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DIFFERENT MECHANISMS FOR THE RECEPTOR MEDIATED ANTIMINERALOCORTICOID ACTION OF TWO NEW SPIROLACTONE DERIVATIVES

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SUMMARY: The introduction of a methoxycarbonyl residue in position 7 of spirolactone produced a molecule (ZK 91587) that exhibited dramatically increased affinity for the rat renal mineralocorticoid receptor (MR) that was lacking in RU 26752 with a propyl group in this same 7 position. The binding of ^{3}H-ZK 91587 was specific to MR in renal cytosol and was not obtained with either serum or cytosol from non-target organs such as liver and lung. The RU 26752-receptor complex was more unstable than aldosterone-MR complex at 35° C but underwent complete thermal activation on DNA cellulose. Contrarily, ZK 91587 did not permit thermal activation at all and also rendered the MR highly labile at 35° C. Unactivated aldosterone and RU 26752-MR complexes sedimented largely in the 7 S region during sucrose density gradient centrifugation, and this shifted to 4 S following thermal activation. Paradoxically, under these very conditions, the molybdate stabilized, nonactivated, ZK 91587-MR complex was distributed almost equally into 7 S and 4 S regions which was not altered further by the activation process. If it is assumed that ZK 91587 exerts an antagonist action by inhibiting MR activation, or by MR labilization at body temperature, RU 26752 would seem to act at a step beyond the activation process. These form new tools to dissect receptor structure and function and necessitate a reevaluation of current notions regarding hormone action.

INTRODUCTION: Adrenocortical hormones are generally believed to act by saturating the specific receptor in the cytoplasm of the target organ, followed by activation and transfer of the complex to the nuclear compartment (1-3). Antagonists are supposed to interfere with any or all of these processes but furthermore saturate sites

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Abbreviations:

Aldosterone = 11β,21-dihydroxy-3,20-dioxopregn-4-ene-18-al.
RU 26752 = 7α-propyl-3-oxo-17α-pregn-4-ene-21,17-carbolactone.
ZK 91587 = 7α-methoxycarbonyl-15β,16β-methylene-3-oxo-17α-pregn-4-ene-21,17-carbolactone.

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with no affinity for natural mineralocorticoids (3-6). Two newly synthesized derivatives of spirolactone made available to us revealed unexpected differences in the manner in which they modulate receptor mediated processes. These studies challenge the generalizations usually entertained in the design and strategy of hormone antagonists.

MATERIALS AND METHODS: Male, Wistar rats (200-250 g) were adrenalectomized bilaterally under ether anesthesia and maintained on pellet food and water in a climate controlled animal house. Animals were sacrificed 5-7 days after surgery, the organs were liberally perfused with the initial buffer, and a lipid free cytosol was obtained after centrifugation at 105,000 g for 60 min at 4° C.

For binding and competition studies, 0.2 ml cytosol, in 0.01 M Tris-HCl, pH 7.4, was incubated for 120 min at 2-4° C with the desired amount of the tritiated steroid alone or in presence of an excess of a non-radioactive steroid of choice. To remove free radioactivity, an additional incubation was permitted for 10 min at 2-4° C in presence of 0.2 ml of 2.5% activated charcoal-0.25% dextran T-70 (Sigma) which was finally sedimented at 3000 g (10 min 4° C). The supernatant fluid was quantitatively transferred to scintillation vials and counted in presence of 10 ml ACS (Amersham) in a Packard Spectrometer equipped for background and quench correction. The amount bound was calculated as CPM/mg protein (Bradford method), as described before (5,6).

For denaturation studies, renal cytosol was incubated with 10 nM tritiated steroid as above and left at 35° C for various periods of time immediately prior to the charcoal treatment (5,6). Bound radioactivity was calculated as CPM/mg protein (5,6).

Activation was assessed by incubation at 25° C for 45 min, after the 2 h equilibration with 10 nM tritiated steroid at 4° C. 200 µl samples were thereafter mixed with 100 µl DNA-cellulose (1.4 mg calf thymus DNA/ml cellulose) and incubated at 4° C with occasional agitation. After three washes with 1 ml Tris buffer, the samples were suspended in the ACS scintillation fluid overnight and then counted as above (5,6).

For sedimentation analysis, 5-20% linear sucrose gradients were prepared in 10 mM Tris buffer, pH 7.5, containing 30 mM KCl (low salt gradient). Renal cytosol in this same buffer was incubated (2 h 4° C) with 10 nM tritiated steroid alone or with 500 fold excess of cold, homologous steroid to assess specificity. After an additional incubation (45 min 20° C) in the presence or absence of 10 mM sodium molybdate, all samples were charcoal treated as above to remove unbound radioactivity and 0.4 ml aliquots were layered onto the gradients, precooled at 4° C. Following centrifugation for 18 h at 4° C in a Spinco SW rotor at 40,000 rpm, samples of 8 drops were collected with a Gilford Densiflow apparatus. Charcoal resistant radioactivity was thereafter estimated in 5 ml scintillation fluid. Bovine serum albumin (4.6 S) and human gamma globulin (7.1 S) were used as references to estimate sedimentation coefficients (6,7).

^{3}H-RU 26752 (50 Ci/mM; lot X3025A) and the corresponding radioinert steroid (lot 7) were kindly furnished by Dr. D. Philibert, Roussel-Uclaf, Romainville). ^{3}H-ZK 91587 (lot ES 2096-2; 87 Ci/mM)

and the corresponding cold steroid were kindly provided by Dr. W. Losert, Schering, Germany. 1,2,^{3}H-aldosterone (45 Ci/mM; batch 57) was purchased from Amersham, U. K. Radiochemical purity exceeded 97% in thin layer chromatography for all steroids.

These studies were aided by UER Broussais, Université Pierre et Marie Curie (70 51 R-12), Paris, and by the American Heart Association, Virginia Affiliate.

RESULTS AND DISCUSSION: Data in Fig. 1 show that the two derivatives of spirolactone differ substantially in the occupancy of the renal mineralocorticoid receptor (MR). ZK 91587-MR complex formation reached a maximum within the first 30-60 min and remained unaltered for at least 8 h thereafter. By contrast, RU 26752 labelled renal MR maximally only after 2 h and the antagonist began to dissociate soon after. In other words, the chemical nature of the substitution in position 7 of the spirolactone ring was directly related to the affinity for MR. In other experiments, specifically bound radioactivity could not be observed when liver or lung, that are not target organs for mineralocorticoids, were used in place of the kidney (data not shown). Similarly, neither antagonist was able to label serum transcortin.

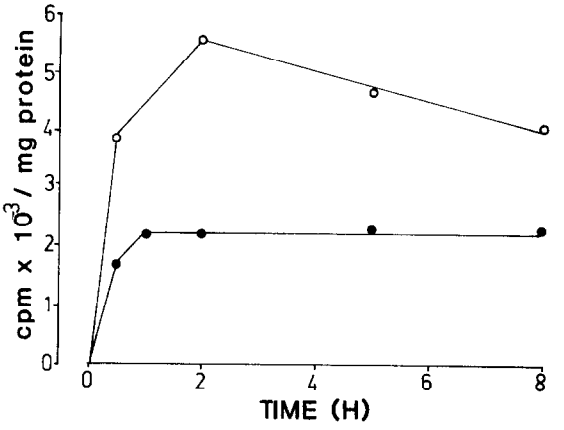

Fig. 1. VARYING AFFINITIES OF TWO SPIROLACTONE DERIVATIVES FOR THE MINERALOCORTICOID RECEPTOR IN RAT KIDNEY.

Renal cytosol was incubated with 100 nM of either ^{3}H-RU 26752 (O) or ^{3}H-ZK 91587 (●), alone or with 1000 fold excess of the homologous, unlabelled steroid to account for the nonspecific binding which was subtracted from the former to obtain antagonists specifically bound to the receptor. All determinations are average of three concurrent samples.

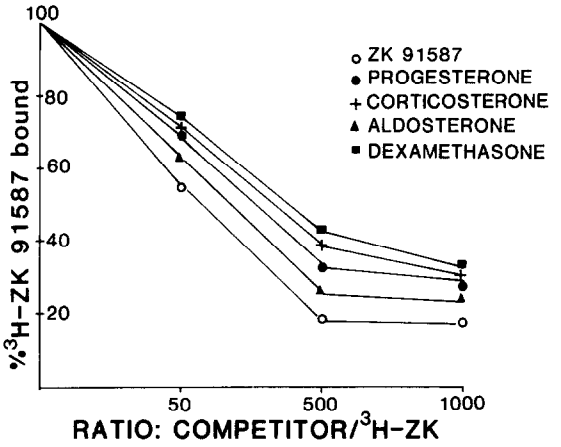

Fig. 2. EVIDENCE FOR THE SPECIFICITY OF ANTAGONIST BINDING TO THE MINERALOCORTICOID RECEPTOR.

Kidney cytosol incubated with 20 nM ^{3}H-ZK 91587 alone (O) is expressed as 100% binding, after removal of free radioactivity. Varying concentrations of a battery of radioinert steroids were added, separately, during incubation with the antimineralocorticoid to assess % displacement of ^{3}H-ZK 91587. All values are an average of three concurrent determinations.

The specificity of binding was further confirmed in studies where ^{3}H-ZK 91587 was competed with increasing concentrations of a number of nonradioactive ligands. Data in Fig. 2 show that bound antagonist, expressed as 100% in absence of any competing steroid, was displaced in the order: ZK 91587 > aldosterone > progesterone > corticosterone

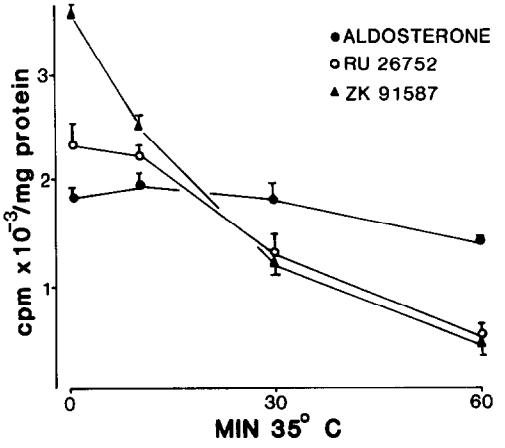

Fig. 3. DENATURATION OF RENAL MINERALOCORTICOID RECEPTOR DEPENDS UPON THE NATURE OF THE OCCUPYING STEROID.

Renal cytosol was incubated, in triplicate, with 10 nM of either ^{3}H-aldosterone (●), ^{3}H-RU 26752 (O), or ^{3}H-ZK 91587 (▲). All samples were brought to 10 mM sodium molybdate and left at 35° C for the indicated lengths of time. Remaining radioactivity is shown ± the standard error.

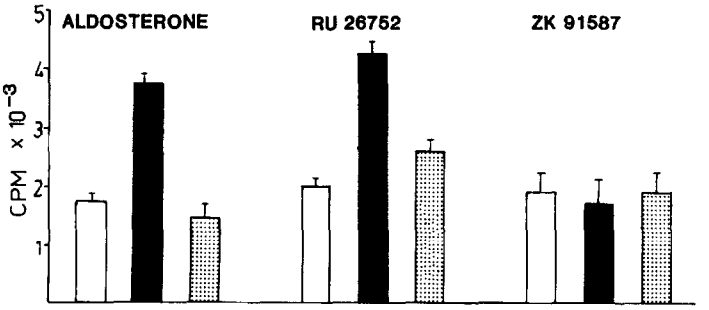

Fig. 4. PARADOXICAL DIFFERENCES IN THE ANTAGONIST DEPENDENT ACTIVATION OF THE MINERALOCORTICOID RECEPTOR.

Following 2 h equilibration at 4° C in presence of 10 nM tritiated steroid, activation was accomplished by additional incubation for 45 min at 25° C. Radioactivity bound to DNA-cellulose was finally assessed as in the Methods section. Each point is an average of three experiments, in triplicate, ± the standard error. (□) 4° C control; 25° C without (■) or with (▥) molybdate.

> dexamethasone. This limited displacement with dexamethasone would also confirm that ZK 91587 is not saturating the glucocorticoid receptor. Some competition with progesterone may seem surprising at first but it must be remembered that progestins are potent aldosterone antagonists and saturate a distinct subspecies of MR (8,9). Similar results were observed earlier with ^{3}H-RU 26752 (10).

Data in Fig. 3 show that the molybdate stabilized MR was largely stable at 35° C for at least 60 min in presence of aldosterone. Very rapid initial denaturation of MR was however observed with ZK 91587 and 90% of either antagonist dissociated by 60 min under these conditions, again confirming the importance of the 7α substitution.

As further evidence for differences in the action of these two antihormones, data in Fig. 4 show that RU 26752-MR complex could be heat activated, assessed by binding to DNA cellulose, just as well as the aldosterone-MR complex. By contrast, ZK 91587 did not permit thermal activation at all under these very conditions.

In our original analysis of the mineralocorticoid receptor on sucrose gradients, data in Fig. 5a show that the unactivated, molybdate stabilized, RU 26752-MR complex sedimented in the 7 S region in low salt buffer, as with the glucocorticoids (11). The antihormone-

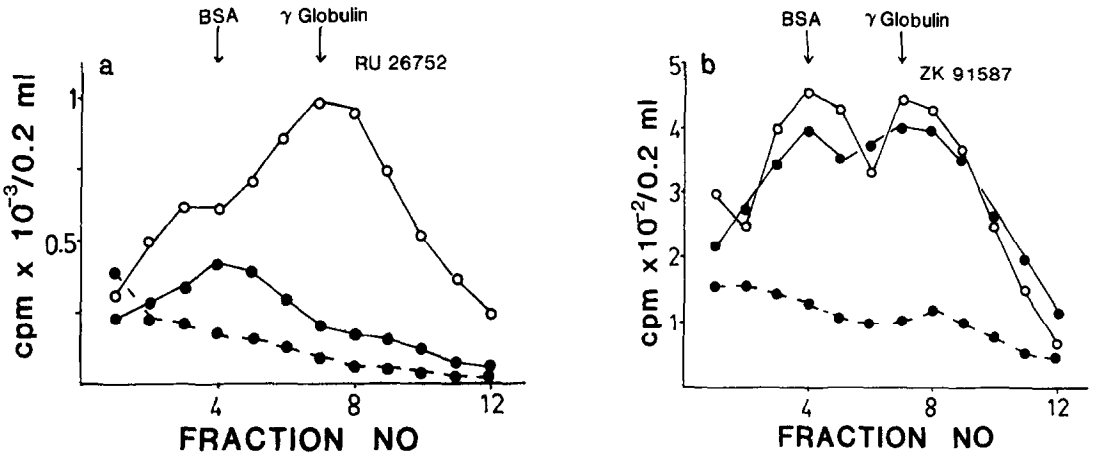

Fig. 5. ANALYSIS OF THE MINERALOCORTICOID RECEPTOR ACTIVATION BY SUCROSE DENSITY GRADIENT CENTRIFUGATION.

Cytosol was incubated with 10 nM tritiated steroid alone (○) or with 500 fold excess of the cold homologue (--●--). Activation was performed as for Fig. 4 without (●) or with (○) 10 mM molybdate. (a) RU 26752; (b) ZK 91587; BSA = bovine serum albumin.

MR complex shifted to the 4 S region following thermal activation in absence of molybdate (Fig. 5a), as with the agonist aldosterone (not shown). Surprisingly, the nonactivated, molybdate stabilized, ZK 91587-MR complex in low salt buffer showed two peaks of equal magnitude in the 7 S and the 4 S regions and thermal activation did not alter this profile any further (Fig. 5b). The mineralocorticoid receptor specificity was confirmed in all these cases by complete elimination of binding in presence of a 500 fold excess of the corresponding radioinert hormone or antihormone (Fig. 5a,b).

Data presented here permit a number of important conclusions. The introduction of a propyl residue in the 7 position of spirolactone confers a specificity for the MR in RU 26752, without affecting the ability of the receptor to undergo activation. Contrarily, when a methoxycarbonyl residue is introduced in this same 7 position, the affinity for the unactivated MR at 4° C increases dramatically but the ZK 91587-MR complex then assumes a conformation that is highly unstable at 35° C and that can not be heat activated. Therefore, neither of these properties of the receptor can be used per se to

screen agonists from antagonists. Such differences may be uniquely important in the design of derivatives for the genesis of receptor variants endowed with the desired physiological function, more so in view of the clinical application of spirolactones.

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