the antagonist saturate the same species

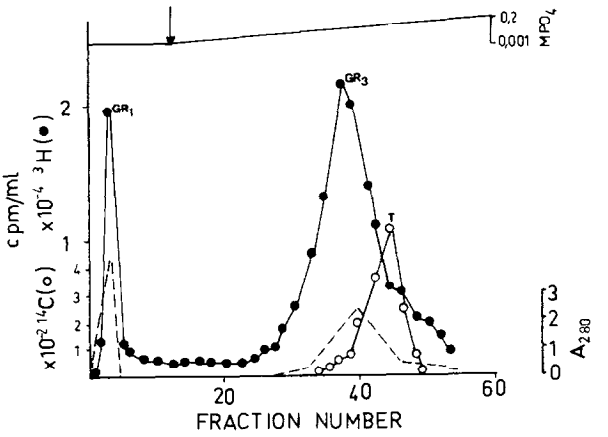

Fig. 3. ION EXCHANGE SEPARATION OF ^{3}H-RU 38486 BINDERS IN RAT LIVER.

4 ml liver cytosol in 0.001 M phosphate buffer pH 7.5 was incubated with 10^{-7} M ^{3}H-RU 38486 (●); 2 ml rat blood serum was incubated with 0.5 µCi ^{14}C-corticosterone (○). After 60 min at 4°C, both samples were treated with an equal volume of charcoal (50 mg/ml) as in Methods. The samples were thereafter mixed and loaded onto a column (1 x 25 cm) of DEAE-cellulose-52 (Whatman). After an initial prewash with 0.001 M phosphate, protein elution was begun (at arrow in the figure) with a linear gradient between 60 ml each of 0.001 M and 0.02 M phosphate. 1 ml samples were counted in ACS and the absorbance was determined manually (----) as in (6,7).

of the GR complex, extrapolations from one steroid system to another are hazardous when one considers that an antagonist specific species is present in the estradiol receptor system (11). The antagonist action is more potent than cortexolone which exhibits weak antiglucocorticoid action in only selected tissues (1-3,8). In addition, being devoid of any inherent agonist or antagonist activity, RU 38486 fulfills the definition of an "ideal" antagonist

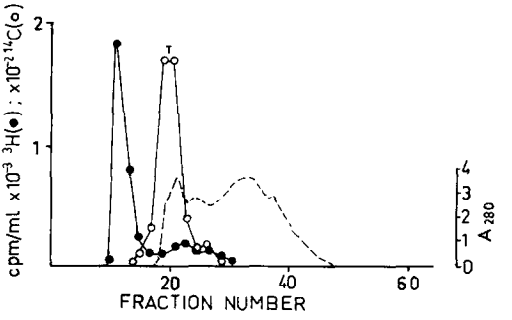

Fig. 4. MOLECULAR FILTRATION OF ^{3}H-RU 38486 BINDERS IN RAT LIVER.

2 ml liver cytosol and 1 ml serum were equilibrated with ^{3}H-RU 38486 (●) and ^{14}C-corticosterone (○), respectively, as in legend to Fig. 3. Samples were loaded onto 1 x 130 cm columns of Ultrogel-ACA-44 (Pharmacia) equilibrated and eluted with 0.01 M phosphate containing 0.1 M NaCl. Radioactivity and absorbance (----) were assessed as for Fig. 3 and (6,7).

more truly than other materials currently in vogue in contemporary endocrinology. Finally, the nature of receptor activation and acceptor function (9,13), prerequisites for steroid hormone action in general, can at long last be analyzed more precisely in an effort to dissect hormone specific events from those that merely follow the cascade of intracellular events.

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