

SHORT COMMUNICATION

ALDOSTERONE RECEPTOR ASSAY IN RAT KIDNEY CYTOSOL

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SUMMARY

A method has been developed to measure aldosterone receptors using rat kidney cytosol preparations. Addition of 0.1 M Na molybdate to homogenization buffer markedly diminished receptor inactivation, allowing for more accurate assessment of affinity and total number of receptors. Statistical analysis of Scatchard plots was used to resolve curvilinear plots into high affinity (type I) and a low affinity (type II) components. Aldosterone and deoxycorticosterone compete most effectively for binding to type I sites, whereas dexamethasone competes most effectively for binding to type II sites. Molybdate does not alter the location of the 8.0S peak on density gradient analysis. Assay of type I sites revealed 47.7 ± 1.7 fmol/ml cytosol protein in male adrenalectomized rats. Constant of dissociation (K_D) was measured at 5.08×10^{-10} M. Non-adrenalectomized male rats had 24.4 ± 3.0 fmol/mg protein type I sites. Addition of molybdate to homogenization buffer combined with statistical analysis of curvilinear Scatchard plots allows for accurate and reproducible measurement of high affinity aldosterone receptors in rat kidney cytosol.

INTRODUCTION AND METHODS

Aldosterone binding proteins (ABP) have been identified in the cytoplasm of rat and human kidney tissue [1-3]. Quantitation of the number and affinity of ABP has been complicated by the presence of two classes of sites; a high affinity, low capacity protein, thought to be the physiologic aldosterone receptor, and a low affinity, high capacity protein. Funder *et al.* have labelled these type I and type II sites respectively [4]. Measurement of aldosterone receptors has also been complicated by their instability in a cytosol preparation [5]. For this reason, most studies have utilized tissue slice incubations, a technique which limits the number of incubations and renders quantitative analysis difficult because of high variability.

Because sodium molybdate has been effective in preventing glucocorticoid receptor inactivation [6], we have stabilized aldosterone receptors in cytosol preparations by the addition of sodium molybdate to homogenization buffer.

Male Sprague-Dawley rats weighing 150-250 gm were used. Adrenalectomy was performed under ether anesthesia 1-3 days prior to assay. On the day of the assay, kidneys were perfused with iced isotonic solution (NaCl = 0.133 M, KH_2PO_4 = 0.006 M, CaCl_2 = 0.001 M, Tris-HCl = 0.005 M, MgCl_2 = 0.0005 M, glucose = 0.005 M, pH = 7.4) via a catheter in the abdominal aorta, again using ether anesthesia. Kidneys were removed and minced and all subsequent procedures were performed at 4°C. Minces were homogenized in TSM buffer (Tris-HCl = 0.1 M, sucrose = 0.25 M, Na molybdate = 0.1 M, pH = 7.4) using a motor driven Teflon pestle and glass vial. The homogenate was centrifuged at 100,000 g for 1 h, and the supernatant fraction was regarded as cytosol.

Cytosol incubations were performed with [^3H]-aldosterone (85 Ci/mmol, New England Nuclear) in concentrations ranging from 1.63×10^{-10} M to 5.33×10^{-8} M in a total

volume of 300 μl . Parallel incubations with excess unlabelled aldosterone (1.33×10^{-5} M) were performed with each concentration of [^3H]-aldosterone. Incubation for 40 min at 22°C was followed by a charcoal separation step. Dextran-coated charcoal [10 gm of activated charcoal (Sigma), 250 mg Dextran T-80 (Pharmacia) per liter of TSM buffer], 0.5 cc, was added to each incubation tube, vortexed, and centrifuged at 3000 g for 10 min. A 0.5 cc portion of the supernatant fraction was counted.

Nonspecific binding was defined as [^3H]-aldosterone bound in the presence of 1.33×10^{-5} M unlabelled aldosterone. Specific binding was calculated by subtracting nonspecific binding from total binding. Cytosol protein was determined by the method of Lowry [7], and curvilinear Scatchard plots were analyzed by the method of Priore and Rosenthal [8]. This analysis allowed for determination of the slope and x-intercept of both type I and type II lines.

The effect of molybdate in blocking receptor inactivation was assessed by preparing cytosol in homogenization buffer with (TSM) or without (TS) the addition of Na molybdate. Cytosol was also prepared in TS buffer with 0.1 M NaCl. Cytosols were allowed to stand at 22°C for 0, 20, 40, and 60 min, then incubated with 1.33×10^{-9} M [^3H]-aldosterone.

Binding of various steroids to ABP was assessed by incubating cytosol prepared in TSM buffer with 3.3×10^{-10} M or 3.3×10^{-8} M [^3H]-aldosterone along with increasing concentrations of unlabelled steroids. For each steroid, six concentrations were used, each point representing the mean of four experiments. Duplicate incubations were performed with 1.33×10^{-5} M unlabelled aldosterone and specific binding was expressed as percent of total specific binding seen in the absence of competing unlabelled steroid.

Gradient analysis of receptors in TS and TSM buffer was performed. Cytosol was incubated with 3.3×10^{-9} M [^3H]-aldosterone, and 400 μl was added to a pellet of dextran charcoal, vortexed, and centrifuged at 3000 g for 10 min. A volume of 200 μl of cytosol was then layered on to a linear 10-30% sucrose gradient (5.0 ml). Gradients

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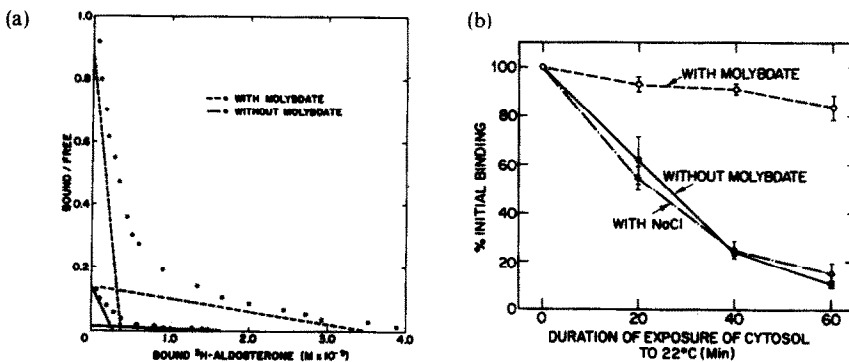


Fig. 1. Effect of molybdate upon $[^3\text{H}]$ -aldosterone binding to ABP. (a) Scatchard analysis of ABP with or without Na molybdate. (b) Inactivation of ABP at 22°C . Cytosol prepared with or without 0.1 M Na molybdate or 0.1 M NaCl was allowed to stand at 22°C for 0 to 60 min prior to incubation with $[^3\text{H}]$ -aldosterone. Specific binding is expressed as the % of binding seen at 0 time, and values given are the means of three experiments \pm SEM. Initial binding with TSM buffer was 40.4 fmol/mg protein, with TS buffer was 12.2 fmol/mg protein, and with 0.1 M NaCl was 11.7 fmol/mg protein.

were centrifuged at 300,000 g for 14.5 h at 4°C using a SW65 rotor in a Beckman L2-65B ultracentrifuge, then separated into two drop fractions and counted. Bovine serum albumin was layered on a separate gradient as a standard marker.

RESULTS AND DISCUSSION

Scatchard analysis of binding with TSM buffer showed markedly increased binding when compared to experiments using TS buffer (Fig. 1). Statistical analysis for type I sites in adrenalectomized rats ($n = 7$) revealed 46.7 ± 1.7 fmol/mg cytosol protein using TSM buffer, and 35.1 ± 2.6 fmol/mg protein using TS buffer ($n = 8$) ($P < 0.005$). In addition, the apparent affinity of type I sites for aldosterone was markedly enhanced. Using molybdate, the measured K_D was 5.08×10^{-10} M, which was significantly less than that for type I sites using TS buffer, $K_D = 3.23 \times 10^{-9}$ M ($P < 0.001$). Using TSM buffer, intact animals had 24.4 ± 3.0 fmol/mg protein type I sites, a significant decrease compared to adrenalectomized animals ($P < 0.001$), while affinity was unchanged. The results of inactivation experiments are also shown in Fig. 1. During one hour at 22°C , cytosol prepared with molybdate had only a 16% decrease in binding capacity, while cytosol prepared without molybdate lost 90% of binding capacity.

The ability of various unlabelled steroids to compete for

$[^3\text{H}]$ -aldosterone binding sites is shown in Table 1. Gradient analysis using both TS and TSM buffer revealed a major peak with an 8.0S sedimentation coefficient (Fig. 2).

Nielsen *et al.* have shown that glucocorticoid receptors in thymocyte cytosol preparations appear to be inactivated by a phosphatase enzyme system, and that sodium molybdate and other phosphatase enzyme inhibitors are effective in preventing this inactivation [6]. In this study, sodium molybdate has also been shown to protect ABP from inactivation.

The apparent increase in affinity seen with molybdate can be explained by the observation that filled receptors are more resistant to inactivation than are unoccupied receptors [5]. Incubations with smaller amounts of aldosterone result in more receptor inactivation, increasing the slope of the Scatchard plot and decreasing the calculated affinity [9].

The equal affinity of aldosterone and DOC for binding to type I sites has been seen in some other aldosterone receptor systems [4, 10]. Glucocorticoids and progesterone had about 30% of the affinity of aldosterone in this study, whereas other cytosol assays have found progesterone and corticosterone to be 28% and 25% as effective as aldosterone [11], and dexamethasone to be 50% as active as aldosterone in competing for $[^3\text{H}]$ -aldosterone binding [5]. Higgins *et al.* found dexamethasone and cortisol both dis-

Table 1. Inhibition of $[^3\text{H}]$ -aldosterone binding to high affinity (type I) and low affinity (type II) sites by nonradioactive steroids

Steroids	Type I sites		Type II sites	
	Concentration (nM)	Relative inhibition	Concentration (nM)	Relative inhibition
Aldosterone	3.0 ± 0.05	1.00	54.0 ± 0.05	1.00
Deoxycorticosterone	3.0 ± 0.02	1.00	23.1 ± 2.6	2.34
Spirolactone	9.2 ± 1.4	0.33	228 ± 9.0	0.24
Progesterone	10.8 ± 0.6	0.28	61.8 ± 3.6	0.87
Corticosterone	11.2 ± 0.4	0.27	55.0 ± 2.2	0.98
Dexamethasone	11.3 ± 0.6	0.27	8.6 ± 0.3	6.28
Estradiol	553 ± 50	0.005		

Cytosol was incubated with 3.3×10^{-10} M $[^3\text{H}]$ -aldosterone (80–85% of binding is to high affinity sites) or 3.3×10^{-8} M $[^3\text{H}]$ -aldosterone (75–80% of binding is to low affinity sites). Concentrations of nonradioactive steroids required to displace 50% of bound $[^3\text{H}]$ -aldosterone are listed.

Relative inhibition = aldosterone concentration at 50% displacement/steroid concentration at 50% displacement.

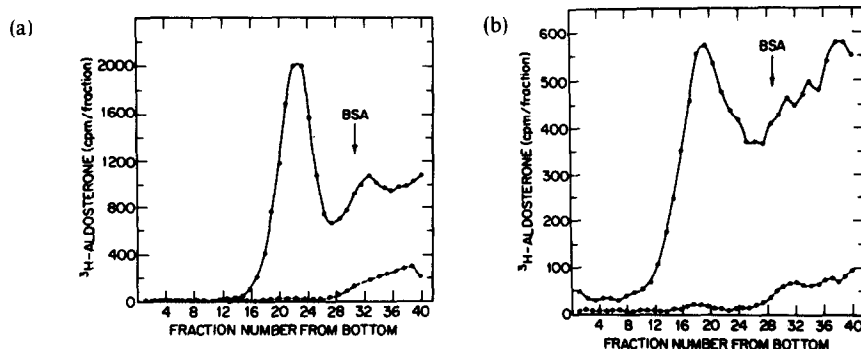


Fig. 2. Sucrose density gradient analysis of ABP. Cytosol was incubated with 3.3×10^{-9} M [3 H]-aldosterone using either TSM (a) or TS (b) buffer. The major peak is at 8.0S for both experiments. Closed circles represent total binding, open circles represent nonspecific binding. Protein concentration for both cytosols was 25 mg/ml. Calculated sedimentation coefficient of BSA = 4.3S.

placed [3 H]-aldosterone about 10% as well as unlabelled aldosterone [10]. Corticosterone had 10% of the affinity of aldosterone in a slice assay preparation [4].

The binding affinities of aldosterone and other steroids do not correspond closely with their potency in mineralocorticoid bioassay systems. In such systems, DOC has about 10% of the activity of aldosterone and glucocorticoids are 1 to 2% as active [12]. Part of this discrepancy can be explained by increased binding of DOC and corticosterone to corticosteroid binding globulin *in vivo* compared to aldosterone [4, 13].

The smaller numbers of receptors in intact animals compared to adrenalectomized animals is not surprising. This smaller number is most probably secondary to *in vivo* binding of receptors to aldosterone, rendering these receptors unavailable for *in vitro* binding. Receptor measurements using molybdate stabilized cytosol must be interpreted cautiously, for addition of molybdate may alter the behavior of receptors as is seen for glucocorticoid receptors [14]. The use of a cytosol preparation for assessment of renal aldosterone receptors should allow for easier determination of receptor number and affinity during physiologic and pathologic alterations.

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