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EVIDENCE FOR AN ANTAGONIST SPECIFIC RECEPTOR THAT DOES NOT BIND MINERALOCORTICOID AGONISTS

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SUMMARY: Kinetics of association - dissociation, competition and chromatography on two different resins, all revealed the presence of a new binding site which: specifically accepts 7-alpha-propyl spirolactone (^{3}H-RU-26752), has little affinity for aldosterone, is present only in the target tissue (rat kidney), and is wanting in a non-target organ (liver). The presence of such sites could explain syndromes of mineralocorticoid excess where even trace amounts of an unusual aldosterone analogue, with little affinity for the classical mineralocorticoid receptor, can nevertheless produce hypertension through the intervention of an entirely new and abundant receptor system. This new molecule thus forms a novel tool to understand the nature and function of the soluble mineralocorticoid receptor in target organs.

INTRODUCTION: Spirolactone (7 alpha-(Acetylthio)-17 alpha-hydroxy-3-oxopregn-4-ene-21 carboxylic acid gamma-lactone 7 acetate) derivatives antagonize the effects of mineralocorticoids in target tissues and are commonly employed clinically to treat various syndromes of hormone excess such as Conn's disease, hypertension (1,2). Since, in excess, they inhibit the binding of tritiated aldosterone to kidney cytosol in vitro, it is generally assumed that the antagonistic action of spirolactones proceeds through the specific mineralocorticoid receptor (MR) in the target tissue (3-5). Recently, the availability of the 7-alpha propyl derivative (^{3}H-RU-26752) of spirolactone, procured through special synthesis, has provided a new tool to investigate these relationships. We report here a specific anti-mineralocorticoid binding site in rat kidney which is physicochemically distinct from the conventional MR in various target organs.

MATERIALS AND METHODS: Male, Wistar rats (150-200 g) were bilaterally adrenalectomized 5-7 days prior to use and maintained on laboratory pellet food and water ad libitum. Animals were sacrificed by exsanguination, the organs were perfused with the initial buffer, and a clear cytosol prepared by centrifugation at 105,000 g.

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For binding and competition studies, 0.5 ml 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 non-radioactive 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 of ACS fluid (Amersham) and counted in a Packard scintillation spectrometer. The 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 remove the remaining traces of carbon. Rat blood serum was similarly equilibrated with ^{14}C-corticosterone as a marker in double labelled chromatography that we had previously established (6-8).

Ion exchange chromatography on DEAE-cellulose-52 columns was performed as previously described in detail (6-8). Briefly, for Fig. 3, 2 ml renal cytosol and 1 ml serum were labelled, respectively, with ^{3}H-RU-26752 (10^{-7}M) and 0.2 µCi ^{14}C-corticosterone, charcoal treated as above, and finally mixed just prior to chromatography. The mixture was then loaded onto a DEAE-52 column (0.5 x 25 cm) equilibrated in the same initial buffer as the cytosol (0.001 M phosphate pH 7.5). After passage of 30 ml of the initial buffer, a linear gradient was begun (at the arrow in the figure) between 30 ml each of 0.001 and 0.2 M phosphate, at a flow rate of 60 ml/h. All manipulations were carried out at 4°C. Fractions of 1 ml were processed for either radioactivity in the ACS fluid or for protein and conductivity measurements. For further details see (6-8) and figure legends.

For gel filtration on Ultrogel-ACA-44 columns, shown in Fig. 4, 4 ml cytosol and 2 ml serum were labelled as above and loaded onto the resin bed (1 x 130 cm). Equilibration and elution were carried out in the initial buffer (0.01 M phosphate + 0.1 M NaCl pH 7.4) in cold (4°C) at a flow rate of 30 ml/h. Fractions of 1.5 ml were collected of which 1 ml were processed for either radioactivity or protein quantitation. Further details have been already described (6-8) and also indicated in the legend.

^{3}H-RU-26752 (50 Ci/mM), >97% pure, as well as the corresponding cold compound were kindly furnished by Roussel-Uclaf, Romainville, France. The purity of the material, synthesized by Roussel, was checked by paper chromatography. 4-^{14}C-corticosterone (52 mCi/mM; batch 12) was purchased from Amersham, G.B. The radioinert steroids used in this study were purchased from Sigma, U.S.A. All reagents were high purity, analytical grade, mostly from Merck. DEAE-cellulose-52 was a product of Whatman and Ultrogel-44 that of LKB; ACS fluid was purchased from Amersham, G.B. Rats were raised and furnished by IFFA-Credo, France. Finally, due to its known lability upon storage, the purity of 1,2-^{3}H-aldosterone (45 Ci/mM; batch 34, Amersham, G.B.) was checked by paper chromatography and exceeded 98% during the entire course of investigation.

RESULTS AND DISCUSSION: The evidence for this new binding site comes from both saturation and competition studies in target and non-target tissues. Data in Fig. 1 show that both ^{3}H-RU-26752 and ^{3}H-aldosterone labelled two sets of sites in rat kidney. Whereas the initial slope was parallel with both materials, ^{3}H-RU-26752 later labelled another, more abundant site which exhibited little affinity for aldosterone. In other experiments, both sites were wanting when a non-target tissue (liver) was used, clearly suggesting a specific, physiological role in the kidney.

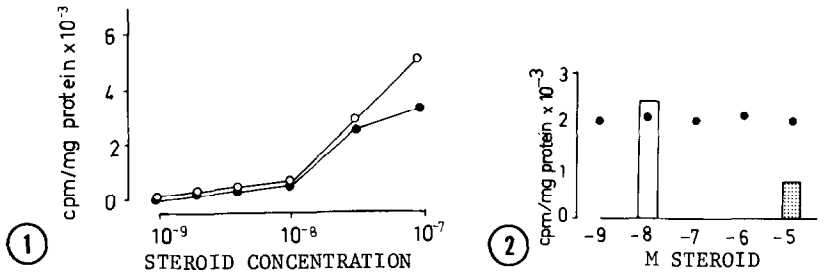

Fig. 1. Binding of the Agonist and the Antagonist to Rat Kidney Mineralocorticoid Receptor.

Renal cytosol was incubated in triplicate in presence of either ^{3}H-aldosterone (●) or ^{3}H-RU-26752 (○), alone or with one thousandfold excess of the homologous , unlabelled molecule to account for the non-specific binding which was subtracted from the receptor bound steroid.

Fig. 2. Lack of Competitive Displacement of the Agonist by the Antagonist in Rat Kidney.

Renal cytosol was incubated alone with ^{3}H-RU-26752 (□), and in presence of either cold RU-26752 (⊡) or cold aldosterone (●) at the indicated concentrations of the competitor. All samples were run in triplicate and calculated as CPM/mg protein.

Data in Fig. 2 establish that ^{3}H-RU-26752, bound to kidney cytosol, could not be displaced by aldosterone and this was confirmed when ^{3}H-aldosterone was allowed to compete with an excess of unlabelled RU-26752 (not shown). In still other experiments, incubation of renal, but not hepatic, cytosol with ^{3}H-RU-26752 plus ^{3}H-aldosterone resulted in more bound counts than incubation with just either analogue alone. The relative abundance of anti-mineralocorticoid, as compared to the agonist binding, site appears to be at least 3:1 in rat kidney; additional experiments are necessary for similar determinations in other mineralocorticoid targets (parotid, heart, intestine).

Physicochemical characterization of these anti-mineralocorticoid binders was next attempted by techniques that we had previously established to reveal the agonist binders in the kidney (6-8). Data in Fig. 3 show that ^{3}H-RU-26752 labelled exclusively an MR_3 component peaking in 0.2 M phosphate, pH 7.5, region with a peak conductance of 4.1 miliSiemens at 20°C. ^{3}H-aldosterone complexes eluted in MR_1 and MR_2 positions under these conditions, in 0.001 and 0.006 M phosphate (0.2 & 2.3 mSiemens) respectively.

Data in Fig. 4 confirm the separate identity of these two types of binding sites. ^{3}H-RU-26752 was bound to a heavier (H = 120,000) and a lighter (L =

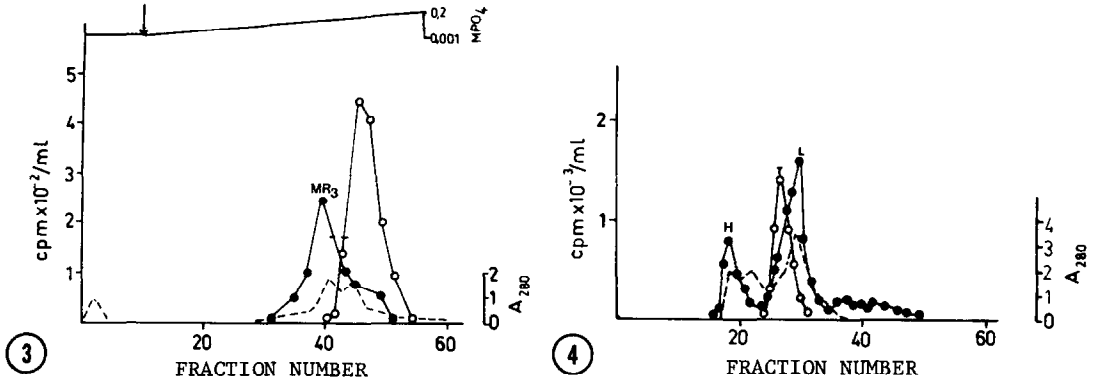

Fig. 3. Ion Exchange Separation of ^{3}H-RU-26752 Binding Proteins from Rat Kidney.

Sample preparation, equilibration, and elution were carried out as in Methods and (6-8). ------A_{280}; (●) ^{3}H; (○) ^{14}C.

Fig. 4. Molecular Filtration of ^{3}H-RU-26752 Binding Proteins.

For sample preparation, equilibration, and elution, see Methods and (6-8). ------A_{280}; (●) ^{3}H; (○) ^{14}C.

45,000) molecular weight component neither of which coeluted with rat serum transcortin (T = 63,000) that was preferentially labelled with ^{14}C-corticosterone; ^{3}H-aldosterone, however, labels 67,000 and 113,000 dalton entities (7).

Collectively, data presented here show a more abundant, tissue specific anti-mineralocorticoid binding site that is physicochemically distinct from a less abundant agonist binder, and that is wanting in a non-target organ. However, since non-radioactive spirolactone can, in very high doses, totally displace labelled aldosterone from MR, studies in the past have perpetuated the fallacious notion of a single receptor capabale of expressing both the agonist and the antagonist action; to some extent this was due to the inavailability of a suitable ligand, such ^{3}H-RU-26752.

Heterogeneity of the sort demonstrated here has just as clearly been described with oestradiol both for the cytoplasmic receptor (9) and the nuclear acceptor (10) and has, in general, been a topic of much discussion for all classes of steroid hormones (11). Although the nature and the physiological role of receptor heterogeneity in vivo remain speculative, an Argusoid model (12) attempts to explain the biological role of deoxycortico-

sterone and its 18-hydroxy derivative which, by virtue of low affinity for MR_2, and minimal concentrations in vivo, could not otherwise produce syndromes of mineralocorticoid excess despite an overwhelming concentration of aldosterone that preferentially saturates MR_2, in the intact organism (1,8,12). An actual demonstration of physiological role in this system awaits defined, MR dependent model systems in vitro.

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REFERENCES

(1) E. J. Ross, Aldosterone and Aldosteronism, Llyod-Duke, London, 1974.
(2) L. E. Ramsay in "Antihormones", M. K. Agarwal (ed), Elsevier/North Holland, Amsterdam, New York, pp 137-152, 1979.
(3) G. Wambach and A. Helber in "Hormone Antagonists", M. K. Agarwal (ed), Walter de Gruyter, Berlin, New York, pp 293-306, 1982.
(4) G. Wambach and J. Casals-Stenzel in "Adrenal Steroid Antagonism", M. K. Agarwal (ed), Walter de Gruyter, Berlin, N.Y., pp 291-314, 1984.
(5) L. E. Ramsay and G. T. McInnes in "Adrenal Steroid Antagonism", M. K. Agarwal (ed), Walter de Gruyter, Berlin, N.Y., pp 315-340, 1984.
(6) M. K. Agarwal, Nature (Lond) 254: 623-625 (1975).
(7) M. K. Agarwal, Biochem. J., 154: 567-575 (1976).
(8) M. K. Agarwal, FEBS Letters, 85: 1-8 (1978).
(9) R. L. Sutherland, L. C. Murphy, M. S. Foo, M. D. Green, A. M. Whybourne and Z. S. Krozowski, Nature (Lond) 288: 273-275 (1980).
(10) K. Sudo, F. J. Monsma Jr. and B. S. Katzenellenbogen, Endocrinology, 112: 425-434 (1983).
(11) M. K. Agarwal, Multiple Molecular Forms of Steroid Hormone Receptors, Elsevier/North Holland, Amsterdam, New York, 1977.
(12) M. K. Agarwal, FEBS Letters, 178: 1-4 (1984).