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GLUCOCORTICOID AND MINERALOCORTICOID RECEPTORS FOR ALDOSTERONE

G. ROUSSEAU*, J. D. BAXTER†, J. W. FUNDER‡, I. S. EDELMAN and G. M. TOMKINS
Department of Biochemistry and Biophysics, Cardiovascular Research Institute and Department of Medicine, University of California, San Francisco, U.S.A.

SUMMARY

Specific binding of aldosterone and dexamethasone by rat kidney and hepatoma tissue culture (HTC) cell cytosol has been studied. In cytosol of HTC cells, aldosterone and dexamethasone bind to a single class of sites with affinities that correspond with their potencies as inducers of tyrosine aminotransferase.

Kidney cytosol, however, contains two classes of specific aldosterone binding sites. The higher affinity sites bind aldosterone with an affinity (equilibrium, dissociation, constant) which is similar to the plasma concentration of aldosterone required for antinatriuresis. The lower affinity aldosterone-binding sites are present at a higher concentration than the higher affinity sites and also bind dexamethasone with a high affinity. We have tentatively identified these two classes of binding sites in renal cytosol as "mineralocorticoid" and "glucocorticoid" receptors, respectively.

INTRODUCTION

STEROID hormones appear to exert many of their effects via the induction of specific proteins in target tissues. Consistent with this, the mineralocorticoid action of aldosterone in rat kidney and toad urinary bladder requires *de novo* synthesis of protein[1, 2]. Furthermore, aldosterone behaves like a glucocorticoid in hepatoma tissue culture (HTC) cells since it induces tyrosine aminotransferase (TAT)[3] and stimulates cell adhesiveness[4].

The induction of specific proteins by steroids appears to be initiated by binding of the hormone to cytoplasmic "receptors"[5].

Aldosterone receptors have been detected in the cytoplasmic fraction of rat kidney[6, 7], duodenal mucosa, spleen, liver and brain[8], and salivary gland[9]. Nuclear binding of aldosterone has been shown in the same organs[1, 8–11] and in the toad bladder[12–14]. Recent studies[15] indicate that cytoplasmic binding of aldosterone is necessary for the subsequent specific nuclear localization of the hormone. In these systems, a close correlation was demonstrated in dose-response relationships for binding and for mineralocorticoid effect.

HTC cells contain cytoplasmic glucocorticoid receptor proteins, which appear to be involved in enzyme induction[16, 17]. In cytoplasmic extracts of HTC cells, steroids (dexamethasone, cortisol, corticosterone, progesterone) which influence TAT induction bind to the same receptor. Moreover, these binding proteins are similar in various glucocorticoid-responsive tissues[18].

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The present study was designed to distinguish between mineralocorticoid and glucocorticoid receptors for aldosterone in HTC cells and rat kidney. Based on Scatchard analysis[19] rat kidney cytosol contains two classes of specific binding sites for aldosterone. Dexamethasone binds with high affinity to at least one of these classes, i.e. those sites present at a higher concentration with a lower affinity for aldosterone. In contrast, HTC cell cytosol contains a single class of specific binding sites with differing affinities for aldosterone and dexamethasone.

MATERIALS AND METHODS

The source and purity of the reagents has been described elsewhere[11, 16, 17]. [1,2-^{3}H]-aldosterone was 44–50 Ci/mmole and [1,2,4-^{3}H]-dexamethasone was 9–12 Ci/mmole. Determination of protein and of radioactivity were performed as previously reported[17, 18, 20].

Kidneys from rats adrenalectomized and maintained on saline for one to two weeks were perfused *in situ* with ice-cold phosphate-buffered saline (0·1M NaCl and 0·025 potassium phosphate, pH 7·6) and homogenized in one volume of 0·02 M N-tris (hydroxymethyl) methylglycine (Tricine), 0·002M $CaCl_2$, 0·001M $MgCl_2$, pH 7·4, using a motor-driven tissue grinder. Growth and harvesting of HTC cells was performed as described elsewhere[21]. The cells were washed with ice-cold phosphate-buffered saline and homogenized by the same procedure as the kidneys. The HTC cell and kidney homogenates were centrifuged at 100,000 g for 1 h and the supernatant cytoplasmic fractions (cytosol) were used for the binding studies.

Binding reactions were performed at 0°C by incubating radioactive steroids in homogenization buffer with aliquots of cytosol (final dilution 4:1, v/v). The amount of bound steroid was determined by the charcoal assay described earlier [17, 18]. The data reported here are corrected for non-specific binding by subtracting from the total amount of steroid bound, the "background" value determined in parallel incubations containing 500–10,000-fold excess of the appropriate non radioactive steroid[17].

RESULTS

(a) *Binding of aldosterone by* HTC *cell cytosol*

HTC cell cytosol contains a single class of specific receptors which reversibly bind dexamethasone with high affinity (K equilibrium (dissociation), $K_d = 3 \times 10^{-9}$ M at 0°C)[17]. Since aldosterone also induces TAT in HTC cells[3], it should compete with dexamethasone for specific binding if these steroids share a common receptor involved in the induction mechanism. To test this inference, HTC cell cytosol was incubated in the presence of [^{3}H]-dexamethasone with and without aldosterone (Fig. 1). The results are shown in the form of a double-reciprocal plot and the common intercept on the ordinate suggests that both steroids bind to the same site. From these data, aldosterone binds to the receptors with an affinity (K_d, 0°C) of 3×10^{-8}M. By direct measurement of [^{3}H]-aldosterone binding, saturable and reversible interaction was found (Fig. 2). The linear Scatchard plot of these data (inset of Fig. 2) indicates that aldosterone binds to a single class of sites, with an affinity ($K_d = 2{\cdot}5 \times 10^{-8}$ at 0°C) which is in good agreement with the value obtained in the competition experiments. The total concentration of binding sites (indicated by the intercept on the abscissa of the Scatchard plots) was the same for [^{3}H]-aldosterone and [^{3}H]-dexamethasone and, in parallel experi-

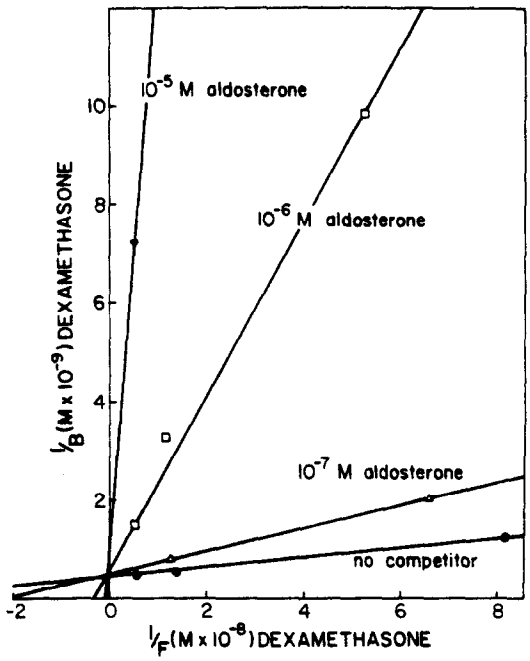

Fig. 1. Competitive inhibition by aldosterone of specific dexamethasone binding in HTC cell cytosol. HTC cell cytosol (5·9 mg protein/ml) was incubated at 0°C in the presence of various concentrations of [^{3}H]-dexamethasone with or without competing nonradioactive aldosterone. Incubations containing a 1000-fold excess of nonradioactive dexamethasone were run in parallel for background determination. After 90 min, specific dexamethasone binding was determined by the charcoal assay.

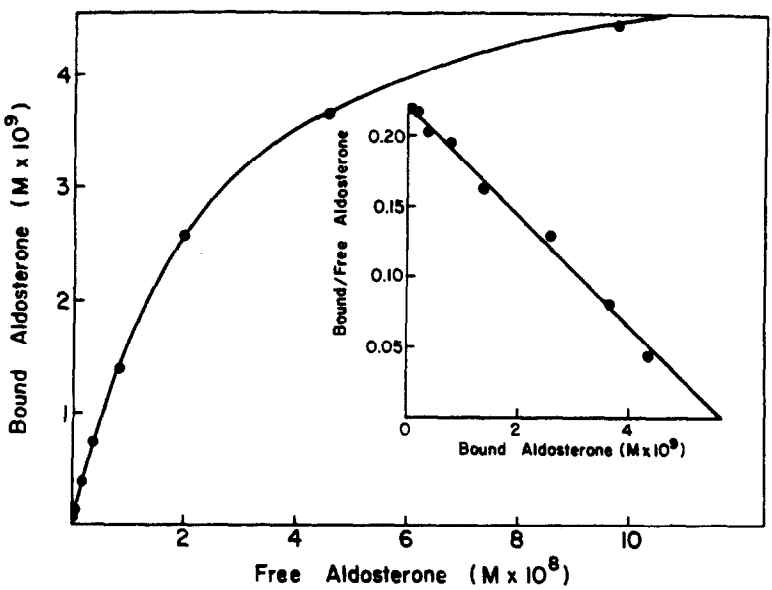

Fig. 2. Aldosterone binding by HTC cell cytosol. Specific binding of various concentrations of [^{3}H]-aldosterone by HTC cell cytosol (4·8 mg protein/ml) was determined by the charcoal assay (see Legend of Fig. 1). The inset shows the Scatchard plot of the data.

ments, for [^{3}H]-cortisol, [^{3}H]-corticosterone and the anti-inducer[22] [^{3}H]-progesterone. In addition, as shown in Fig. 3, dexamethasone prevented [^{3}H]-aldosterone from occupying its binding sites. The anti-inducers progesterone and 17α-methyl-testosterone also competed with [^{3}H]-aldosterone for all the binding sites. These results indicate that in HTC cells, aldosterone interacts with the cytoplasmic glucocorticoid receptor system which probably mediates TAT induction.

(b) *Binding of dexamethasone and aldosterone by rat kidney cytosol*

As in HTC cells, we find specific saturable binding of [^{3}H]-dexamethasone in the cytosol of adrenalectomized rat kidney (Fig. 4). The linear Scatchard plot (inset of Fig. 4) is consistent with a single set of binding sites, with an affinity (K_d, 0°C) of 10^{-8}M. Progesterone and aldosterone exhibit the properties of competitive inhibitors of the binding of dexamethasone in renal cytosol (Fig. 5). There are obvious similarities between these findings and those in HTC cells described above.

In contrast to the results obtained with [^{3}H]-dexamethasone, [^{3}H]-aldosterone appears to bind specifically to two classes of sites in kidney cytosol, as shown by the Scatchard analysis (Fig. 6). From the two linear regressions we have estimated the affinity and the concentration of sites in the first component to be about (K_d, 0°C) 5×10^{-9}M and $1{\cdot}3 \times 10^{-13}$ moles/mg protein, respectively, and in the second component to be about (K_d, 0°C) $6{\cdot}5 \times 10^{-8}$M and $4{\cdot}1 \times 10^{-13}$ moles/mg protein, respectively. It should be emphasized that accurate determinations of the affinity constants and concentrations of binding sites from the data in Fig. 6 require a more detailed analysis[23–25]. Since the experimental curve on the Scatchard plot corresponds to the sum of two straight lines describing the two populations of sites, the slopes and intercepts of these straight lines differ slightly

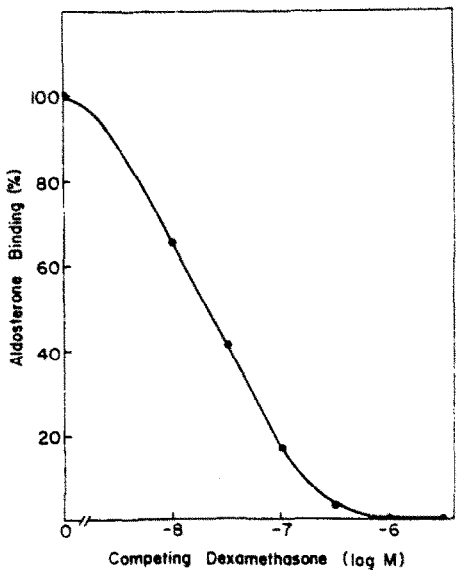

Fig. 3. Competition of dexamethasone with aldosterone for specific binding in HTC cell cytosol. Cytosol was incubated with 10^{-7}M [^{3}H]-aldosterone with or without various amounts of nonradioactive dexamethasone and specific binding was measured by the charcoal assay (see legend of Fig. 1).

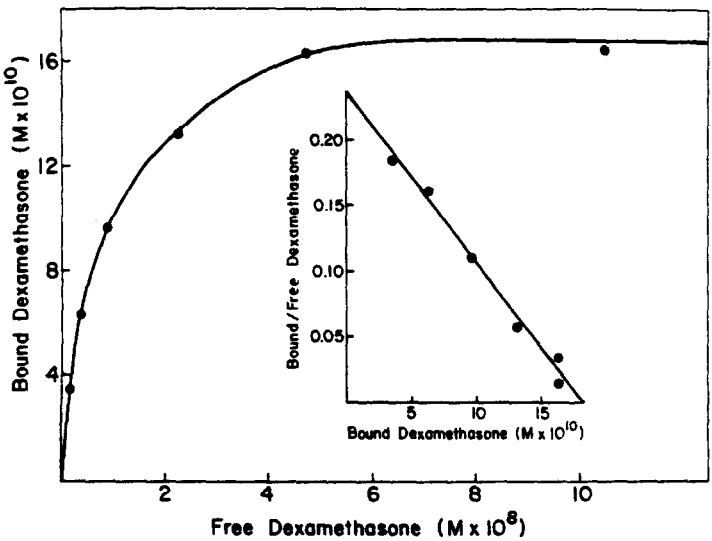

Fig. 4. Dexamethasone binding by rat kidney cytosol. Specific binding of various concentrations of [^{3}H]-dexamethasone by rat kidney cytosol (6·3 mg protein/ml) was determined using the charcoal assay (see legend of Fig. 1). The inset shows the Scatchard plot of the data.

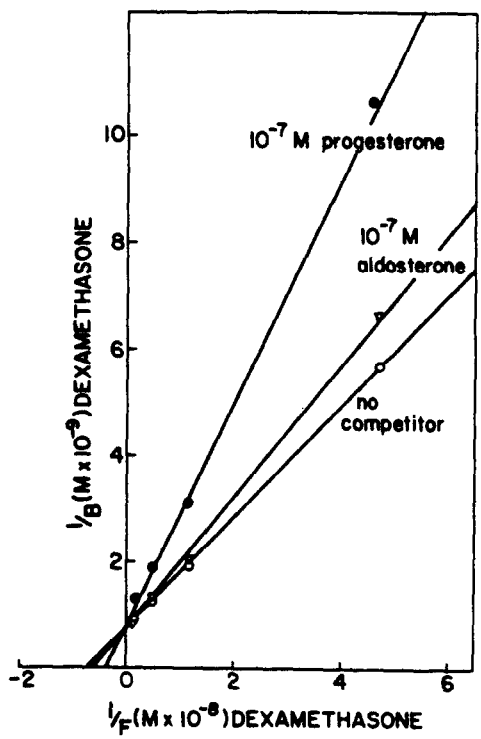

Fig. 5. Competitive inhibition by progesterone and aldosterone of dexamethasone binding by rat kidney cytosol. Cytosol (2·5 mg protein/ml) was incubated in the presence of various concentrations of [^{3}H]-dexamethasone with or without competing 10^{-7}M non-radioactive progesterone or aldosterone. Binding assays were performed as reported in the legend of Fig. 1.

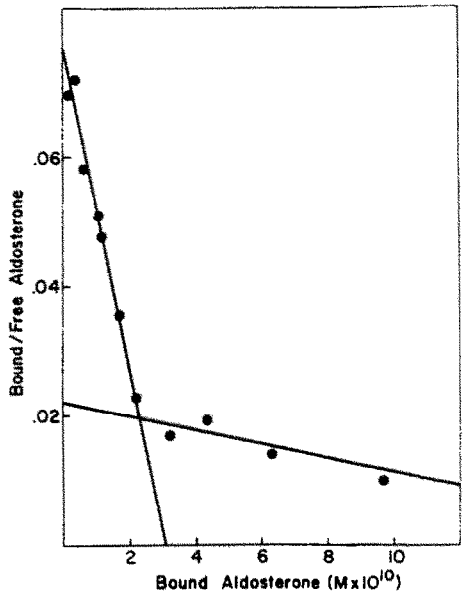

Fig. 6. Specific binding of aldosterone by rat kidney cytosol. Cytosol (6·3 mg protein/ml) was incubated with various concentration of [^{3}H]-aldosterone. The amount of specifically bound steroid was determined as in the experiment shown in Fig. 1. The data are plotted by the Scatchard technique.

from the linear regressions depicted in Fig. 6. For example, the affinity and concentration of sites for the class of receptors which bind aldosterone with higher affinity would be somewhat higher and lower, respectively, than our estimate.

The concentration of the lower affinity aldosterone-binding sites corresponds to that of dexamethasone (Table 1). Dexamethasone, however, prevents all the specific binding of [^{3}H]-aldosterone to renal cytosol. At a concentration of [^{3}H]-aldosterone of 5×10^{-8}M (which saturates the higher affinity sites and almost half of the lower affinity sites, Fig. 6) 3×10^{-6}M dexamethasone eliminated all specific aldosterone binding. These results indicate that dexamethasone impairs the binding of [^{3}H]-aldosterone to both classes of sites. The failure to detect a second class of [^{3}H]-dexamethasone binding sites (cf. Fig. 4) could be a consequence of the low affinity of dexamethasone for the higher affinity aldosterone-binding sites and of the relative low concentration of these sites. The data shown in Fig. 4 represent results obtained at a maximum concentration of [^{3}H]-dexamethasone of 10^{-7}M whereas 3×10^{-6}M dexamethasone was used in the competition experiments.

Table 1. Binding of aldosterone and dexamethasone to rat kidney cytosol*

Steroid	Concentration of receptor sites (moles/mg protein $\times 10^{13}$)	Equilibrium (dissociation) constant (0°C) ($\times 10^8$M)
Aldosterone (I)	1·3	0·5
Aldosterone (II)	4·1	6·5
Dexamethasone	3·9	1·0

*Cell-free binding of [^{3}H]-aldosterone and [^{3}H]-dexamethasone at various concentrations was determined by the charcoal assay[17, 18]. Affinity constants and concentration of receptor sites (mean of two experiments) were calculated from Scatchard plots of the data. I and II denote the two classes of aldosterone-binding sites.

DISCUSSION

Steroids that are active in promoting hepatic glycogen deposition are active inducers of TAT in the liver[26]. All of these steroids interact with the dexamethasone receptors[17. 18]. Similar receptors are present in rat liver[18] and in glucocorticoid-sensitive lymphoid tissue[27. 28]. Aldosterone has been classified as an optimal inducer in HTC cells because it induces TAT to maximal levels[3]. The potency of four optimal inducers (determined by the steroid concentration required to produce half-maximal induction) is as follows: dexamethasone > corticosterone > cortisol > aldosterone[18]. The relative affinities of these steroids for the glucocorticoid receptors (K_d, 0°C = 3×10^{-9}M for dexamethasone, 4×10^{-9}M for corticosterone, 10^{-8}M for cortisol and $2{\cdot}5 \times 10^{-8}$M for aldosterone) correspond to their relative potencies as inducers.

Rat kidney cytosol has at least two classes of specific aldosterone binding sites of unequal capacity. The higher affinity aldosterone-binding sites probably represent mineralocorticoid receptors since the concentration of aldosterone required for saturation is very near the plasma concentration required for maximal antinatriuresis in the adrenalectomized rat[1]. The lower affinity aldosterone-binding sites probably represent glucocorticoid receptors, since the concentration of these sites is the same as the concentration of the dexamethasone-binding sites. This inference is supported by the ability of aldosterone to competitively inhibit binding of ^{3}H-dexamethasone to renal cytosol (Fig. 5).

It is of interest that dexamethasone has approximately the same affinity for cytosol receptors in the liver[18] lymphoid tissue[28] and kidney. In addition, aldosterone has a lower affinity than dexamethasone for binding to this class of sites in both HTC cells and kidney. Plasma transcortin probably does not contribute to the binding reactions described here since transcortin has a very low affinity for aldosterone[29] and for dexamethasone[18].

Results comparable to those reported above have been obtained with intact cells using an *in vitro* kidney slice technique (Funder, Feldman and Edelman, unpublished observations). They found two classes of cytoplasmic aldosterone-binding sites with affinities (K_d) at 0°C of 4×10^{-9}M and 10^{-8}M, and at 25°C of 2×10^{-9}M and 10^{-8}M but only one set of dexamethasone-binding sites with an affinity (K_d) at 25°C of 5×10^{-9}M.

A tentative conclusion from these experimental results is that there are mineralocorticoid and glucocorticoid receptors in the cytoplasmic fraction of rat kidney, both of which bind aldosterone. The latter receptors are similar to those we find in HTC cells.

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DISCUSSION

Leaf: When looking at the saturation of binding sites in the toad bladder we found two populations of binding sites distinguished by difference in binding affinity. The sites with the highest binding affinity we estimated to be completely saturated at concentrations of aldosterone too low to affect sodium transport in this tissue. Thus we have never understood the role of this population of binding sites. I gather than the affinity of the sites you have been examining is not so high as to raise this difficulty?

Rousseau: We have not studied the binding of aldosterone in the toad bladder. The Scatchard analysis of our data indicates that if binding sites with such a high affinity for aldosterone exist in the rat kidney, their concentration must be so small (below 2×10^{-11}M) that they could not be distinguished from the other high-affinity binding sites.

Funder: In reference to Dr. Leaf's point, I would like to ask permission of the Chairman to show one slide. [Fig. Kidney cytosol] After 20 min. incubation of rat kidney slices at 37°C, we found two sets of high-affinity aldosterone binding sites. The affinity of the smaller group is consistent with the maximum anti-natriuretic effect seen at a plasma concentration of 5×10^{-9}M. As can be seen from this figure, there is no suggestion under the circumstances of a set of binding sites with a still higher affinity and lower capacity.

Jørgensen: The steroid hormones must pass the cell membrane to reach the binding sites in the cytosol and in the nuclei. Is it possible that the cell membrane forms a barrier for the steroids?

Rousseau: I do not know of any evidence for an active transport of steroids into the cells and the current concept is that these hormones freely diffuse through the cell membrane. However, energy-dependent outward transport of cortisol from mouse fibroblasts and adrenal gland cells in culture has been reported (Gross S. R. *et al.*: *J. Cell Biol.* **44** (1970) 103. Still, the question could be raised whether the first step in the mechanism of action of steroid hormones consists of a specific

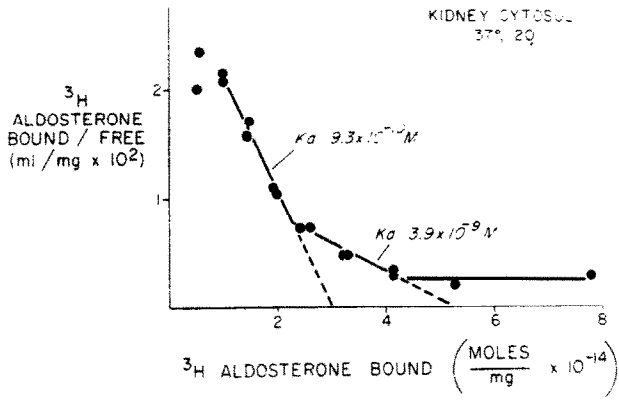

Fig. 7.

interaction with the cell membrane as seems to be the case for several polypeptide hormones. In HTC cells, specific binding of dexamethasone appears to occur inside the cell rather than at the cell surface; i.e. the specific binding components found in the cytoplasmic fraction are not released from the membrane during fractionation procedures but occur naturally inside the cell. We think this because organic mercurials, which do not readily enter the cells, do not inhibit the binding of dexamethasone by intact HTC cells although they can prevent the formation of the steroid-receptor complex in cell-free extracts (Levinson B. B. *et al.*, *Science*, (in press).

Jørgensen: Thus, in the interpretation of your results you imply that the cell membrane does not discriminate between the various steroids you have used?

Rousseau: Yes.