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THE INITIATION MECHANISM IN THE ACTION OF ALDOSTERONE ON SODIUM TRANSPORT

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SUMMARY

Induction of protein synthesis at the transcriptional level apparently mediates the regulation of transepithelial Na^+ transport by aldosterone. The present communication summarizes the evidence suggesting that initiation of gene activation is a consequence of a series of interactions between aldosterone and a set of protein "receptors". Stereospecific aldosterone-binding proteins (ABP) have been isolated from cytosol and nuclear fractions of adrenalectomized rat kidneys. The nuclear binding system has been resolved into two components, a 3S complex that is soluble in 0·1 M tris-3mM-$CaCl_2$ after pre-treatment with 2·2 M sucrose (i.e. released by "osmotic shock") and a 4S complex tightly bound to chromatin that is released by extraction with 0·3 M KCl. Time-course studies after intravenous injection of ^{3}H-aldosterone into adrenalectomized rats revealed that the cytosol ABP complex (8·5S and 4·5S in glycerol density gradients) appears first and followed soon thereafter by the formation of the 3S soluble and 4S chromatin bound intranuclear complexes. Based on studies with kidney slices and reconstituted cytosol and nuclear fractions, a model has been constructed that proposes that the cytosol ABP complex gives rise to the 3S soluble nuclear complex which in turn results in formation of the 4S chromatin-bound species.

1. INTRODUCTION

THE EXPECTATION that hormonal regulation of transepithelial Na^+ transport is initiated by non-covalent binding of aldosterone to a specific receptor system is based on a variety of results obtained in previous studies: (1) Crabbé[1] recovered all of the [^{3}H]-aldosterone from the isolated toad bladder as the native steroid after the effect on Na^+ transport was complete. (2) Unmetabolized aldosterone is extracted readily from subcellular fractions of rat kidney and from the toad bladder by CH_2Cl_2[2–4]. (3) Radioautographs, by the dry mount technique, showed that [^{3}H]-aldosterone was selectively localized over the nuclei of toad bladder epithelial cells in contrast to the inactive steroid [^{3}H]-progesterone which was randomly distributed between the nuclear and cytoplasmic regions[2, 5]. (4) Induction of protein synthesis initiated by steroidal modification of transcription appears to mediate the action on Na^+ transport[6]. These results suggest that aldosterone-receptor complexes react with the chromatin of target cells to initiate the action. The present paper will review some of the current evidence as to the nature and distribution of these receptors.

2. STUDIES ON THE URINARY BLADDER OF THE TOAD

In addition to the radioautographic evidence cited above of nuclear localization of [^{3}H]-aldosterone in toad bladder epithelium, studies on the time-course of uptake of [^{3}H]-aldosterone revealed that intracellular accumulation of aldosterone reached a steady level 30–60 min before the onset of the effect on Na^+ transport was apparent[2]. Sharp *et al.*[3] used a displacement technique to

distinguish specifically bound from non-specifically adherent aldosterone. With one exception (progesterone) the ability of a steroid to displace aldosterone correlated with its activity on Na^+ transport. In cell fractionation studies, the nuclei of toad bladder epithelium contained 60% of the aldosterone-binding sites [7]. The specificity of these sites was indicated by the findings that agonists (9α-fluorocortisol, DOC, cortisol) and the antagonist (spirolactone) displaced the nuclear [^{3}H]-aldosterone but inactive steroids (testosterone and cholesterol) did not. Recent quantitative radioautographic studies carried out by R. Bogoroch (unpublished results) indicate the existence of mineralocorticoid-binding systems in both the nuclear and extranuclear regions of toad bladder epithelium. Table 1 lists the distribution of grains in sections of toad bladder epithelium exposed to [^{3}H]-d-aldosterone ($5{\cdot}3 \times 10^{-8}$ M) alone or in the presence of cold estradiol-17β ($5{\cdot}3 \times 10^{-6}$ M) or 9α-fluorocortisol ($5{\cdot}3 \times 10^{-6}$ M). Incubation with estradiol-17β had no significant effect on the grain counts. In contrast, 9α-fluorocortisol reduced the nuclear grain count to 32% of the control value and the cytoplasmic grain count to 57% of the control. These results are in accord with the evidence obtained in the rat kidney system of both cytoplasmic and nuclear stereo-specific aldosterone binding systems [9].

Table 1. Distribution of [^{3}H]-aldosterone in toad bladder epithelial cells*

Competitive steroid (100 : 1)	Grains/nucleus	Grains/cytoplasm
none	2·66	0·79
Estradiol-17β	2·75	0·96
9α-fluorocortisol	0·84	0·45

*Toad bladders were exposed to [^{3}H]-aldosterone ($5{\cdot}3 \times 10^{-8}$ M) for 30 min with or without added steroid ($5{\cdot}3 \times 10^{-6}$ M). Average of 200 cells counted per section. R. Bogoroch and I. S. Edelman, (unpublished results).

3. STUDIES ON THE RAT KIDNEY

The distribution among the conventional cell fractions (nuclei, mitochondria, microsomes and cytosol) of the rat kidney of [^{3}H]-aldosterone and aldosterone metabolites was determined [4]. Within physiological plasma concentrations (i.e. 10^{-8} M), the nuclear fraction contained the highest concentration of [^{3}H]-aldosterone compared to the other fractions. Thus at a plasma concentration of 4×10^{-11} M, the distributions of [^{3}H]-aldosterone were: 31×10^{-6} moles/mg protein in nuclei, 11×10^{-16} moles/mg protein in cytosol, 6×10^{-16} moles/mg protein in mitochondria and 4×10^{-16} moles/mg protein in microsomes. Nuclear uptake of [^{3}H]-aldosterone was competitively inhibited by 9α-fluorocortisol but not by 6α-methylprednisolone or estradiol 17β. In the other cell fractions uptake of [^{3}H]-aldosterone was unaffected by 9α-fluorocortisol. That nuclear accumulation of aldosterone involved binding to proteins was suggested by the accelerated release of [^{3}H]-aldosterone from pre-labeled nuclei on incubation with proteolytic enzymes of broad substrate specificity (i.e. pronase and chymotrypsin). In contrast, incubation of pre-labeled nuclei with DNAase, RNAase, lipase, phospholipase D or neuraminidase had no effect on the rate of release of [^{3}H]-aldosterone.

These results imply the existence of intranuclear binding proteins that are specific for mineralocorticoids. Fanestil [8] obtained additional evidence that

the cytosol and nuclear fractions of rat kidney contain mineralocorticoid-specific binding mechanisms with spirolactone, a competitive antagonist of the action of aldosterone on urinary Na^+ and K^+ excretion. Injection of excess spirolactone (SC14266) in the adrenalectomized rat inhibited uptake of [^{3}H]-aldosterone by the nuclear and cytosol fractions but had no effect on plasma concentration of [^{3}H]-aldosterone or on mitochondrial or microsomal binding.

Further progress was made by Herman *et al.*[9] on the isolation and characterization of the aldosterone binding substances in the cytosol and nuclear fractions of the rat kidney. The cytosol aldosterone-binding substances were identified as proteins since binding activity was lost on exposure to wide-spectrum proteases but not by nucleases, lipase or phospholipase. Treatment with SH reagents (e.g. parahydroxymercuribenzoate) also destroyed binding activity. The nuclear binding substances were identified as proteins by similar criteria. Partial purification of these cytosol and nuclear aldosterone-binding proteins (ABP) were achieved with Sephadex chromatography and by precipitation with $(NH_4)_2SO_4$. The specificity of these binding species was indicated by the findings that 9α-fluorocortisol, deoxycorticosterone, 6α-methylprednisolone and estradiol-17β impaired the formation of cytosol and nuclear aldosterone-protein complexes in direct proportion to their potencies as mineralocorticoids. In renal homogenates, moreover, spirolactone inhibited binding of [^{3}H]-aldosterone to cytosol or nuclear ABP at concentration ratios that inhibit the action of aldosterone *in vivo*. The stereoisomer, 17α-isoaldosterone was found to be physiologically inert in the toad bladder system at $1{\cdot}4 \times 10^{-7}$ M and had no effect on urinary Na^+: K^+ ratios at a dose of 1 μg/150 g. body wt in the adrenalectomized rat[10]. ^{3}H-17α-isoaldosterone did not bind to renal ABP nor did the cold stereoisomer inhibit the formation of ^{3}H-aldosterone labeled nuclear ABP at a concentration ratio of 10:1.

Cytosol and nuclear stereospecific ABP have also been identified in other rat tissues[10]. The quantity of these binding species was greatest in the known target tissues, kidney and intestinal mucosa, and significantly less in spleen, liver and brain. The nuclear species of ABP defined in the studies cited above [9, 10] were obtained by centrifuging nuclear fractions through 2·2 M sucrose and then extracting soluble nuclear proteins by resuspending the pellets in 0·1 M tris HCl-3mM $CaCl_2$ (pH $\simeq$ 7·5). This procedure is equivalent to osmotic shock and leaves an additional amount of [^{3}H]-aldosterone bound to nuclear components. Swaneck *et al.*[11] defined the distribution of [^{3}H]-aldosterone in rat kidney nuclei and found that 28% was bound to the protein released by osmotic shock, 55% was firmly bound to chromatin and 17% was associated with the residual (chromatin-free pellet). Based on differential susceptibility to DNAase, RNAase, trypsin and chymotrypsin, distribution in C_3Cl density gradients, chemical analysis of 0·3 M KCl extracts of labeled chromatin and glycerol density centrifugation, the binding substance was identified as a 4S acidic chromosomal protein. Chromatin ABP proved to be stereospecific and in competition studies, the order of affinities for the [^{3}H]-aldosterone binding sites were d-aldosterone > 9α-fluorocortisol > cortisol > estradiol-17β = progesterone = 17α-isoaldosterone. These affinities are in accord with the relative potencies of these steroids as mineralocorticoids. In addition, spirolactone blocked the formation of the chromatin-aldosterone complex at the molar ratio that inhibited the mineralocorticoid response. The procedures employed to date, therefore, have identified three stereo-specific, mineralocorticoid binding proteins, one recoverable from the

cytosol fraction, the second from nuclei subjected to osmotic shock and the third from chromatin by high salt elution.

4. A THREE-STEP HYPOTHESIS

The definition of the cytosol, tris-soluble nuclear and chromatin-bound ABP's raises the question of their interrelationships. In recent studies, (D. Marver, D. Goodman and I. S. Edelman, unpublished observations) the sedimentation properties and the time-course of formation of the cytosol and intranuclear steroid-protein complexes have been determined *in vivo* and in kidney slices *in vitro*.

The sedimentation properties of the cytosol and tris-soluble nuclear ABP's were characterized in glycerol density gradients as earlier studies demonstrated that glycerol (20–30% v/v) stabilized the complexes. Labeled cytosol ABP was prepared from adrenalectomized rat kidneys after intravenous injection or after incubation of unlabeled renal cytosol fractions with [^{3}H]-aldosterone *in vitro* (5×10^{-9} M, at 0°C for 30 min). Two characteristic peaks were obtained, 8·5S and 4·5S, that were eliminated when the binding sites were occupied by cold d-aldosterone or 9α-fluorocortisol. In 0·1 M tris HCl, 1·5 mM EDTA and 0·4 mM KCl, all of the cytosol ABP is recovered as a 4·5S species, suggesting that the 8·5S form is an aggregate of the 4·5S species. In density gradients with or without high salt (i.e. 0·4 M KCl), tris-soluble nuclear ABP sedimented at 3S. Swaneck *et al.*[10] recovered a 4S complex by extraction of chromatin with 0·3 M KCl. A uniform 0·3 M KCl background was required to demonstrate this complex; in glycerol density gradients free of KCl, the steroid-protein complexes formed high molecular weight aggregates. These results, therefore imply the existence of three distinct species of ABP's, a 4·5S cytosol, a 3S tris-soluble nuclear and a 4S chromatin bound species.

The sequence of formation of the three forms of ABP's was defined *in vivo* by intravenous injection of [^{3}H]-aldosterone into adrenalectomized rats and removing kidneys at 2 min intervals. At the earliest time point, 2 min post injection the cytosol complex is at the peak or beginning to decline. In contrast, the 3S soluble and the 4S chromatin bound forms attained their maximum specific activities 10 min post-injection and declined, in parallel, thereafter. To resolve the relationship between the 3S and 4S intranuclear forms these studies were repeated in kidney slices incubated in [^{3}H]-aldosterone at 25°C. The aldosterone-specific binding sites in cytosol were saturated in 10–15 min; the tris-soluble nuclear species formed at a slower rate and increased linearly over a 40 min period; the chromatin-bound appeared at the slowest rate after a 5 min lag period. These results are consistent with the possibility that the 4·5S cytosol complex gives rise to the 3S nuclear complex which in turn generates the 4S chromatin-bound complex.

A further test of this hypothesis was obtained in reconstitution experiments (D. Marver and I. S. Edelman, unpublished observations). Washed unlabeled rat kidney nuclei were mixed with pre-labeled cytosol for varying intervals of time. Concomitant with the fall in cytosol content of [^{3}H]-aldosterone-protein complex, there was an early appearance of the 3S soluble nuclear complex followed by the later appearance of 4S chromatin-bound form. The sequence of events therefore appears to be a transfer or conversion of the cytosol 4·5S complex to the 3S nuclear complex which in turn gives rise to the 4S chromatin-bound form.

Funder *et al.* (J. Funder, D. Feldman and I. S. Edelman, unpublished observations) recently compared cytosol donor and nuclear acceptor activity in rat kidney and parotid gland slices. Their findings indicate that the parotid gland contains about 20 times more capacity for formation of the chromatin-bound species than the kidney. The relationship between the quantitative steady state levels of aldosterone-ABP complexes in intranuclear compartments and steroid regulation of transcription remains to be studied. In any case, the parotid gland may prove to be an advantageous tissue in which to study the determinants of cytosol donor and nuclear acceptor activity.

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DISCUSSION

Pasqualini: Do you have any data about the metabolism of aldosterone in the cytosol binding fraction? How much of unchanged aldosterone is present in this fraction?

Edelman: All of the bound steroid is unmetabolized native aldosterone.

Pasqualini: In a series of *in vivo* and *in vitro* studies that we carried out on guinea pig foetal kidney, we found the formation of [^{3}H]-aldosterone receptors in this foetal tissue (Pasqualini J. R. and Sumida C., *C. R. Acd. Sci.* (*Paris*) **273** (1971) 1061). We observed that after incubation of [^{3}H]-aldosterone with foetal kidney (10μCi($2{\cdot}2\times10^{-10}$ mol) of [1,2-^{3}H]-aldosterone (gr. of tissue) the aldosterone bound in the total nuclear extracts are 1·5–1·8 times that bound in the cytosol. We also found that after incubation of [^{3}H]-aldosterone with purified nucleus of the same tissue, the specific activity per mg of protein in the macromolecules complexes of the different nuclear extracts was higher than in the corresponding extracts after incubation of the total cell. These data suggest the possibility that the [^{3}H]-aldosterone macromolecule complexes in the nucleus can be also formed directly from unbound aldosterone. (Pasqualini J. R., Sumida C. and Gelly C.: *J. Steroid Biochem.* **3** (1972) in press).

Edelman: I am not familiar with the binding properties of the foetal kidney but in the kidney of the adult rat studied *in vitro*, formation of the intranuclear complexes requires the presence of cytosol *d*-aldosterone-receptor complexes. We have no evidence that the cytoplasmic to nuclear transfer steps involve steroid metabolizing enzyme systems.

Pasqualini: It is possible that the mechanisms of formation of the aldosterone-macromolecule complexes are different in the foetal period.

Edelman: We will hear more about the determinants of aldosterone binding to

specific sites in specific tissues in the talks to be given by Dr. Funder and Dr. Rousseau.

Handler: What's the evidence that the nuclear soluble product becomes the protein that binds to the chromatin?

Edelman: The appearance of the tris-soluble nuclear complex precedes the appearance of the chromatin-bound species in the *in vitro* experiments.

Handler: The 3S component seems to be continuing to increase.

Edelman: In both kidney slice and reconstitution experiments, the 3S complex is generated more rapidly than the chromatin-bound 4S complex. If this is a system in series, this would imply that the 3S complex is a precursor of the 4S product.