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INTERACTION OF [^{3}H]-ALDOSTERONE WITH RAT KIDNEY PLASMA MEMBRANES

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SUMMARY

The interaction of [^{3}H]-aldosterone with isolated rat kidney plasma membranes has been studied by centrifugation and gel filtration methods. The Scatchard plot analysis of data indicated the affinity of aldosterone for membrane sites, expressed as equilibrium constant K_{diss}, to be 1.3×10^{-8} M and the concentration of binding sites (n) 1.69×10^{-13} mol/mg protein. The high concentration of unlabelled aldosterone present in the incubation medium, or injected into rats 45 min before killing, decreased the amount of labelled aldosterone bound to plasma membranes. Gel filtration on Sephadex G-200 and disc electrophoresis on polyacrylamide gel of solubilized [^{3}H]-aldosterone–renal plasma membranes complex showed that the hormone was bound to the high molecular weight protein component of the plasma membranes. The proteinaceous nature of membrane component which bound aldosterone was determined using delipidated plasma membranes.

INTRODUCTION

The mechanism of aldosterone action upon kidney is not known. One of the hypothesis, put forward by Feldman *et al.* [1], suggested the initial intracellular binding of aldosterone to cytoplasmic protein receptors and subsequent transfer of the hormone–receptor complex into the nucleus where it binds to chromatin. The next step is binding of nuclear–aldosterone complex to DNA, a process that activates transcription by stimulating the synthesis of m-RNA. The newly synthesized protein is responsible for the subsequent effect on sodium transport.

It has been assumed that steroid hormones penetrate into their target cells by passive diffusion across membrane lipids layers [2]. However there is evidence which indicates that the role of the cell membrane in the case of transmembrane passage of steroids into the cell should not be neglected. Using electron microscope authoradiography Williams and Baba[3] have shown that following the injection of labelled aldosterone into the rat aorta most radioactivity can be found in the proximal and distal tubules of the kidney, bound mainly to plasma membranes and mitochondria of their cells. Gross *et al.*[4] have demonstrated the energy-dependent outward transport of cortisol from mouse fibroblasts and adrenal gland cells. Recently Harrison *et al.*[5–7] have proposed a specific mechanism associated with the cell membrane which transports steroid hormones into their target cells where they can bind to the intracellular receptors.

In order to contribute to the understanding of aldosterone action on kidney, experiments were designed to study the interaction of [^{3}H]-aldosterone with isolated rat kidney plasma membranes.

MATERIAL AND METHODS

Male rats of the Fischer strain aged 3–4 months were used. The rats were adrenalectomized 7–10 days before killing and were kept on standard laboratory diet and saline *ad libitum*.

Following killing the kidneys were removed and placed into STE buffer (0.25 M sucrose, 0.01 M triethanolamine–HCl and 0.005 M EDTA), pH 7.6. Plasma membranes were isolated by differential centrifugation using the method of Fitzpatrick *et al.*[8]. The final membrane pellet was suspended into STE buffer. All operations were done at 0°.

The protein concentration of the plasma membranes was determined by the method of Lowry *et al.*[9] and the activity of the characteristic marker enzyme, Na-K-ATP-ase, by the method of Kinsolving *et al.*[10].

Binding assay. The standard binding assay consisted of incubating 0.5 ml of freshly prepared renal plasma membranes (0.2–0.3 mg protein) at 37° for 30 min in STE buffer containing 0.1 μCi or 0.2 μCi [^{3}H]-aldosterone (S.A. 103 Ci/mmol, Amersham, England). The binding was performed over the aldosterone concentration range from 1.8×10^{-9} M to 2.36×10^{-8} M. The unlabelled aldosterone (Sigma, U.S.A.) was added to the concentration desired. Following incubation the membranes were centrifuged for 20 min at 13,300 g. The supernatants were removed and the excess liquid on the tube wall wiped by tissue paper. In order to remove trapped radioactivity the membrane pellet was washed in 0.5 ml of STE buffer, left to stand at room temperature for 15 min and centrifuged again for 20 min at 13,300 g. Then the supernatants were discarded, the tubes wiped again and the pellets dissolved in Soluene-100

(Packard Inst., U.S.A.). The radioactivity of the samples was determined by the liquid scintillation technique in a Nuclear Chicago liquid scintillation counter Mark II.

To determine the specificity of [^{3}H]-aldosterone binding to plasma membranes three methods were used. In the first one the membrane pellet obtained after the first centrifugation was suspended into 0.5 ml of STE buffer containing high concentration (10^{-5} M) of unlabelled aldosterone, spironolactone (generous gift of the Searle Co., Chicago, IL.), 9α-fluorohydrocortisone or progesterone, respectively. In the second method the same substances were present in the concentration of 10^{-6} M in the binding assay. In the third method the large amount of unlabelled aldosterone, spironolactone or 9α-fluorohydrocortisone (100 μg) was injected into rats 45 min before killing.

Gel filtration. Following the standard binding assay (aldosterone concentration in the incubation medium was 5.6×10^{-9} M) membranes associated with [^{3}H]-aldosterone (3–4 mg protein) were solubilized with 0.01 M Tris–HCl buffer containing 0.1 M NaCl and 1.2% Lubrol-WX detergent (Sigma, U.S.A.), pH 7.6, and were subjected to gel filtration on Sephadex G-200 column (1.5 × 25 cm, Vo = 6.8 ml) equilibrated and eluted (1.5 ml/h) with 0.02 M Tris–HCl buffer containing 0.15 M NaCl and 0.12% Lubrol-WX, pH 7.6. Fractions of 0.75 ml were collected and assayed for protein and radioactivity. Protein determination in those fractions had to be modified since Lubrol detergent interfered with the Lowry method [9] of protein determination. The modification of the method involved the addition of an anionic detergent, sodium dodecyl sulphate (SDS, 0.5%), in the alkali reagent. Similarly Dulley and Grieve [11] used 0.5% SDS to remove the interference of Triton X-100 in the Lowry method of protein determination.

Delipidation of plasma membranes. Immediately after isolation plasma membranes were suspended into the mixture of 96% alcohol and ether (3:2, v/v)—1 ml/g of original tissue, homogenized and then shaked for 4 h at 4°. Then the membranes were centrifuged for 15 min at 33000 *g*, and the pellet resuspended by homogenization into the mixture of 96% alcohol and ether (3:1, v/v) and shaken again for 18 h at 4°. Following the extraction of lipids delipidated membranes were suspended into STE buffer and subjected to the standard binding assay with [^{3}H]-aldosterone and gel filtration on Sephadex G-200.

Disc electrophoresis was performed on 10% polyacrylamide gel [12] using plasma membranes solubilized with 0.01 M Tris–HCl buffer containing 0.1 M NaCl and 1.2% Lubrol-WX detergent, pH 7.6. The electrophoresis was run in Tris–glycine buffer containing 0.12% Lubrol-WX, pH 8.7.

RESULTS

The electron microscopic examination of isolated plasma membranes, used in this study, showed that they were free of gross contamination by subcellular particles and other types of membranes (Fig. 1). Enzy-

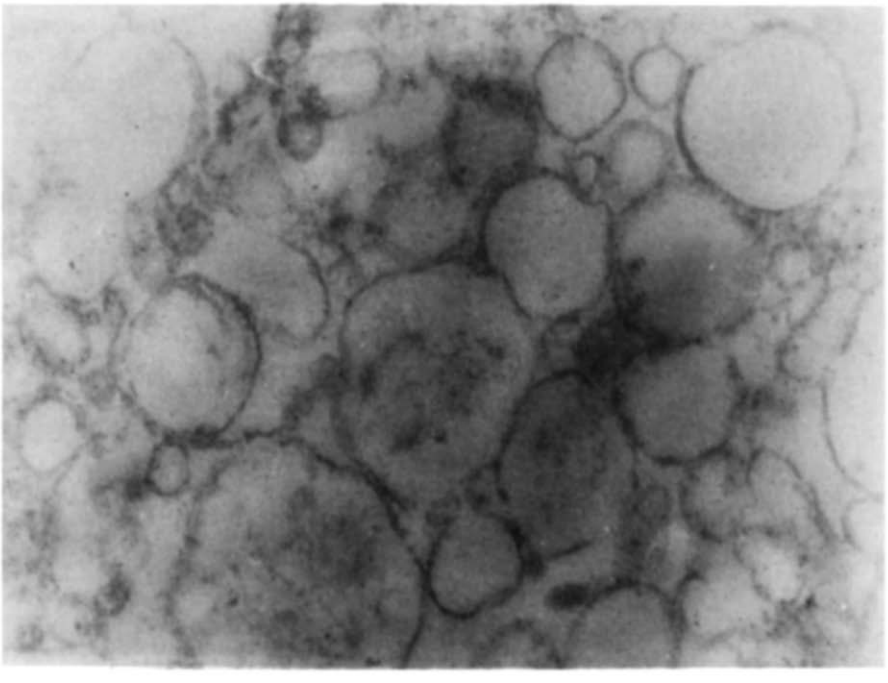

Fig. 1. Electron micrograph of isolated rat kidney plasma membranes. Specimens for electron microscopy were fixed for 1 h in 5% glutaraldehyde and 1% OsO_4, respectively. Dehydration was done in acetone and the embedding in Durcopan ACM resins. Those sections were doubly stained with uranil acetate and lead citrate and viewed in electron microscopy type Opton M-9, Zeiss. Magnification 40,000 ×

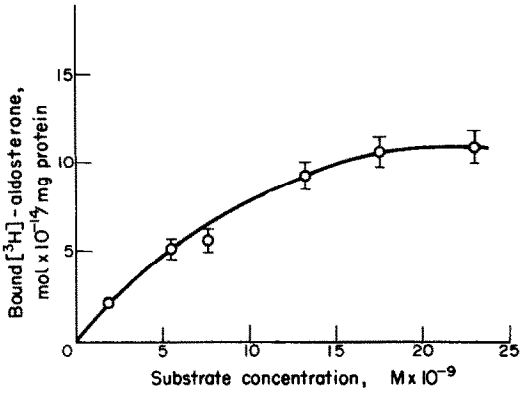

Fig. 2. Binding of [^{3}H]-aldosterone as a function of aldosterone concentration in the incubation medium. Isolated membranes were incubated for 30 min at 37° in STE buffer pH 7.6 containing [^{3}H]-aldosterone over the aldosterone concentration range from 1.8×10^{-9} M to 2.36×10^{-8} M. Following incubation membranes were centrifuged for 20 min at 13,300 *g*, supernatants removed, pellets washed in 0.5 ml STE buffer and left to stand at room temperature for 15 min. Then membranes were centrifuged again, supernatants discarded, pellets dissolved in Soluene-100 and assayed for radioactivity. Each point is the mean of 6–9 observations. Bars represent S.E.

matic analyses of plasma membranes revealed enrichment of the characteristic marker enzyme activity, Na-K-ATP-ase (39.08 ± 0.93 μmol/h/mg protein in membranes vs 5.8 ± 0.88 μmol/h/mg protein in homogenate—the results are the means of 10 preparations ± S.E.).

The binding of [^{3}H]-aldosterone to rat renal plasma membranes was studied over the aldosterone concentration range from 1.8×10^{-9} M to 2.36×10^{-8} M. Figure 2 illustrates the effect of increasing aldosterone concentrations in the binding assay upon the binding of hormone to isolated plasma membranes. As can be seen from plotted results binding approached saturation at 2.36×10^{-8} M aldosterone concentration. The Scatchard plot analysis of data indicated the affinity of aldosterone for plasma membrane sites, expressed as equilibrium constant K_{diss}, to be 1.3×10^{-8} M and the concentration of binding sites (n) 1.69×10^{-13} mol/mg protein (Fig. 3). Pellets from the standard binding assay (aldosterone concentration in the incubation medium was 5.6×10^{-9} M) resuspended in STE buffer containing high concentrations (10^{-5} M) of unlabelled aldosterone, spironolactone, 9α-fluorohydrocortisone or progesterone, respectively did not cause displacement of [^{3}H]-aldosterone bound to plasma membranes. The same substances, except for unlabelled aldosterone, present in the incubation medium (10^{-6} M) or injected into rats (100 μg) 45 min before killing did not exhibit decrease of [^{3}H]-aldosterone binding to plasma membranes. The high amount of unlabelled aldosterone present in the standard binding assay or injected into rats 45 min before killing decreased the amount of labelled aldosterone bound to plasma membranes by 31% and 15%, respectively (Table 1.).

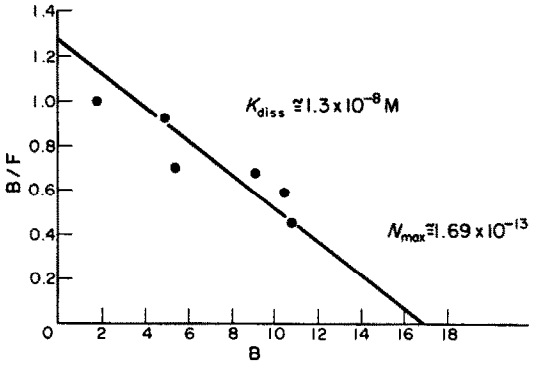

Fig. 3. The Scatchard plot analysis of data presented in Fig. 2. B = bound [^{3}H]-aldosterone (mol/mg protein), B/F = bound [^{3}H]-aldosterone (mol/mg protein)/free [^{3}H]-aldosterone (molar). The line of best fit shown in the figure was calculated by least squares analysis; the K_{diss} is 1.3×10^{-8} M and the concentration of binding sites (n) 1.69×10^{-13} mol/mg protein.

Table 1. The effect of high concentration of unlabelled aldosterone upon the binding of [^{3}H]-aldosterone to rat kidney plasma membranes

Treatment	[^{3}H]-aldosterone bound to plasma membranes (d.p.m./mg protein)	per cent
Standard incubation procedure—5.6×10^{-9} M aldosterone	13328 ± 776* (8)	100
Unlabelled aldosterone (10^{-6} M) added to the standard incubation medium	9175 ± 186† (6)	69
Unlabelled aldosterone (100 μg) injected into rats 45 min before killing	11380 ± 280‡ (3)	85

* = Mean ± S.E., ( ) = number of observations. † = Significant difference compared to standard incubation procedure, $P < 0.01$. ‡ = Significant difference compared to standard incubation procedure, $P < 0.05$.

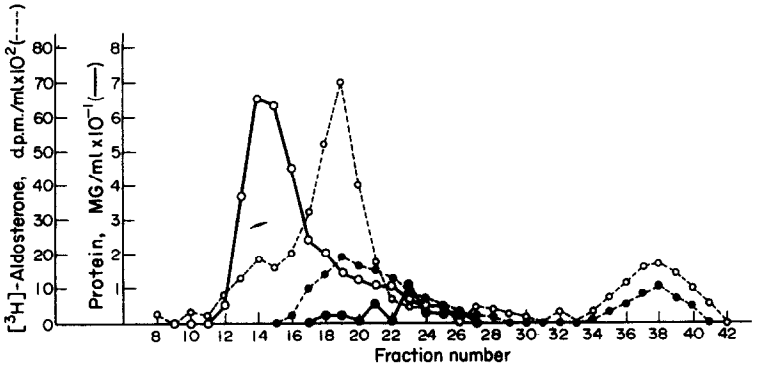

Fig. 4. Gel filtration chromatography of solubilized [^{3}H]-aldosterone–renal membranes macromolecular complex. Plasma membranes were incubated with 5.6×10^{-9} M [^{3}H]-aldosterone in STE buffer, pH 7.6, and were solubilized with 0.01 M Tris–HCl buffer containing 0.1 M NaCl and 1.2% Lubrol-WX detergent, pH 7.6 Dissolved membranes were applied to Sephadex G-200 column which was equilibrated and eluted with 0.02 M Tris–HCl buffer containing 0.15 M NaCl and 0.12% Lubrol-WX detergent, pH 7.6. Fractions of 0.75 ml were collected and assayed for protein and radioactivity. ○—intact plasma membranes, ●—delipidated plasma membranes.

In order to ascertain that the binding of [^{3}H]-aldosterone to kidney plasma membranes was not the physical entrapment into membrane vesicles, but a [^{3}H]-aldosterone–macromolecular complex, the plasma membranes associated with labelled aldosterone were solubilized with Tris–HCl buffer containing NaCl and Lubrol-WX detergent and subjected to gel filtration on Sephadex G-200. As shown in Fig. 4 one protein and two [^{3}H]-aldosterone peaks were eluted. Seventy eight percent of [^{3}H]-aldosterone was eluted with the proteins of the protein peak and the rest as free [^{3}H]-aldosterone (this was checked in experiment where [^{3}H]-aldosterone alone in Tris–HCl buffer containing NaCl and Lubrol-WX detergent was subjected to gel filtration). The free [^{3}H]-aldosterone peak is probably the result of dissociation or degradation process due to solubilization with Lubrol-WX detergent. Although gel filtration of [^{3}H]-aldosterone–plasma membranes complex on Sephadex G-200 did not enable better separation of proteins that bound aldosterone, it indicated that the aldosterone binding macromolecules were present in the kidney plasma membrane fraction. Figure 4 also displays results obtained in experiment where delipidated plasma membranes were used. One protein and two [^{3}H]-aldosterone peaks were eluted, the same as in the case of intact plasma membranes. However those peaks were much lower because during delipidation membrane proteins became slightly denatured and therefore hardly soluble. Thus a small amount of soluble protein entered the Sephadex column. However these results indicate the possibility that the [^{3}H]-aldosterone was not trapped or dissolved in the lipid component of the plasma membranes.

Following gel filtration membrane proteins associated with labelled aldosterone were subjected to disc electrophoresis on polyacrylamide gel, which revealed more than one protein band (Fig. 5). The low electro-

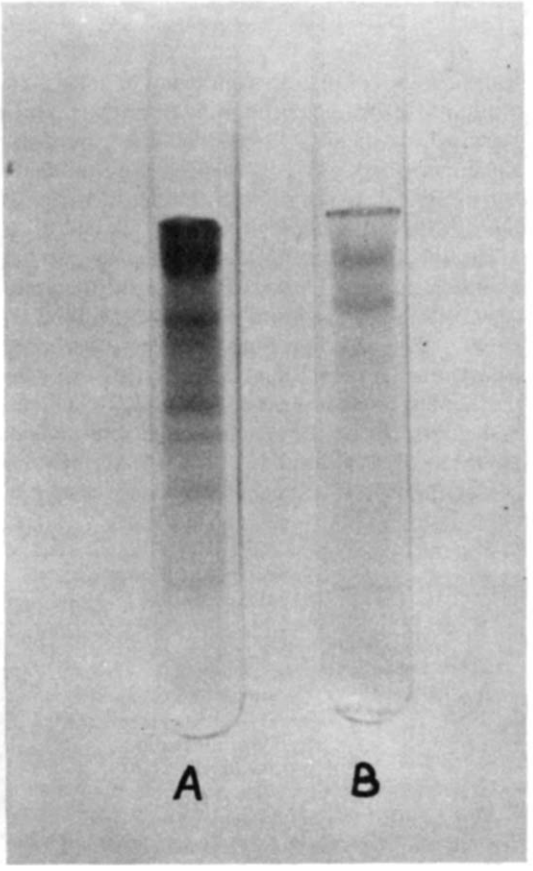

Fig. 5. Disc electropherograms of membrane proteins solubilized with 0.01 M Tris–HCl buffer containing 0.1 M NaCl and 1.2% Lubrol-WX detergent, pH 7.6. The system for electrophoresis was 10% polyacrylamide gel and Tris–glycine buffer, pH 8.7. The amount of protein applied was 75 μg. A—all protein components of plasma membranes, B—proteins associated with [^{3}H]-aldosterone, obtained by gel filtration on Sephadex G-200.

phoretic mobility and the Ve/Vo ratio point to the high molecular weight of protein which binds aldosterone.

DISCUSSION

The results presented in this paper revealed the affinity of aldosterone for the rat kidney plasma membranes with $K_{diss} = 1.3 \times 10^{-8}$ M and the concentration of binding sites of 1.69×10^{-13} mol per milligram of membrane proteins (Fig. 3). The amount of [^{3}H]-aldosterone bound to plasma membranes could be decreased by the high concentration of unlabelled aldosterone. Membrane component which binds aldosterone seems to be a high molecular weight protein(s) (Figs. 4 and 5). The lack of the competition for aldosterone binding sites by aldosterone competitive inhibitors (spironolactone and 9α-fluorohydrocortisone) might argue against the biological relevance of aldosterone binding to renal plasma membranes in terms of its action on sodium transport. It is possible that the nature of aldosterone interaction with kidney plasma membranes is different from aldosterone binding to the renal intracellular receptors where from it can be displaced by its agonists and antagonists. Although our results do not provide explanation for the physiological role of aldosterone binding to kidney plasma membranes they indicate that the role of kidney plasma membrane should not be neglected in the mechanism of aldosterone action upon the kidney.

Specific receptors for aldosterone have been demonstrated in the nucleus and the cytoplasm of the rat kidney [13,14]. The affinity of aldosterone for the kidney nucleus, expressed as equilibrium constant K_{diss}, was 6×10^{-9} M [13]. Two classes of receptors for aldosterone have been demonstrated in kidney cytoplasm—one type with high affinity ($K_{diss} = 5 \times 10^{-9}$ M and $n_{max} = 1.3 \times 10^{-13}$ mol/mg protein) and the other type with low affinity ($K_{diss} = 6.5 \times 10^{-8}$ M and $n_{max} = 4.1 \times 10^{-13}$ mol/mg protein) [14]. It is possible that the plasma membrane provides a driving force for pulling the bound aldosterone into the cell before its attachment to cytoplasmic receptors. Similarly Erdos *et al.*[15] tried to explain the binding of estradiol to the nonspecific cell membrane receptors of mouse vagina prior to its binding to specific intracellular receptors.

It is also possible that aldosterone binding to renal plasma membranes is involved in the mechanism of potassium excretion independent of sodium reabsorption. This hypothesis is supported by the data suggesting that the antinatriuretic and kaliuretic components of the response to aldosterone may be separable [16].

At the present time the results presented in this paper should be taken with scrutiny although they clearly suggest that the interaction of aldosterone with renal plasma membranes might be the early event in the transmembrane movement of aldosterone across the cell membrane of the kidney. Further studies are needed to establish the protein component of membrane which binds aldosterone and the physiological role of aldosterone interaction with its target cell membrane.

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