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ACTIVATION OF MINERALOCORTICOID AGONIST AND ANTAGONIST SPECIFIC RECEPTORS FROM RAT KIDNEY

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SUMMARY: The kinetics of saturation, as well as of denaturation, confirm the existence of two distinct mineralocorticoid receptor populations one each for the agonist aldosterone (MR_2) and the antagonist RU 26752 (MR_3) in rat kidney. Receptor activation in vitro was dependent upon the buffer, progressed just as well in the presence of the agonist and the antagonist, and was inhibited by molybdate. These necessitate a reassessment of both the importance of receptor activation in vitro and its possible contribution to hormone action in vivo.

INTRODUCTION: It is generally accepted that the initial step in adrenocorticoid hormone action consists of the formation of a complex between the ligand and its specific cytoplasmic receptor (1,2). The "activation" (3) of this hormone-receptor complex leads to nuclear transfer, and eventual amplification of target specific genes (4,5). Hormone antagonists either compete with the agonist for receptor occupancy and/or interfere with the activation process (6). Availability of synthetic derivatives in tritiated form has already allowed the discovery of antagonist specific binders in both the estrogen receptor (7) and the mineralocorticoid receptor (8) populations, contrary to the classical view where all ligands would compete for the one and the same receptor species. The evidence presented here challenges the notion that antagonists exert their action via interference of activation of the mineralocorticoid receptor (MR), and furthermore questions the relevance of this phenomenon in vivo.

MATERIALS AND METHODS: Male, Wistar rats (150-200 g) were bilaterally adrenalectomized 5-7 days prior to organ ablation and maintained on laboratory pellet food and water ad libitum. Animals were sacrificed under ether anaesthe-

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sia by exsanguination, the organs were perfused with the initial buffer, and a lipid free cytosol was prepared by centrifugation at 105,000 g.

Binding and denaturation studies were performed on 0.5 ml aliquots of cytosol (0.01 M Tris-HCl, pH 7.4) incubated for 120 min with the desired quantity of the steroid at 4°C. Free radioactivity was removed by further incubation (10 min 4°C) in presence of 0.5 ml of 2.5% charcoal-0.25% Dextran, followed by centrifugation (1000 g 10 min 4°C). For denaturation studies, the steroid-receptor complexes were incubated at 35°C, for varying lengths of time, just prior to the charcoal treatment. Aliquots of 0.5 ml were counted in the ACS fluid (Amersham) in all cases.

Activation was accomplished by heating the cytosol at 25°C for 45 min, following the 2 h equilibration at 4°C, either in presence or absence of 10 mM sodium molybdate. 200 µl samples were mixed with 100 µl of DNA-cellulose (1.4 mg native calf thymus DNA/ml cellulose), and incubated for 45 min at 4°C with occasional agitation. The samples were washed thrice with 1 ml Tris buffer and the pellet was suspended overnight in the scintillation fluid prior to counting, as described before (3).

For chromatography, the cytosol in 0.002 M phosphate buffer, pH 7.5, was incubated with 100 nM tritiated RU 26752 and charcoal treated (50 mg powder per ml cytosol). Activation was accomplished as above when necessary and the cytosol was then brought to 20 mM with sodium molybdate to arrest further MR transformation. Rat blood serum was incubated with 0.25 µCi of ^{14}C-corticosterone (60 min 4°c), charcoal treated (50 mg powder/ml), and finally mixed with the cytosol just prior to chromatography on DEAE-cellulose, described previously in detail (1,2,8). Briefly, 2 ml cytosol and 1 ml serum was loaded on to DE-52 (Whatman) columns (0.5 x 25 cm) equilibrated with 0.002 M phosphate. After passage of 30 ml of this intial buffer, a linear gradient was begun with 30 ml each of the 0.002 M and 0.2 M phoshate pH 7.5, at 4°C at a flow rate of 30 ml/h. Fractions of 1 ml were counted in the ACS fluid.

Radioactivity was measured by mixing upto 1 ml of the aqueous sample with 10 ml ACS fluid and corrections for quenching, background, and spill of ^{14}C into the tritium channel in double labelled studies, were made routinely (1,2,8).

^{3}H-RU 26752 (50 Ci/mM; reference X3025A), and the corresponding cold steroid, were kindly provided by Roussel-Uclaf, France. 4-^{14}C-corticosterone (52 mCi/mM; batch 12) and 1,2,^{3}H-aldosterone (45 Ci/mM; batch 34) were purchased from Amersham, G.B. The radiochemical purity exceeded 97% in all cases. All other chemicals were high purity reagents, mostly from Merck. Animals were purchased from IFFA-Credo, France.

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RESULTS AND DISCUSSION: Data presented in Fig. 1 confirm and extend earlier observations, and show that the kinetics of receptor saturation in rat kidney cytosol depends upon the nature of the ligand. Aldosterone-MR complex formation reached a maximum by 60 min at 4°C and remained constant until 8 h thereafter. Receptor-RU 26752 (7 alpha-(Acetylthio)-17 alpha-hydroxy-3-oxo-pregn-4-ene-21 carboxylic acid gamma-lactone 7 acetate) complex, however,

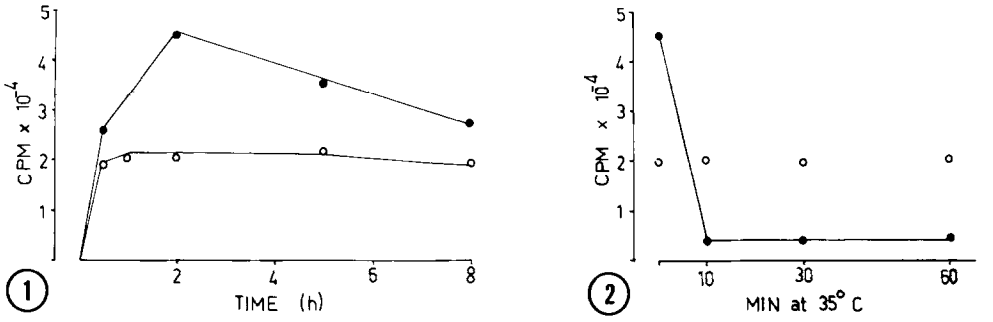

Fig. 1. Different Saturation Characterstics of the Agonist vs the Antagonist Bound Receptors in Rat Kidney.

Renal cytosol was incubated with 100 nM of either ^{3}H-RU 26752 (●) or ^{3}H-Aldosterone (○). At the indicated times periods thereafter, 500 µl samples in duplicate were processed for determination of bound radioactivity.

Fig. 2. Denaturation of the Mineralocorticoid Receptor in Rat Kidney Proceeds Faster with the Antagonist than with the Agonist.

Renal cytosol was incubated (2 h 4°C) with 100 nM of either ^{3}H-RU 26752 (●) or ^{3}H-Aldosterone (○). Samples, in duplicate, were then left at 35°C for various periods of time, charcoal treated, and counted in the ACS scintillation fluid.

attained a maximum only after 2 h at 4°C and started to decline progressively soon after. As compared to the agonist, the receptor-antagonist association was therefore definitely slower, and dissociation clearly faster, suggesting that two distinct vector populations are involved. In addition, despite comparable specific activities, RU 26752 was saturating a more abundant MR species, albeit with lower affinity, as compared to aldosterone. These differences became dramatic when non-saturating levels (10 nM) of the antagonist were employed (not shown) where the relative abundance of the agonist vs the antagonist specific vectors was the reverse of that seen with 100 nM steroid concentrations (Fig. 1).

Denaturation assays shown in Fig. 2 confirm these inferences. Although the aldosterone-MR complex was stable at 35°C for at least 60 min, 90% of RU 26752 dissociated from the receptor within 10 min after exposure to 35°C, and the remainder stayed unchanged therefore, confirming receptor heterogeneity (reviews in 9).

Data in Fig. 3 show that incubation at 25°C for 45 min was very effective in activating the receptor, as evident by binding of the ^{3}H-aldosterone-MR to DNA-cellulose that could only be eluted by high salt. Absence of heat activation prior to exposure to DNA-cellulose, or presence of molybdate during incu-

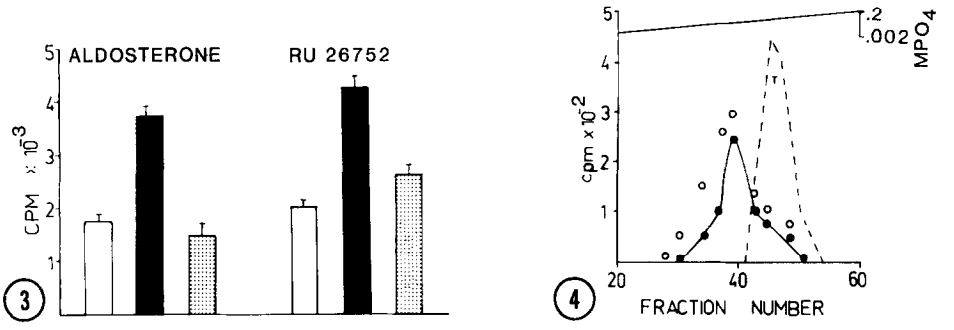

Fig. 3. Activation of the Mineralocorticoid Receptor in Presence of either the Agonist or the Antagonist.

Kidney cytosol was incubated (2 h 4°C) with 30 nM of either ^{3}H-RU 26752 or ^{3}H-aldosterone. Aliquots of samples were then incubated with or without 10 mM sodium molybdate for 45 min at 25°C. 200 µl of the activated and unactivated samples were added to 100 µl DNA-cellulose to assess bound radioactivity (3). Each point represents the average of three experiments in triplicate each time; (□) 4°Control; 25°C without (■) or with (⊡) molybdate.

Fig. 4. Ion Exchange Separation of RU 26752 Bound Mineralocorticoid Receptor from Rat Kidney.

4 ml renal cytosol was equilibrated (2 h 4°C) with 100 nM ^{3}H-RU 26752 and charcoal treated to remove free radioactivity. 2 ml samples each were then incubated for 45 min at either 4°C (●) or 25°C (○). The cytosol was fractionated, separately, on DEAE-cellulose with the aid of a phosphate gradient (8). 1 ml rat blood serum, labelled with ^{14}C-corticosterone, was used as a position marker in each case. Only the relevant portion of the chromatogram has been shown.

bation at 25°C, did not permit MR-agonist activation. Data in Fig. 3 also show that MR activation progressed equally well when RU 26752 was used to saturate the receptor in place of aldosterone. Thus, activation of the agonist (MR_2) or the antagonist (MR_3) specific receptor per se is not a causal event in the action of mineralocorticoid hormones.

Much has been said about the choice of buffer systems in receptor analysis (10). In these earlier investigations it was clearly established that phosphate buffer, the natural ion in the cell, is by far the most suitable to study the receptor in vitro (10), since it inhibits a variety of processes that tend to destabilize and fragment the receptor (reviews in 9,10). Data in Fig. 4 confirm the previous RU 26752 profile on DEAE-cellulose columns. The elution position of RU 26752-MR complex (MR_3) is clearly distinct from that of transcortin (T) in the double labelled chromatography established earlier for precise receptor location; aldosterone eluted in the MR_2 position under these conditions (7). When heated RU 26752-MR was similarly fractionated with the aid of phosphate gradient

on DE-52 columns, the elution profile was almost superimposable on the unheated antagonist-receptor profile, contrary to fractionation with KCl or NaCl containing Tris buffers where activation decreases receptor affinity for DEAE-cellulose (11). In other words, activation may have little relevance in vivo where indigenous phosphate ions would not permit receptor transformations, inducible in vitro by a whole array of manipulations, albeit in non-phosphate buffers (3,11).

The results described here assume importance for several reasons. First, they establish the irrlevance of activation as a possible causal event in receptor mediated hormone action, especially in vivo. In any event it is not possible to employ the activation process to screen out agonist from antagonist or inactive steroids. Second, the spcificity of ligands for their respective receptor moities makes it possible to visualize MR purification by methods that require activation as an obligatory step for affinity chromatography on DNA cellulose (12). Third, phosphate buffers may be used most effectively to distinguish those events that form solely an academic exercise in vitro from those that are probably relevant to receptor mediated hormone action in vivo. Finally, the extensive use of spirolactone derivatives in various clinical situations (13) makes it imperative that renal mineralocorticoid receptor populations be characterized more carefully.

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