# Evidence for an unusual multifunctional protoreceptor in hormone action

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*Summary.* Contrary to the techniques of mere association, column chromatography has revealed a 'protoreceptor' that accepts aldosterone agonists and antagonists only in the physiological target, the kidney, and is absent in non-targets, liver and serum; it is furthermore different from the aldosterone specific receptor in renal cytosol.

In an initial step, corticosteroid hormones appear to bind to their target-specific receptor with subsequent translocation of the complex to nuclear acceptor sites[2]. 'Antihormones' may act either by reversing this sequence of events or via association with other cellular vectors[3]. Progesterone opposes aldosterone action in patients with Addison's disease, inhibits the mineralocorticoid mediated natriuretic diuresis[4], and antagonises receptor agonist association in competition studies[5]. Contrary to the idea of a unitary vector by Scatchard analysis, receptor polymorphism was demonstrated for mineralo- and glucocorticoids during physical separation[6], and this seems to be true of the receptor for all 5 classes of steroid hormones[2]. In the present study, an 'ideal' synthetic progestin[7] R-5020 (promegestone 17-*α*-methyl), was used to demonstrate that mineralocorticoid antagonism by an antihormone may proceed via saturation of that subpopulation in the kidney that does not bind aldosterone.

*Materials and methods.* Male Wistar rats (150–200 g) were bilaterally adrenalectomized 2–3 days prior to use; food and 1% NaCl were provided ad libitum after surgery. Blood was obtained by aortic cannulation, allowed to clot at 37 °C (30 min), then at 4 °C (60–90 min), and finally centrifuged (3000 × g) to obtain serum. The desired organ was perfused with the initial buffer and the cell sap was obtained by centrifugation at 105,000 × g (50 min).

Organ cytosol (in 0.01 M Tris-HCl, pH 7.4), or serum, in 0.5 ml aliquots was incubated (60 min, 4 °C) in presence of the desired amount of the radioactive steroid alone or in presence of a known excess of a cold steroid. Free steroid was removed by the addition of 0.5 ml (50 mg/ml) of activated charcoal (Sigma C-5260), further incubation (10 min, 4 °C), and centrifugation (15 min, 4000 × g). Aliquots of 0.5 ml were mixed with 10 ml Scintix (Isotec, France) and counted in a Packard Tricard Scintillation Spectrometer[2,3,6]. Protein was quantitated by the Biuret method. Non-specific binding in all cases was calculated by determination of radioactivity in presence of 1000-fold excess of cold, homologous steroid and was subtracted from the corresponding value obtained with tritiated hormone alone. Each sample (cold + radioactive or radioactive alone) was studied in triplicate at all points shown in figure 1.

For chromatography on DEAE-cellulose-52, the cell sap was loaded onto the resin (1 × 25 cm) and, after a low ionic prewash with 0.001 M phosphate, pH 7.5, elution was begun at the arrow with a linear gradient between 60 ml each of 0.001 M and 0.2 M of this buffer. 1-ml samples were processed for radioactivity and absorbance was recorded manually. 5 ml kidney cytosol with $10^{-8}$ M $^3$H-aldosterone alone or with $10^{-6}$ M cold progesterone, 2 ml serum with 0.5 μCi of $^{14}$C-progesterone; the 3 determinations were carried out separately and superimposed (figure 2, a). 4 ml kidney cytosol with $10^{-7}$ M $^3$H-progesterone and 2 ml serum with 0.5 μCi $^{14}$C-progesterone (figure 2, b).

*Materials and methods (Contd).* 4 ml renal cytosol with $10^{-7}$ M $^3$H-progesterone + $10^{-5}$ M cold R-5020 (figure 2, c). 4 ml kidney cytosol was incubated with $10^{-7}$ M $^3$H-progesterone plus $10^{-8}$ M $^3$H-R-5020 (figure 2, d) (Agarwal[2,3,6]).

Data in figure 1, a, show that the saturation characteristics and the quantitative binding of $^3$H-progesterone were similar in both the liver and the kidney. However, far greater quantities of R-5020 could be bound to renal as opposed to hepatic cytosol. Data in figure 1,b, establish that progesterone nearly totally abolished $^3$H-aldosterone binding to kidney cytosol whereas R-5020 was far less effective than progesterone. This is surprising since R-5020 is supposedly an 'ideal' progestagen[7], the kidney is not a primary target for progestative hormones and only poorly binds other synthetic progestagens[4,5]. In view of receptor multiplicity[2,3], and the above disparity, do these molecules (R-5020, aldosterone and progesterone) bind to the same component?

Data in figure 2, a, confirm the previously reported aldosterone ($10^{-8}$ M) binding to the mineralocorticoid specific receptor (MR), $MR_1$ and $MR_2$, and the glucocorticoid type of receptor (GR), all of which were nearly totally eliminated in presence of $10^{-6}$ M cold progesterone, and all of

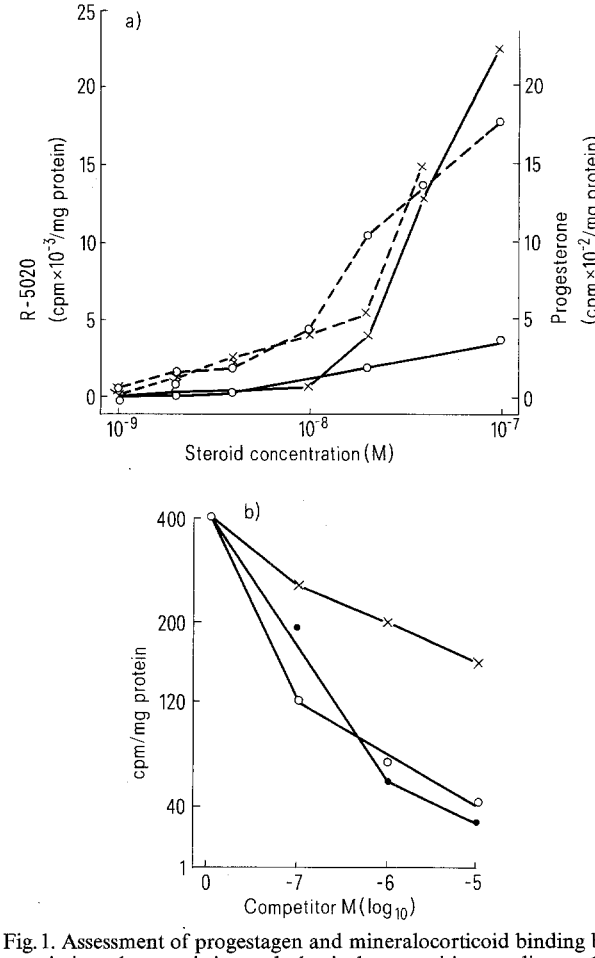



Fig. 1. Assessment of progestagen and mineralocorticoid binding by association characteristics and classical competition studies. *a* ○, liver; × kidney; --------, progesterone; ——, R-5020; *b* $10^{-8}$ M $^3$H-aldosterone with cold aldosterone (●), progesterone (○), R-5020 (×).

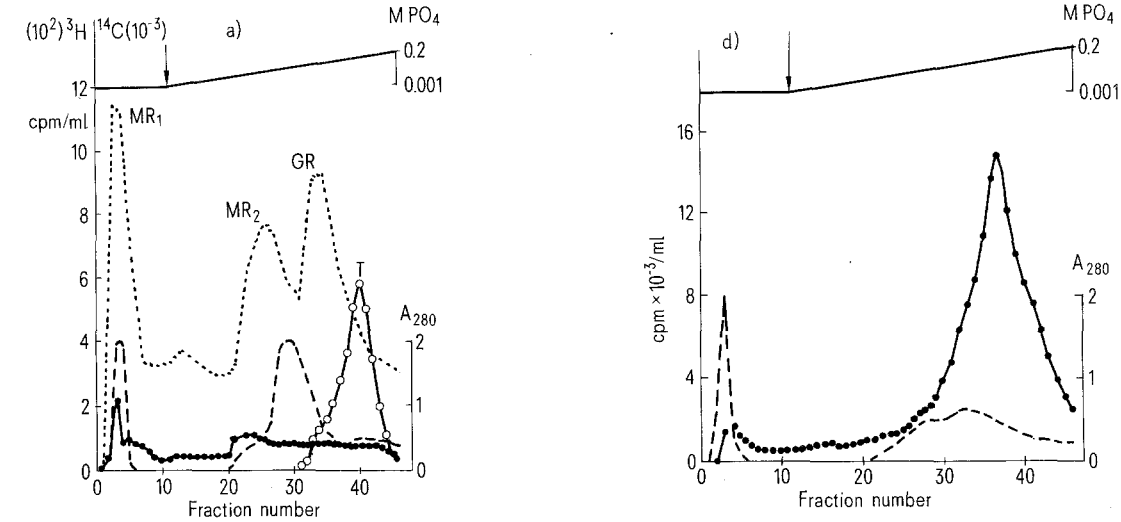
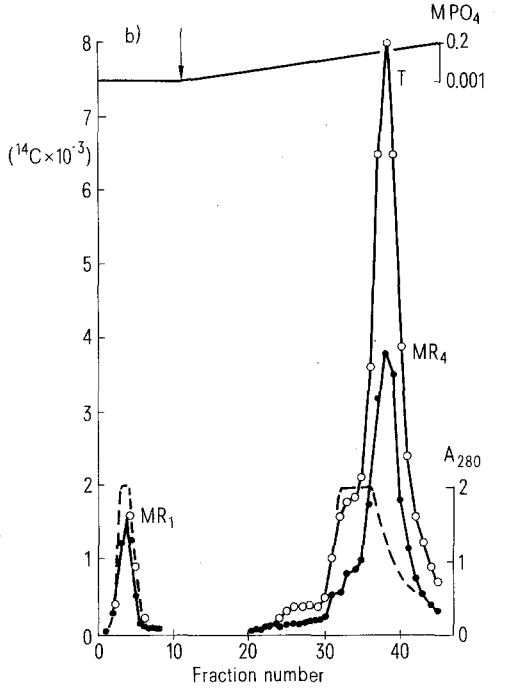
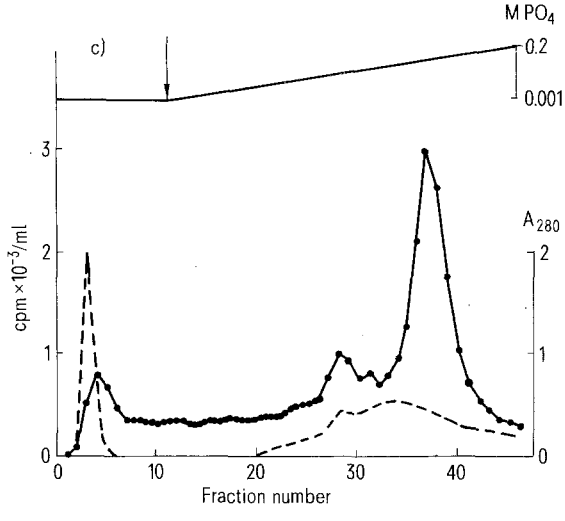



Fig. 2. The nature of aldosterone and progesterone binding to renal receptor separated on DEAE-cellulose-52 column. For details see text and Agarwal[2,3,6]. --------, $A_{280}$; ●—●, $^3H$; ○—○, $^{14}C$.




which were clearly distinct from blood serum transcortin (eluted in position T). This would confirm the results in figure 1, b, and fortify the argument that progesterone excess provokes inhibition of aldosterone-receptor binding leading to an antidiuretic effect at the physiological level[4]. Data in figure 2,b, show, however, that in the physiological range of $10^{-7}$ M (and $10^{-8}$ M, not shown), progesterone was bound only to $MR_1$ and $MR_4$ components (eluting with serum bound $^{14}C$-progesterone) and that this was reduced by a factor of $> 10$ in the presence of $10^{-5}$ M nonradioactive R-5020 (figure 2, c) suggesting that both progestins were binding to the same component which is not transcortin since R-5020 does not bind to the corticosteroid binding globulin (CBG = transcortin) as shown below. This was further confirmed by a mixing experiment (figure 2, d) where the amount of $MR_4$ bound radioactivity in the presence of $10^{-7}$ M $^3H$-progesterone + $10^{-8}$ M $^3H$-R-5020 concurrently, prior to chromatography, was intermediate between that obtained with either $10^{-7}$ M $^3H$-progesterone (figure 2, b), or the latter plus $10^{-5}$ M unlabelled R-5020 (figure 2, c) and this was less than that obtained with either $10^{-7}$ M $^3H$-progesterone or $10^{-8}$ M $^3H$-R-5020 alone, due to the difference in affinity (ratio at $10^{-7}$ M progesterone = 1, R-5020 = 5; at $10^{-8}$ M 1 and 20, respectively); please note the difference in scale in the figures.

Data in figure 3, a, show $^3H$-R-5020 ($10^{-7}$ M) binding to $MR_1$ and $MR_4$ entities. Although the latter coeluted with transcortin bound $^{14}C$-corticosterone (figure 3, a), blood serum bound $^3H$-R-5020 did not label $MR_4$ (figure 3, b) and no R-5020 binding could be observed in this position in the liver which is not a target for either progestatives or mineralocorticoids. In further studies, incubation with $10^{-8}$ M $^3H$-aldosterone + $10^{-8}$ M $^3H$-R-5020 lead to an additive effect with a profile $MR_1 > MR_2 < GR < MR_4$ which could be resolved only partially due to superimposition of various peaks, but ruling out competition for the same component, and further establishing that an antagonist (R-5020) may bind to the vector while leaving other sites intact for concurrent attachment of the agonist (aldosterone).

Briefly, $MR_4$ accepts selected mineralocorticoid agonists (deoxycorticosterone) and antagonists (progesterone $<<$ R-5020) exclusively in the target organ (kidney), but not in a non-target organ (liver) or serum, and it may be a backup

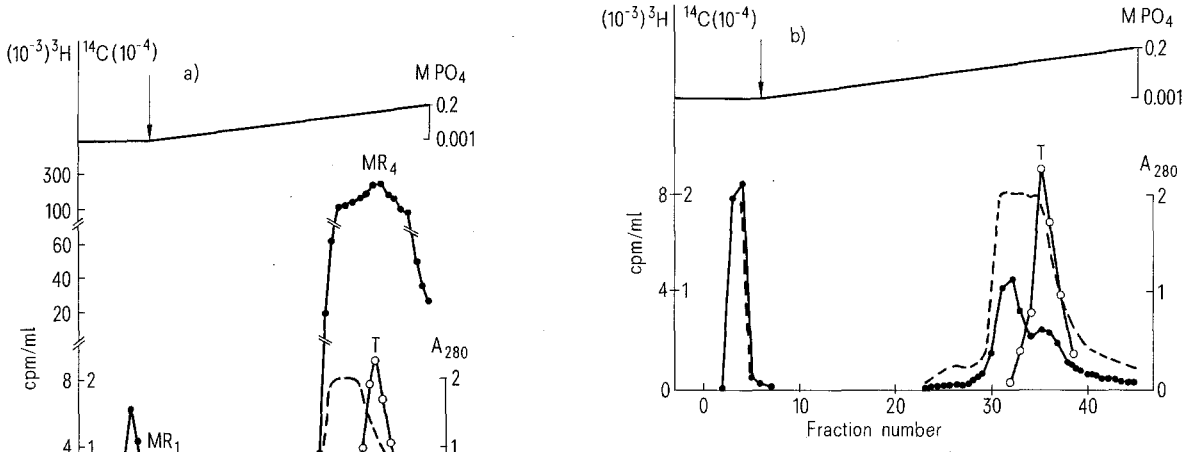



Fig. 3. Separation of renal R-5020 binders on DEAE-52 columns. 4 ml renal cytosol (a) or undiluted serum (b) was incubated with $10^{-7}$ M $^3$H-R-5020; 2 ml fresh serum with 0,5 µCi $^{14}$C-corticosterone was used for cochromatography as for figure 2, b, and Agarwal[2,3,6]. --------, $A_{280}$; ●—●, $^3$H; ○—○, $^{14}$C.

mechanism of greater significance than the agonist specific component ($MR_2$) that has a low capacity. The name protoreceptor seems appropriate for its multifunctional role although receptor maturation from a proreceptor can not be ruled out[8] without receptor purification. Finally, the discordance between physical separation vs mere competition establishes that the Scatchard type of analysis is not adequate by itself for the interpretation of receptor activity and may even be misleading, so that its widespread use needs some caution.

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## Changes in total and polymerized tubulin of the medial basal hypothalamus and adenohypophysis of castrated or hormone-injected rats[1]

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*Summary.* Treatment of orchidectomized rats with LH, FSH or prolactin decreased the tubulin content of the medial basal hypothalamus (MBH), whereas FSH or prolactin augmented it in the adenohypophysis (AH). After castration, negative correlations existed between serum LH and total or polymerized MBH tubulin, whereas in the AH positive correlations were found. After estradiol-progesterone treatment of spayed rats a significant correlation was found between serum LH and the percentage of AH tubulin in the polymerized form.

In a previous study[2], we observed that orchidectomy decreased the concentration of tubulin, the protein constituting microtubules, in rat medial basal hypothalamus (MBH), whereas testosterone administration augmented it in MBH and adenohypophysis (AH); likewise estradiol treatment resulted in a marginal increase of MBH tubulin. It seemed feasible that these changes in tubulin levels could be associated with modification of transport and/or release of materials of neuroendocrine significance, and the present experiments were performed to examine further hormone-related changes of MBH and AH tubulin by determining: 1. the effects of gonadotrophins and prolactin on tubulin levels in acutely castrated rats; 2. the changes in the equilibrium between the polymerized and depolymerized forms of tubulin following acute or sustained stimulation of the hypothalamic hypophyseal axis.

*Material and methods.* Adult Wistar rats (180–220 g) were kept under light between 0700 and 2100 h daily and were given access to chow and water ad libitum. 3 experiments were performed. Experiment 1: to assess the effects of FSH, LH and prolactin on MBH and AH tubulin levels, acutely orchidectomized rats received 2 s.c. injections of 100 µg of FSH (NIH-FSH-S12) or LH (NIH-LH-S19) 3 h and 18 h after castration, and the animals were killed 3 h later. Prolactin (NIH-S-P12, 200 µg) was injected according to the same schedule. Controls received the vehicle alone (1 ml of saline). Experiment 2: to examine the effects of a sustained rise in gonadotrophin levels on the polymerized and depolymerized forms of MBH and AH tubulin, male rats were castrated or sham-operated under ether anesthesia at 0800 h, and were killed 96 h later. Experiment 3: to examine the changes in the polymerized and depoly-