AN IN VITRO STUDY OF MINERALOCORTICOID BINDING PROTEINS FROM RAT KIDNEY

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1. Introduction

Various studies have shown that specific proteins of target cells bind added steroids: oestrogens [1,2] testosterone [3,4] and progesterone [5,7]. These proteins have been isolated from the cytosol of cell homogenates. When the active steroid has bound to the cytosol receptor protein, the complex migrates into the nucleus, as initially shown by Jensen [2] and Gorski [1] where it encounters another acceptor the nature of which is still unknown but may be a protein or DNA itself [7]. A similar succession of events appears to be involved in the mechanism of action of aldosterone at peripheral receptor sites. The studies of Edelman [8] and Swaneck [9, 10] led to the isolation of proteins obtained from the nuclear and cytoplasmic fractions of the rat kidney that bind tritiated aldosterone in vivo and these proteins have also been found in homogenates of other tissues of the rat.

Alberti [11] extracted protein-bound aldosterone from nuclei of the mucosal cells of the toad bladder by sonication. Rousseau [12] showed, both in vivo and in vitro, that there were two classes of renal cytosol receptors for aldosterone. The type I receptors have a high affinity for aldosterone while type 2 receptors have a high affinity for glucocorticoids.

The present study shows that tritiated aldosterone binds to proteins of the cytosol when incubated with rat-kidney slices. The binding of tritiated aldosterone to soluble proteins of the nucleus and to the chromatin has also been observed; this binding is reduced in the presence of active steroids and not in the presence of inactive steroids.

2. Experimental

2.1. *Material and methods*

Male albino rats of the Wistar strain, 100–150 g in weight, were adrenalectomised 3–4 days prior to the experiments. They were fed ad libitum and given 0.9% NaCl (w/v) to drink. [1,2-^{3}H]aldosterone (44–50 Ci/mmol) was obtained from New England Nuclear. Unlabeled aldosterone, deoxycorticosterone, 9α-fluorocortisol were obtained from Ciba-Geigy (Basle) and spironolactone from Searle (Chicago). Centrifugation was carried out in a Martin Christ refrigerated centrifuge. All radioactive samples were counted for 10 min at 0°C in a liquid scintillation spectrometer (Packard Company) with 38–45% efficiency. The proteins were determined by the method of Lowry [14], using bovine serum albumin as a standard.

2.2. *Incubations*

The rats were killed by decapitation and the kidneys were removed, decapsulated, and cut into fine slices of 275 μm thickness. The slices from two kidneys were collected, placed into flasks containing 4 ml of a Krebs–bicarbonate buffer solution with glucose (1 g/l) Trasylol^R (200 U/EKI) and undiluted [1,2-^{3}H]aldosterone (5.2 nM). During all these manipulations the flasks were kept in an ice bath. The flasks were then placed in a shaking incubator (Braun) for 1 hr at 24°C in an atmosphere of 95% oxygen, 5% CO_2.

2.3. *Isolation of the nuclear pellet*

At the end of the incubation period the medium

was aspirated and the kidney slices were rinsed with iced incubation buffer and homogenised with 6 vol of 2.2 M sucrose, 3 mM in $MgCl_2$ using a Potter-Elvehjem Teflon homogeniser. This procedure and other subsequent steps were carried out at 4°C. The homogenate was filtered through 4 layers of gauze; the filtrate was brought to a volume of 10 ml with the same sucrose $MgCl_2$ solution and then centrifuged at 100 000 *g* for 30 min. The floats and supernatants were discarded and the nuclear pellets were gently resuspensed in 10 ml of sucrose and recentrifuged at 100 000 *g* for 30 min.

2.4. *Preparation of the soluble nuclear proteins*

From the washed nuclei, albumin and globulins were extracted by the addition of 0.15 M NaCl, pH 8, followed by centrifugation for 10 min at 19 000 *g*, the operation being repeated twice. The supernatant contained the soluble nuclear proteins.

2.5. *Preparation of the chromatin*

Chromatin was subsequently extracted from the pellet free of soluble proteins, either with distilled water or in 2 M NaCl [13]. The pellet was manually homogenised by hand with a Potter-Elvehjem homogeniser and centrifuged at 10 000 *g* for 30 min. The supernatant contains the chromatin or the desoxyribonucleoproteins. The extraction was repeated until the extract was no longer viscous.

2.6. *Preparation of the cytosol*

After incubation as described above, the kidney slices were homogenised in 6 vol of 0.25 M sucrose, 3 mM in $MgCl_2$. The homogenates were centrifuged at 130 000 *g* for 60 min and the supernatant constituted the cytosol fraction.

2.7. *Separation of free and bound aldosterone*

One-ml aliquots of cytosol, nuclear fraction and chromatin were passed through a 20 × 1 cm column of Sephadex G-50 (fine mesh, particle size : 20–80 μm). Protein-bound aldosterone was recovered in the excluded volume of the column. The aldosterone–protein complex was also precipitated from the supernatant fractions by addition of an equal volume of saturated $(NH_4)_2SO_4$.

The 50%-saturation $(NH_4)_2SO_4$ solutions were incubated for 30 min at 4°C and then centrifuged at 20 000 *g* for 15 min, re-suspended in a freshly prepared solution of glycerol (200 g/l) (pH approx. 7.4) containing 0.02 M ammonium bicarbonate and assayed for radioactivity and proteins as described.

3. Results and discussion

3.1. *Binding of aldosterone to nuclear and cytosolic proteins*

The specific activity of the nucleoprotein–aldosterone complex was 130 ± 12 fmol/mg protein (mean ± standard deviation). That of the protein-aldosterone complex in the cytosol was 13 ± 2.5 fmol/mg protein; $n = 12$.

3.2. *Chromatography on Sephadex*

In the nuclear fraction, after separation of the bound form from the free aldosterone on Sephadex G-50, the bound material was divided into two portions and rechromatographed on columns of Sephadex G-100 or G-200 respectively.

In the first case the bound hormone was totally excluded. In the second case, only 15% of the radioactivity was present in the excluded volume, indicating an association to a complex of a molecular weight exceeding 10^6. The fraction excluded is lower if the pH is less than 6 or at higher ionic strength. The

Table 1
Effect of various steroid hormones on the binding of [1,2-^{3}H]aldosterone in the soluble nuclear proteins of rat kidneys

	Binding (%)
Aldosterone	100
Aldosterone + 17 β-oestradiol (1:80)	105
Aldosterone	100
Aldosterone + deoxycorticosterone (1:50)	40
Aldosterone	100
Aldosterone + 9α-fluorocortisol (1:80)	35
Aldosterone	100
Aldosterone + spironolactone (1:10 000)	65

The results are expressed in % of the values obtained in the presence of [1,2-^{3}H]aldosterone only. Dose ratios in parentheses.

remaining radioactivity (85%), although originally bound, was eluted in a volume equal to that occupied by the free aldosterone in a separate control chromatography, indicating that the bound aldosterone dissociates into protein and free aldosterone during the 2 hr necessary for the elution.

Neither dithiothreitol or dimethylsulfoxide at 10% (w/v) concentration, nor Trasylol^{R} (200 U/EKI) (a trypsin inhibitor) stabilised the complex. In contrast, the use of higher concentrations of glycerol (200–250 g/l) increased the stability of the complex but this slowed down the elution considerably.

3.3. *Specificity of the binding to soluble nuclear proteins*

The kidney slices were incubated in the presence of 0.26 nmol [1,2-^{3}H]aldosterone and of an excess of the following steroids (dose ratio in parentheses): deoxycorticosterone (1:50), 9α-fluorocortisol (1:80), 17β-oestradiol (1:80) or spironolactone (1:10 000). This resulted in reduced binding except with oestradiol (table 1). These results confirm those obtained in vivo by Swaneck [10]. These experiments of Swaneck and ours indicate that there is a relationship between the capacity of the steroids to bind to these proteins and their characteristics either as mineralocorticoids or as competitive inhibitors of mineralocorticoids.

Table 2
Distribution of tritiated aldosterone

Fraction	10^{-2} × radioactivity (cpm)
Total nuclear fraction	1300
Nuclear proteins soluble in 0.15 M NaCl	
1st extraction	757
(fraction insoluble in 0.5 sat. $(NH_4)_2SO_4$	(392)
2nd extraction	212
3rd extraction	119
Non-solubilized material extracted in 2 M NaCl	204
(fraction insoluble in 0.5 sat. $(NH_4)_2SO_4$	(164)

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