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SUBCELLULAR LOCALIZATION OF ALDOSTERONE-INDUCED PROTEINS IN TOAD URINARY BLADDERS

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Summary

Paired toad urinary hemibladders were incubated with [^{35}S]methionine in the presence (experimental) or absence (control) of aldosterone. Short-circuit current was used to monitor aldosterone-induced Na^+ transport. Protein synthesis in epithelial cell subcellular fractions (cytosolic, microsomal, mitochondrial) was evaluated by gradient polyacrylamide gel electrophoresis and autoradiography. Aldosterone-induced proteins were identified in the cytosolic and microsomal fractions (70 000 and 15 000 daltons, respectively). These results represent the first demonstration of aldosterone-induced proteins in subcellular fractions of epithelial cells derived from single toad urinary hemibladders.

Aldosterone stimulates Na^+ transport in a variety of renal epithelia including the toad urinary bladder [1]. The mechanism of action of aldosterone has been subjected to considerable scrutiny, but a complete description of the cellular events involved is still lacking [1,2]. Most of the earlier evidence that aldosterone-induced Na^+ transport is mediated through new protein synthesis rests on inhibitor studies [3,4]. More recently, several putative aldosterone-induced proteins have been identified [5—9]. However, it is not clear which of these candidates, if any, has a role in determining the magnitude and time course of aldosterone-induced Na^+ transport and which represent epiphenomena secondary to the marked cellular metabolic changes that are the result, rather than the cause, of the enhanced Na^+ transport. In addition, the func-

tion(s) of the aldosterone-induced protein(s) is (are) completely unknown. The resolution of these questions is dependent on the development of a model system which allows for the correlation of the hormone's electrophysiological and biochemical effects. This communication describes such a model system and reports the presence of aldosterone-induced proteins in the cytosolic and microsomal fractions of epithelial cells derived from single toad urinary hemibladders.

Dominican toads (*Bufo marinus*) were maintained in the unfed state in distilled water for at least 48 h prior to use. The animals were killed by pithing and the bladders were surgically removed. Paired hemibladders from single animals were mounted as sacs (mucosa inside) on glass tubes, were filled with 2 ml of incubation medium, and were suspended in 5 ml of continuously aerated incubation medium containing either aldosterone (final serosal concentration, $1.8 \cdot 10^{-7}$ M; Sigma) or methanol carrier. Each animal therefore provided both an experimental and a control hemibladder. The incubation medium (pH 7.90 ± 0.05, osmolality 230 ± 10 mosM/kg) contained 1 part methionine-free Medium-199 (Grand Island Biological Company) and 9 parts modified Ringer's solution. The latter consisted of 5 mM Tris, 5 mM glucose, 103 mM NaCl, 3.4 mM Na_2HPO_4, 3.0 mM KCl, 0.6 mM KH_2HPO_4 and 0.75 mM $CaCl_2$.

After 3 h incubation, the hemibladders were transferred to identical (serosal) solutions containing [^{35}S]methionine (specific activity, 600—1400 Ci/mmol, final concentration, 125 μCi/ml; Amersham) and were then incubated for an additional 4 h. Short-circuit current was used to measure net Na^+ transport [10]. The mean aldosterone-induced increase in short-circuit current after 7 h incubation was 220 ± 43% (n = 7). All bladders responded to aldosterone (a typical response is illustrated in Fig. 1).

The epithelial cells were scraped from the underlying connective tissue (using a glass microscope slide), collected by centrifugation (1200 × g, 5 min) and homogenized in a tight-fitting Dounce homogenizer (40 strokes). The homogenate was centrifuged (1200 × g, 5 min) to remove cellular debris and the resulting supernatant was centrifuged (15 000 × g, 20 min) to yield a mitochondrial fraction (pellet). The 15 000 × g supernatant was centrifuged (120 000 × g, 60 min) to yield a microsomal fraction (pellet) and a cytosolic fraction (supernatant). The latter was dialyzed against 5 mM Tris-HCl, pH 7.5, for 16—20 h (using 2000 dalton cut-off tubing) and then lyophilized.

The three subcellular fractions were solubilized in a sodium dodecyl sulfate (SDS)-containing buffer (1% SDS, 1% β-mercaptoethanol, 10% glycerol in 0.05 M Tris-HCl, pH 6.8) and aliquots of each fraction were subjected to electrophoresis in 7.5—30% gradient acrylamide gels [11]. The radioactive proteins in each subcellular fraction were analyzed autoradiographically [12]. Molecular weights were estimated from the relative mobilities of standard protein mixtures in the same system.

The autoradiograms of the mitochondrial fractions derived from aldosterone-treated and control hemibladders were identical in each of six separate experiments. However, differences existed in both the cytosolic and the microsomal fractions. A cytosolic aldosterone-induced protein (~70 000 daltons) was identified in each of 7 experiments and a microsomal aldosterone-induced

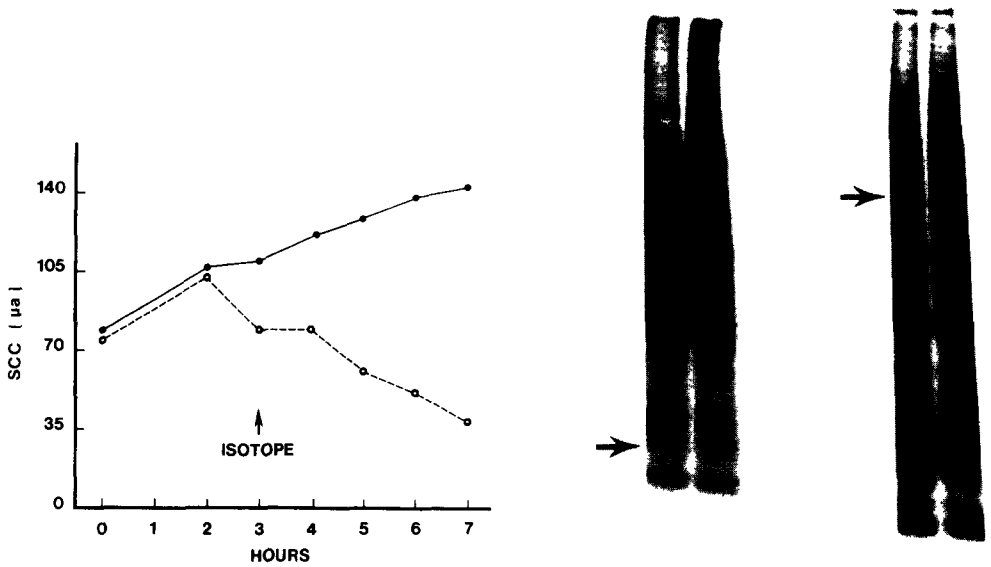

Fig. 1. Short-circuit current (SCC) response to aldosterone. At time zero, one hemibladder (of a pair from a single toad) was treated with aldosterone (final serosal concentration, $1.8 \cdot 10^{-7}$ M) (●———●) and the other was treated with carrier (methanol) (○- - - - -○) (control). After 3 h incubation, both hemibladders were exposed to [^{35}S]methionine (final serosal concentration, 125 μCi/ml) and then monitored for an additional 4 h. Net Na^+ transport was measured by briefly short-circuiting the bladders at hourly intervals. The magnitude (245%) and time course of the aldosterone response in this experiment were typical. (The mean ± S.E. resistances for the entire series were 3657 ± 381 and 4176 ± 165 Ω · cm^2 in the control and aldosterone-treated hemibladders, respectively, at time zero and 5037 ± 736 and 2281 ± 602 Ω · cm^2 in the control and aldosterone-treated hemibladders, respectively, at hour 7.)

Fig. 2. Autoradiographic demonstration of aldosterone-induced proteins. Aliquots of the subcellular protein fractions (containing 100 000—500 000 dpm) were subjected to electrophoresis (75 V for 24 h at 4°C) in 7.5—30% gradient polyacrylamide gel slabs (1 × 90 × 150 mm). The gels were then fixed (20% sulfosalicyclic acid for 45 min), stained (0.25% Coomassie blue in 7% acetic acid for 18 h), destained and dehydrated (50% methanol and 7% acetic acid for 48 h), dried, and exposed to Kodak XR-1 film for 7—10 days. The microsomal aldosterone-induced protein (→) is indicated in the autoradiogram on the extreme left as compared to its adjacent control. The cytosolic aldosterone-induced protein (→) is indicated in the autoradiogram third from the left as compared to its adjacent control. Although there are other (minor differences between the autoradiograms obtained from control and aldosterone-treated bladders, the differences indicated (→) were the only ones consistently seen in all experiments.

protein (~15 000 daltons) was identified in 6 of 7 experiments (Fig. 2). Densitometric tracings of the autoradiograms (Figs. 3 and 4) confirmed these findings.

We have previously reported a specific aldosterone-induced protein (approx. 12 000 daltons) in total epithelial cell homogenates obtained from single toad urinary bladders [5]. This aldosterone-induced protein is most likely the same protein that we have identified in the microsomal fraction in the present experiments. In addition, we have identified at least one other aldosterone-induced protein (in the cytosolic fraction). Other investigators [6,7,9] have also described aldosterone-induced proteins of various molecular weights in cytosolic and/or microsomal fractions obtained from epithelial cell homo-

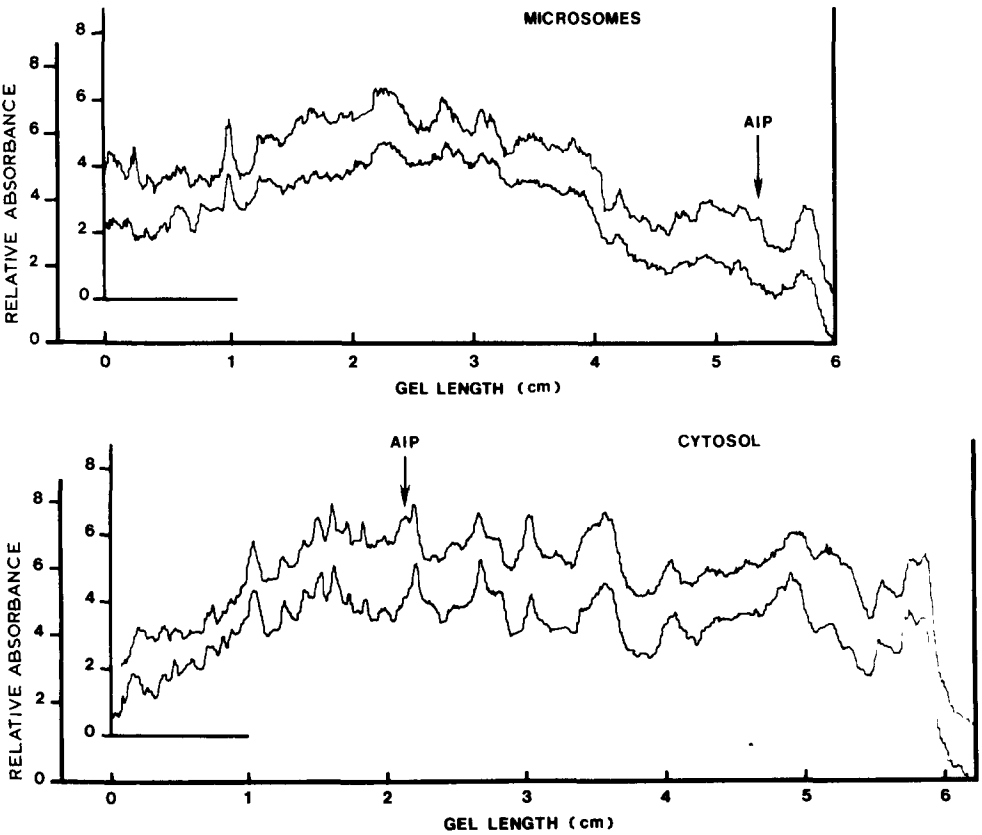

Fig. 3. Densitometric scans of autoradiograms derived from microsomal and cytosolic proteins. Autoradiograms of microsomal proteins (top panel) obtained from aldosterone-treated (upper tracing) and control (lower tracing) hemibladders were scanned at 600 nm using a Teledyne Model 2955 densitometer in the transmittance mode. The abscissa represents distance from the origin (0) in cm. The ordinates represent relative absorbance (the inner and outer ordingates relate to the upper and the lower tracings, respectively). The microsomal aldosterone-induced protein (AIP) is indicated by an arrow in the upper tracing. Autoradiograms of cytosolic proteins (bottom panel) obtained from aldosterone-treated (upper tracing) and control (lower tracing) hemibladders were scanned in a similar fashion. The cytosolic aldosterone-induced protein is indicated by an arrow in the upper tracing.

genates of toad urinary bladders. However, since the latter experiments were performed with pooled homogenates obtained from large numbers of bladders, the physiological response to aldosterone could not be simultaneously monitored. Aldosterone has also been shown to stimulate protein synthesis in all the non-nuclear subcellular fractions derived from rat renal medulla [8]. Detailed analysis of the rat medullary cytosolic fraction revealed an approx. 31 000 dalton aldosterone-induced protein (other subcellular fractions were not analyzed). The analysis of the relationship of these putative aldosterone-induced proteins to one another and of the role of particular aldosterone-induced proteins in aldosterone-induced Na^+ transport awaits further studies.

The analytical procedures described above have resulted in the first detection of aldosterone-induced proteins in subcellular fractions (microsomal and cyto-

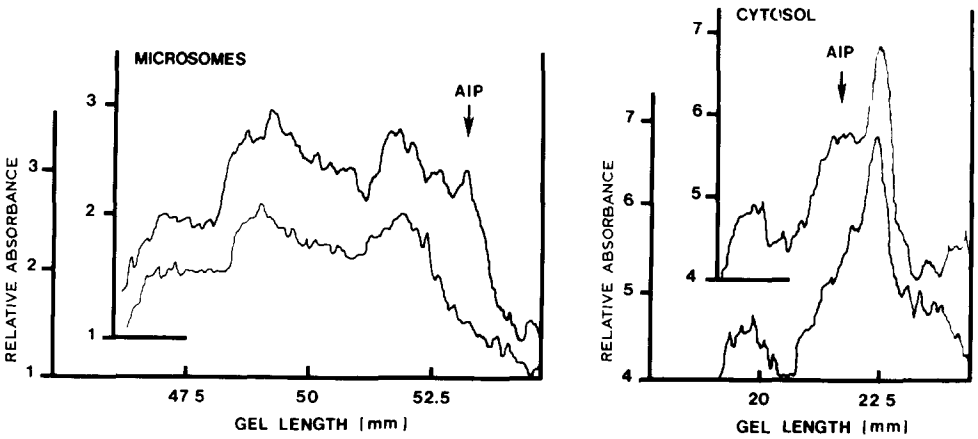

Fig. 4. Expanded densitometric scans of autoradiograms derived from microsomal and cytosolic proteins. Expanded densitometric scans of the relevant portions of the microsomal and cytosolic autoradiograms better define the microsomal (left) and cytosolic (right) aldosterone-induced proteins. The axes are similar to those in Fig. 3. The presence of the two aldosterone-induced proteins was confirmed by comparing the areas (mean ± S.E.) under the relevant portions of the scans derived from control and aldosterone-treated tissue. Microsomes: 3.71 ± 1.22 (control) and 4.56 ± 1.49 (aldosterone), $P < 0.05$, $n = 7$. Cytosol: 2.72 ± 0.36 (control) and 3.46 ± 0.26 (aldosterone), $P < 0.025$, $n = 7$ (Student's t-test). AIP, aldosterone-induced protein.

solic) of epithelial cells obtained from single toad urinary hemibladders. This model system has the major advantage that biochemical changes can be directly correlated with experimentally induced changes in Na^+ transport. Consequently, this approach may prove useful in defining the cellular role of aldosterone-induced proteins in epithelial Na^+ transport.

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