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Cell Biology

Identification of aldosterone-induced proteins in the toad's urinary bladder

(sodium transport/corticosterone/protein synthesis)

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ABSTRACT **Aldosterone (the 8,11-hemiacetal of 11β,21-dihydroxy-3,20-dioxo-4-pregnen-18-al) markedly stimulates sodium transport in a number of epithelial tissues. We have attempted to determine whether aldosterone induces the synthesis of specific protein(s) in the course of its action upon the toad urinary bladder. Paired hemibladders were incubated in media containing either [^{3}H]methionine or [^{35}S]methionine; aldosterone in physiologic concentrations was added to one bath and, after incubation, the intact "mitochondria-rich" (MR) and "granular" (G) mucosal cells were isolated. The ratio (^{3}H/^{35}S) was used to identify proteins whose synthesis was induced in the mucosal cells of the steroid-treated bladders. Using exclusion gel chromatography and isoelectric focusing, we identified several aldosterone-induced proteins in the supernatant (105,000 × *g*) fraction of the MR cell. None was evident in this fraction of the G cell. These proteins have apparent molecular weights ranging from 17,000 to 38,000 and the isoelectric point of the major component is 4.5. Corticosterone (11β,21-dihydroxy-4-pregnene-3,20-dione) induced the synthesis of proteins in the G cells, but none of these proteins was similar in molecular weight to the aldosterone-induced proteins in the MR cell. Our findings support the hypothesis that aldosterone induces the synthesis of specific proteins and indicate that, in this tissue, these proteins are synthesized by the MR cell.**

Aldosterone (the 18,11-hemiacetal of 11β,21-dihydroxy-3,20-dioxo-4-pregnen-18-al) stimulates the active transport of sodium by the kidney tubule and the toad's urinary bladder (1), a tissue widely used as a model for the distal portion of the mammalian kidney tubule. The biochemical processes leading to this physiologically important effect are in dispute. Suggested mechanisms include (*1*) enhancement of the apical membrane permeability to sodium, (2) stimulation of the sodium "pump" on the basal membrane of the mucosal cell, and (3) an increase in the metabolic processes energizing sodium transport (2). Because inhibitors of protein and RNA synthesis block the hormonal stimulation of sodium transport, it has been suggested that aldosterone induces the synthesis of proteins directly related to the sodium transport (3). Isolation and characterization of the steroid-induced proteins, if they exist, should resolve the basic mechanism of aldosterone's effects upon transport.

Using a technique for separating the two major morphologic cell types present in the epithelium of the toad bladder (4), we found that the specific binding of aldosterone is limited to the "mitochondria-rich" (MR) epithelial cell (5). Localization of mineralocorticoid binding in the MR cell, which accounts for only 15–30% of the mucosal cell population, indicates that the initial biochemical events leading to enhanced sodium transport may take place in the MR cell; these data presented a unique opportunity to further explore the effects of aldosterone upon the tissue. Thus, by determining the effects of steroid upon protein synthesis in the MR cell and in the more numerous "granular" (G) cell, we sought not only to obtain enrichment of putative aldosterone-induced protein(s) in our preparation, but also to obtain further evidence that the MR cell was the site of the initial effects of aldosterone. We incubated intact bladders in radioactively labeled amino acids with and without aldosterone, separated the MR and G cells, and chromatographed the labeled proteins. Our results indicate that physiologic levels of aldosterone induce the synthesis of protein by the MR cell.

MATERIALS AND METHODS

Incubation of Isolated Toad Bladders. Toads (*Bufo marinus*) of Colombian origin (Tarpon Zoo, Inc., Tarpon Springs, Fla.) were partially immersed in a solution of 0.6% sodium chloride for 5–8 days before use to suppress the secretion of endogenous aldosterone. Paired hemibladders were incubated at room temperature in two baths of Ringer solution (pH = 7.2–7.6) whose composition was: NaCl, 85 mM; KCl, 4 mM; $NaHCO_3$, 17.5 mM; KH_2PO_4, 0.8 mM; $MgSO_4$, 0.8 mM; $CaCl_2$, 1.5 mM; and glucose, 10 mM. One bath contained either aldosterone or corticosterone (2×10^{-8} M). After 1 hr [^{3}H]methionine (10 Ci/mmol; New England Nuclear Co., Boston, Mass.) was added to one bath, and [^{35}S]methionine (100 Ci/mmol; New England Nuclear Co.) to the other; the final concentration of methionine in each bath was 1 to 3×10^{-6} M. Following an additional hour incubation, the epithelial cells were removed and separated as described below.

Separation of Mitochondria-Rich and Granular Cells. The bladders were mounted as sacs on Luer-lock syringes and filled with and immersed in EDTA-Ringer's solution lacking calcium and methionine. Following incubation in EDTA-Ringer's for 45 min at room temperature the disaggregated mucosal cells from each set of bladders were collected and sedimented at 800 × *g* for 15 min. The cells from the two sets of hemibladders were separately suspended in EDTA-Ringer's, layered over discontinuous gradients of Ficoll (Pharmacia, Piscataway, N.J.), and centrifuged at 27,000 rpm for 45 min in a Beckman SW27 rotor at 4° (4). The bands of material containing mitochondria-rich (MR) cells (density = 1.035 g/cm^3) and granular (G) cells (1.067 g/cm^3) were removed separately, diluted with EDTA-Ringer's, and sedimented. The MR cells from the two sets of hemibladders were then mixed, sonicated with a Branson

Abbreviations: MR cells, "mitochondria-rich" cells; G cells, "granular" cells.

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sonifier for two 15-sec intervals, and centrifuged at 105,000 × *g* for 30 min. The two sets of G cells were treated in the same way.

Sephadex Chromatography of the Cell Extracts. The 105,000 × *g* supernatant fractions of the MR and G cells were each dialyzed against water and lyophilized. The samples were dissolved in a small volume of 0.025 M Tes [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] and chromatographed on a column (2.5 × 80 cm) of Sephadex G-75 equilibrated with Tes, 0.025 M (pH 7.0).

Isoelectric Focusing of Column Eluates. Eluates of the Sephadex column were loaded onto an LKB model 8101 isoelectric focusing column in a solution of 2% carrier ampholytes (pH 3–10). The sample was stabilized with a density gradient of sucrose and was electrofocused for approximately 72 hr. The initial voltage was 250 V and was progressively increased to 600 V, with the power never exceeding 2 W.

Acrylamide Gel Electrophoresis. Samples from the isoelectric focusing column were dialyzed for 24 hr against Tris–glycine buffer (pH 8.9) containing 0.1% sodium dodecyl sulfate. The samples were analyzed by electrophoresis in a discontinuous gradient (4, 10, and 12%) of acrylamide using the same Tris–glycine buffer (pH 8.9) and 0.1% sodium dodecyl sulfate (6).

RESULTS

Separation of MR and G Cells. The mucosal cells were removed from the intact hemibladders, half of which had been exposed to aldosterone, by immersing the tissues in EDTA-Ringer. Centrifugation of these cells on discontinuous gradients of Ficoll yielded two bands of intact mucosal cells greatly enriched in their content of MR and G cells, respectively (4). Rebanding of the MR and G cell preparations showed that the sedimentation properties of the two preparations were reproducible, with little cross-contamination (Fig. 1).

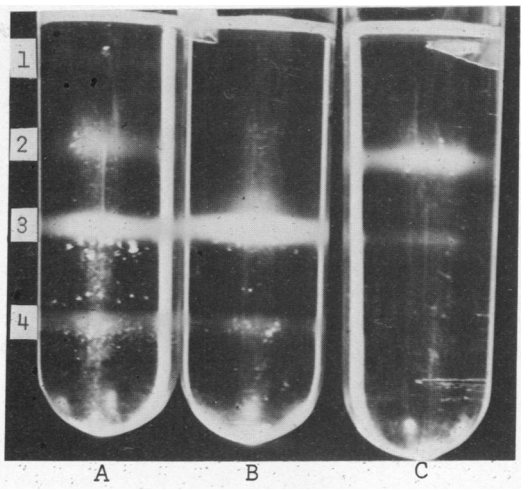

FIG. 1. Density gradient centrifugation of disaggregated toad bladder mucosal cells. Mucosal cells were removed in EDTA-Ringer's from bladders of six toads and layered over a discontinuous gradient of Ficoll (4) in six tubes. Centrifugation at 27,000 rpm for 45 min yielded four bands of material (Tube A). Bands 2 and 3, containing MR and G cells, respectively, were recovered from the remaining five tubes and pooled. The Band 2 and Band 3 preparations were each recentrifuged in a separate tube of the same gradient. The recentrifuged Bands 3 (Tube B) and 2 (Tube C) are compared to one of the original six tubes (A).

Exclusion Gel Chromatography of the Supernatant Fractions. The MR cells prepared from the two sets of hemibladders ([^{3}H]- and [^{35}S]methionine) were mixed and sonicated, and the supernatant fraction was prepared by centrifugation. Exclusion gel chromatography of this preparation consistently yielded a series of fractions which contained a relatively greater amount of that radioactive label to which the aldosterone-treated tissue had been exposed. This enhancement by aldosterone of methionine incorporation was demonstrated in 12 experiments and was observed with either nuclide (^{3}H or ^{35}S) in the aldosterone bath. In the experiment illustrated in Fig. 2A, [^{3}H]methionine was added to the aldosterone-containing bath and the baseline ratio (^{3}H/^{35}S) was 0.55. In those fractions whose elution volumes corresponded to molecular weights ranging from 17,000 to 38,000, this ratio was increased significantly, reaching a value of 2.7. The pattern of elution of the labeled material indicated the presence of at least three components.

The supernatant fraction of the G cells from the same experiment was chromatographed on the same column and the ratio (^{3}H/^{35}S) did not increase above baseline values (Fig. 2B). This indicates that aldosterone did not induce the synthesis of "soluble" proteins in the G cells of the intact bladder, although the large amount of radioactivity appearing in the void volume of the column demonstrates that these cells were actively synthesizing protein.

Isoelectric Focusing of the Sephadex Eluate. Those fractions of the MR cell Sephadex eluate having an elevated (^{3}H/^{35}S) ratio were pooled for analysis by isoelectric focusing. In the analysis illustrated in Fig. 3A, the pooled fractions included the eluate from 240 to 285 ml. Isoelectric focusing of this material yielded several peaks of enhanced ratio, one with a value as high as 11.7 (compared to an initial baseline of 0.55). The peak at pI = 4.5, characteristically the most prominent feature of the isoelectric focusing analysis of the MR cell preparation, was shown in other experiments to correspond primarily to the higher molecular weight fractions of the pooled Sephadex eluate.

Although the Sephadex eluate of the G cell supernatant fraction showed no apparent increases in the (^{3}H/^{35}S) ratio, we pooled those fractions of the G cell Sephadex eluate corresponding to the MR cell fractions with an increased ratio (240–285 ml) and analyzed this material by isoelectric focusing. As shown in Fig. 3B, the material did not yield any fractions with an increased (^{3}H/^{35}S) ratio.

Sodium Dodecyl Sulfate-Acrylamide Gel Electrophoresis of the Isoelectric Focusing Eluate. Isoelectric focusing eluate from the peak at pI = 4.5 was analyzed by electrophoresis in a sodium dodecyl sulfate-acrylamide gel system. The sample contained two bands whose molecular weights were estimated at approximately 12,000 and 20,000.

Glucocorticoid Induction of Protein Synthesis in Mucosal Cells. The effects of corticosterone (11β,21-dihydroxy-4-pregnene-3,20-dione), the natural glucocorticoid of *Bufo marinus* (7), upon the incorporation of labeled methionine into protein were also studied. Using the same number of toads (108) and the same experimental conditions, corticosterone (5×10^{-8} M) was added to the hemibladders incubated in [^{3}H]methionine while the [^{35}S]methionine hemibladders served as a control. The ratio (^{3}H/^{35}S) in the Sephadex eluate of the MR cell preparation is strikingly different from that obtained in the aldosterone-treated tissue—there were no significant increases above baseline in this preparation. On the contrary, the supernatant fraction of the G cells from the corticosterone-treated tissue yielded a series of fractions with elevated ratios (Fig. 4). As indicated by their elution volumes, all these fractions in the G cell prepa-

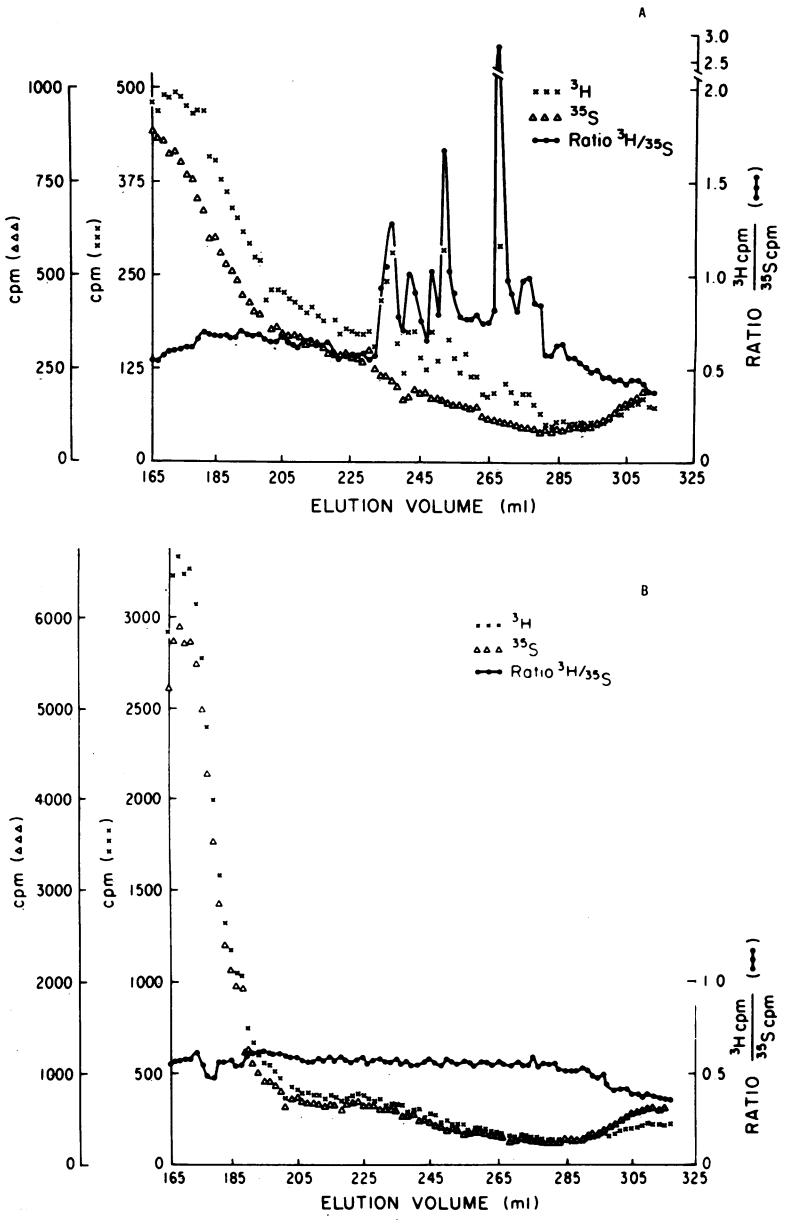

FIG. 2. Aldosterone induction of protein synthesis. Paired hemibladders were incubated in sets of twelve, one set in [^{35}S]methionine and one in [^{3}H]methionine plus aldosterone (2×10^{-8} M). MR and G cells were separated in each set, and the MR cells and G cells were each pooled and sonicated. The MR and G supernatant fractions (105,000 × *g*) of nine such experiments (108 toads) were collected, dialyzed against water, lyophilized, and chromatographed on a column (2.5 × 80 cm) of Sephadex G-75. Fractions of 1.7 ml were collected and the radioactivity in 400 μl aliquots was measured by liquid scintillation counting. (A) MR cell preparation (6.0 mg of protein chromatographed); (B) G cell preparation (8.88 mg of protein).

ration had molecular weights greater than those proteins induced by aldosterone in the MR cell. The fractions of the Sephadex eluates of the MR cells and the G cells with elution volumes of 240–285 ml were pooled and analyzed by isoelectric focusing. There was no evidence for the induction of any proteins similar to those induced in the MR cell by aldosterone.

DISCUSSION

A number of steroid hormones, including estradiol (8), vitamin D_3 (9), progesterone (10), and cortisol (11), induce the synthesis of specific proteins. Experiments with inhibitors of protein and RNA synthesis suggested that a similar sequence of events was involved in the action of mineralocorticoids upon sodium transport (3), but did not exclude the possibility that the observed effects were due to the inhibition of protein degradation, rather than the stimulation of protein synthesis (12). Until the recent experiments of Benjamin and Singer (13), there was no direct evidence for an effect of aldosterone upon the synthesis of a specific protein. However, the protein implicated by these authors may not be related to sodium transport because (*1*) it was obtained with glycogenic (14) concentrations of aldosterone (10^{-6} M), (2) it was also induced by insulin, and (3) it was not induced by high concentrations of dexamethasone, a steroid having potent mineralocorticoid properties in toad bladder as measured both by specific tissue binding (5) and natriferic activity (15).

Our experiments, which clearly demonstrate that aldosterone induces the synthesis of protein(s), indicate that the

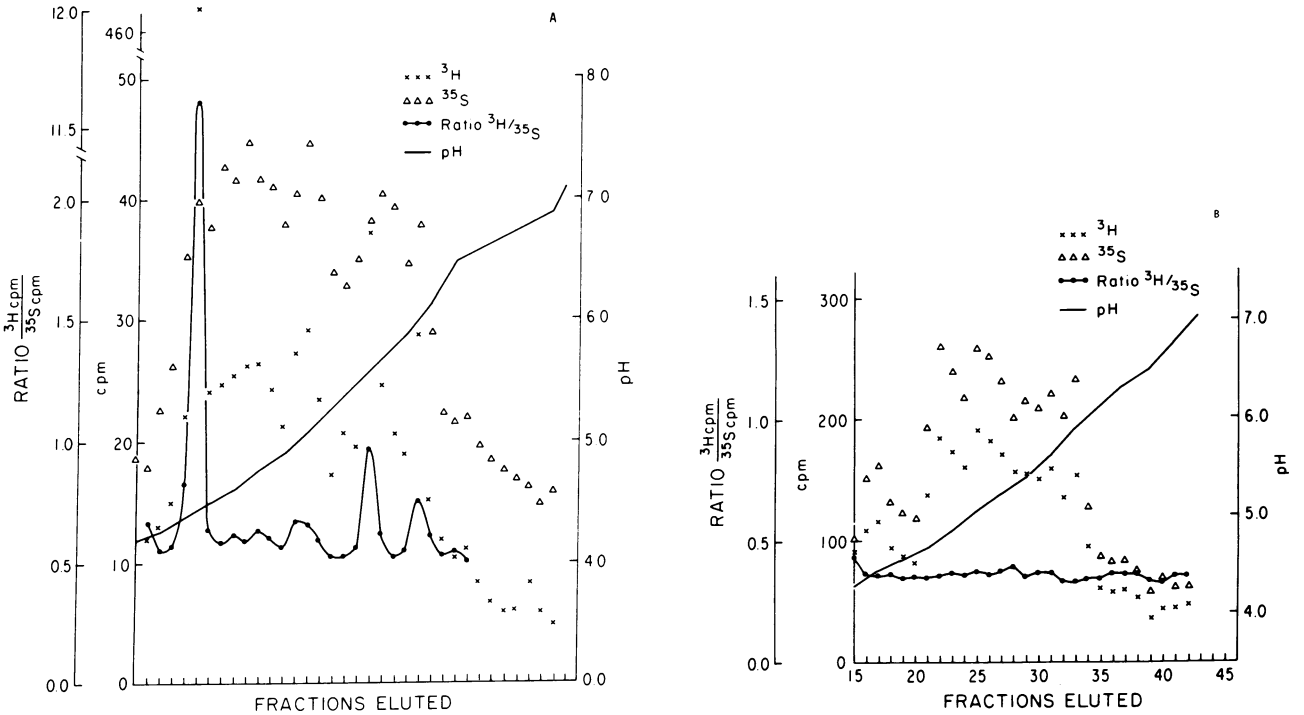

FIG. 3. Isoelectric focusing of eluate from exclusion gel chromatography. Eluates with an enhanced ratio in the experiments in Fig. 2 were pooled, loaded onto an LKB 8101 column with pH 3–10 Ampholine, and electrofocused for 72 hr (600 V). Fractions of 0.6 ml were collected and counted. (A) Fractions from Sephadex G-75 column containing elution volumes 240–285 ml from the MR cell preparation; (B) Fractions from the Sephadex G-75 column containing elution volumes 240–285 ml from the G cell preparation.

basic mode of action—induction of the synthesis of specific proteins—is the same as other steroid hormones. The significance of the fact that the bulk of the induced protein has a single isoelectric point (4.5), even though there is considerable heterogeneity in molecular weight is not clear at this time. The isoelectric point obtained for this induced protein (4.5) has taken on a new significance since our recent observation that aldosterone induces in rat kidney the synthesis of a soluble protein having a similar pI (16).

The apparent limitation of the steroid-induced synthesis of protein to the the MR cell is consistent with our studies of the specific binding of aldosterone in the toad bladder (5) and assures approximately a 4-fold enrichment of induced protein merely by separating the two major morphologic types of mucosal cells. Localization of the initial effects of aldosterone to the MR cell is also consistent with the observations that aldosterone potentiates the effects of vasopressin (2), and vasopressin raises the cyclic AMP levels of the MR cell but not the G cell (4). However, consideration of the MR cell as the primary target of both steroid hormones and neurohypophyseal hormones requires a reexamination of the structure and function of the toad bladder mucosal epithelium. It seems unlikely that all the hormonally induced changes in transepithelial transport occur through the MR cell because (1) the area of this flask-shaped cell exposed to the urine is so small that the tissue resistance should be too great to accommodate the observed fluxes, and (2) there is direct evidence for the movement of water into the G cell

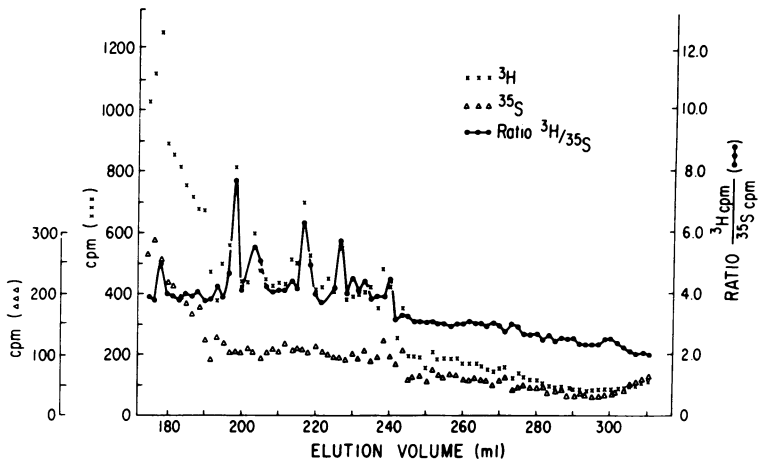

FIG. 4. Corticosterone induction of protein synthesis in the G cell preparation. The experimental conditions were identical to those in Fig. 2 except that aldosterone was replaced by corticosterone. The 105,000 × *g* supernatant fraction (10.0 mg of protein) was chromatographed on a Sephadex G-75 column (2.5 × 80 cm).

during vasopressin-stimulated hydroosmotic flux (17). On the other hand, our evidence that the MR cell is the site of action of both neurohypophyseal and steroid hormones, together with the recent description of the stellate arrangement of G cells around each MR cell (18), suggests the possibility that the MR cells recognize and amplify the hormonal stimuli and, by some device of intercellular cooperation, regulate the transport activity of the more numerous G cells. The recent observations that cyclic AMP causes a considerable increase in the permeability of epithelial membranes to ions (19) and various metabolites, including nucleotides, may be exchanged between contiguous cells (20), indicate that cyclic AMP may have a role in intercellular communication in this epithelium.

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