Aldosterone-induced proteins in primary cultures of rabbit renal cortical collecting system

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Abstract

Primary cultures of immunodissected cells from rabbit kidney connecting tubule and cortical collecting duct were used to study aldosterone's action on transcellular Na^+ flux. Incubation with 10^{-7} M aldosterone stimulated transcellular Na^+ transport which was detected as an increase in benzamil-sensitive short-circuit current. The stimulatory response was consistently noted after 2 h of incubation and stabilized after 6 h. 2D-PAGE was used to identify proteins which were induced concurrently with the increase in transcellular Na^+ flux after an aldosterone incubation of 15 h. Three aldosterone-induced proteins (AIPs; M_r = 100, 70–77 and 46–50 kDa) were found in the membrane and microsomal fractions. Two of these appeared to have more than one isoform. A single heterogeneous AIP (M_r = 77 kDa) was detected in the soluble fraction.

Keywords: Amiloride-sensitive sodium transport; Aldosterone-induced protein; Sodium channel; High resistance epithelium; Benzamil; Amiloride

1. Introduction

A variety of hormones modulate ion transport processes in the distal portions of the nephron. The complex anatomy of the kidney makes it difficult to obtain sufficient amounts of pure distal epithelial tissue to perform correlative structural and functional studies elucidating the mechanisms governing hormonal modulation of transepithelial ion flux. While electrophysiological studies using defined segments of the nephron have provided a wealth of functional data, much of our understanding of the biochemical mechanisms of hormonal action has been derived from high-resistance model epithelia such as the toad urinary bladder and the A6 cell line. To examine hormonal actions in mammalian tissues, an isolated epithelial system analogous to the amphibian models would be very useful.

Aldosterone is one of the major hormones which regulates Na^+ reabsorption in the distal portions of the nephron. As a classic steroid hormone, aldosterone exerts its action via the synthesis of new proteins (aldosterone-induced proteins; AIPs) and several putative AIPs have been identified [1–3]. In amphibian model tissues, a group of these

appear as a constellation of electrophoretically polymorphic (M_r 65 and 70 kDa) and microheterogenous (pI 5.6–6.4) polypeptides by 2D-PAGE analysis [2,3]. The 65–70 kDa AIP has been correlated with aldosterone's natriferic action, is physically associated with an amiloride-sensitive Na^+ channel, and is the only subunit of this channel induced in response to aldosterone [4,5]. While it is clear from both this biochemical data as well as electrophysiological studies that one of the cellular targets of aldosterone's effect appears to be the amiloride-sensitive Na^+ channel, the exact mechanism of this stimulation remains unknown [1,6].

Another impediment to studies of aldosterone's action is the uncertainty regarding the characteristics of the cellular target of the hormone, namely the amiloride-sensitive Na^+ channel. Based on both structural and functional analysis, the channel is not a single entity but a family with some relatively diverse members [6]. The first of these transporters to be described was a renal amiloride-sensitive Na^+ channel which is composed of 5–6 non-identical subunits forming a complex with a molecular weight of 700–800 kDa [7]. One of the subunits appears to be the alpha component of the trimeric G protein, G_{i-3} but the exact identity of the other subunits is unknown. More recently amiloride-sensitive Na^+ channels from mammalian colon, lung and kidney have been identified and

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characterized by expression cloning [8–11]. In these studies, the Na^+ channel properties as well as the amiloride sensitivity appear to reside in a single polypeptide. However, the transport activity can be markedly potentiated by co-expression of two other moderately homogeneous peptides [12,13]. The relationship of the mammalian channels identified by expression cloning to the previously characterized large Na^+ channel complex is unknown.

In order to develop a viable mammalian cultured cell model, immunodissection techniques were used to isolate specific cell types from rabbit kidneys. Connecting tubule and cortical collecting duct cells were isolated using R2G9, a monoclonal antibody which recognizes a cell-surface epitope found only in these nephron segments [14]. Hereafter, we refer to these immunodissected cells as cortical collecting system cells. The primary cultures established from these isolated cells have been shown to exhibit many characteristics of the native tissue [14–16]. At confluence the cultures express a lumen-negative transepithelial potential difference and a moderately high resistance. Removal of apical Na^+ or addition of amiloride to the apical side reversed the potential difference and increased the transepithelial resistance. The cellular monolayer also exhibits functional AVP and PTH receptors.

In the current studies, we demonstrated that the renal collecting system cultures exhibit a normal functional response to aldosterone and have identified AIPs which are induced concurrently with the physiological response.

2. Materials and methods

2.1. Primary cultures of rabbit kidney cortical collecting system

Rabbit kidney connecting tubule and cortical collecting duct cells, hereafter referred to as the cortical collecting system, were immunodissected with MAB R2G9 and set in primary culture on permeable filters (Costar, Badhoevedorp, The Netherlands) as previously described in detail [14–16]. In brief, New Zealand white rabbits (~ 0.5 kg weight) were used. The culture medium was a 1:1 mixture of DME/F12 medium (Gibco, Breda, The Netherlands) supplemented with 5% (v/v) decomplemented fetal calf serum, 50 μg/ml gentamicin, 10 μl/ml nonessential amino acids (Gibco), 5 μg/ml insulin, 5 μg/ml transferrin, 50 nM hydrocortisone, 70 ng/ml PGE1, 50 nM Na_2SeO_3 and 5 pM triiodothyronine, equilibrated with 5% CO_2–95% air at 37°C. All experiments were performed with confluent monolayers between 5–8 days after seeding the cells.

2.2. Determination of transcellular short-circuit current

Cells grown to confluence on filters (area 4.5 cm^2) were incubated at 37°C for indicated time periods in the presence 10^{-7} M aldosterone. Subsequently, filter cups were mounted between two half-chambers (area 0.3 cm^2) and bathed at 37°C with incubation medium containing (in mM): 140 NaCl; 2 KCl; 1 K_2HPO_4; 1 KH_2PO_4; 1 $MgCl_2$; 1 $CaCl_2$; 5 glucose; 5 l-alanine; 10 HEPES/Tris (pH 7.40). The solutions bathing the monolayer were connected via agar bridges and Ag-AgCl electrodes to a voltage-clamp current amplifier (Physiological Instruments, San Diego, CA) and the short-circuit current (I_{SC}) was recorded in the absence and presence of 10^{-6} M benzamil (apical side). The benzamil-sensitive component of I_{SC} was used as an estimate of transcellular Na^+ transport.

Benzamil was dissolved in water, whereas aldosterone was dissolved in ethanol. Final solvent concentrations never exceeded 0.1% (v/v). Results from experiments with vehicle alone were never significantly different from the control.

2.3. Biochemical studies

Cells grown to confluence on filters (area 4.5 cm^2) were incubated at 37°C for 15 h in the presence and absence of 10^{-7} M aldosterone. In some experiments, proteins synthesized during the hormone incubation were radiolabelled by adding [^{35}S]methionine (Amersham, 's-Hertogenbosch, The Netherlands; 125 μCi/ml, 1000–1400 Ci/mmol) to the incubation medium.

At the end of the incubation period the monolayers were washed thoroughly with serum-free medium and the monolayers were scraped from the filter disk with a rubber policeman. The cells were homogenized in a homogenizing buffer (0.25 M sucrose, 2 mM EDTA, 5 mM Tris, pH 7.5) using 40 strokes of a hand-held Dounce homogenizer fitted with a tight-fitting pestle. The cellular homogenate was subjected to a low-speed spin (800 $\times g$; 5 min). This pellet (termed the microsomal pellet) was solubilized as described below and analyzed by 2-dimensional polyacrylamide gel electrophoresis. The supernatant from the low-speed spin was subjected to centrifugation at 120 000 $\times g$ for 1 h to prepare membrane (pellet) and soluble (supernatant) fractions.

The soluble fraction was dialyzed extensively against 5 mM Tris (pH 8.0) to remove the homogenization buffer components. Subsequently the proteins were concentrated by acetone precipitation (10-fold excess of acetone, -20°C overnight). The precipitated proteins were spun out of the acetone/aqueous solution (20 000 $\times g$, 30 min) and air-dried. The precipitate was solubilized in 2-dimensional lysis buffer (9.5 M urea, 2% Triton X100, 200 mM dithiothreitol and 0.8% ampholines).

The microsomal or membrane pellets were either solubilized directly into 2-dimensional lysis buffer or were solubilized in 1% Triton X100; 5 mM Tris (pH 8.0) buffer using 20 strokes of a Dounce hand-held homogenizer. In the latter case, the homogenate was acetone precipitated and the dried precipitate solubilized in 2-dimensional lysis buffer.
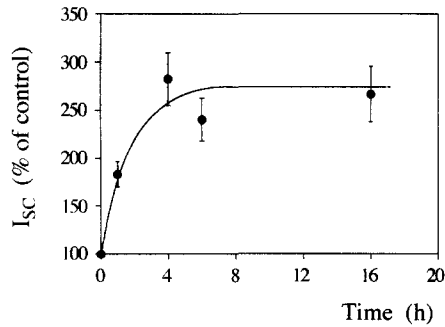
Fig. 1. Time-dependence of aldosterone-induced stimulation of benzamil-sensitive short-circuit current (I_{SC}) across rabbit cortical collecting system in primary culture. Monolayers were incubated in the presence of aldosterone (10^{-7} M, both sides) for indicated time periods. In each experiment the benzamil-sensitive I_{SC} obtained with unstimulated monolayers (19 ± 3 μA·cm^{-2}, $n = 5$) is set at 100%, to which all other values are related.
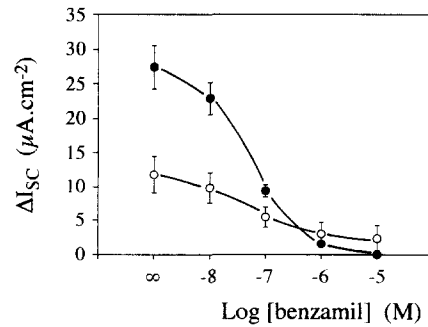
Fig. 2. Dose-dependent effect of benzamil on short-circuit current (I_{SC}) across rabbit cortical collecting system in primary culture. The monolayers were incubated for 15 h in the absence ($\bigcirc$) and presence ($\bullet$) of 10^{-7} M aldosterone. Benzamil was added to the apical compartment. Values are mean $\pm$ SE ($n = 6$).

The solubilized proteins were separated by 2-dimension polyacrylamide gel electrophoresis [2,3,17]. Under the conditions used in these experiments, the first dimension separated proteins with isoelectric points between 4 and 7. The second dimension utilized a linear 5–15% acrylamide gradient which resolved proteins having apparent molecular weights between 250 and 20 kDa. After electrophoresis the separated proteins were blotted onto nitrocellulose or PVDF (Immobilon P^{sq}; Millipore Corp., Bedford, MA) membranes. To detect radiolabelled proteins, the dried membranes were exposed to autoradiography film

(Amersham, 's-Hertogenbosch, The Netherlands). To detect the entire protein complement, the membranes were stained with a colloidal gold stain (Diversified Biotech, Boston, MA).

2.4. Statistical analysis

In electrophysiological experiments, data were assessed from at least three separate immunodissection and subsequent primary culture procedures and expressed as the mean $\pm$ S.E. for n = number of experiments. Statistical differences between the mean values were determined by analysis of variance [18].
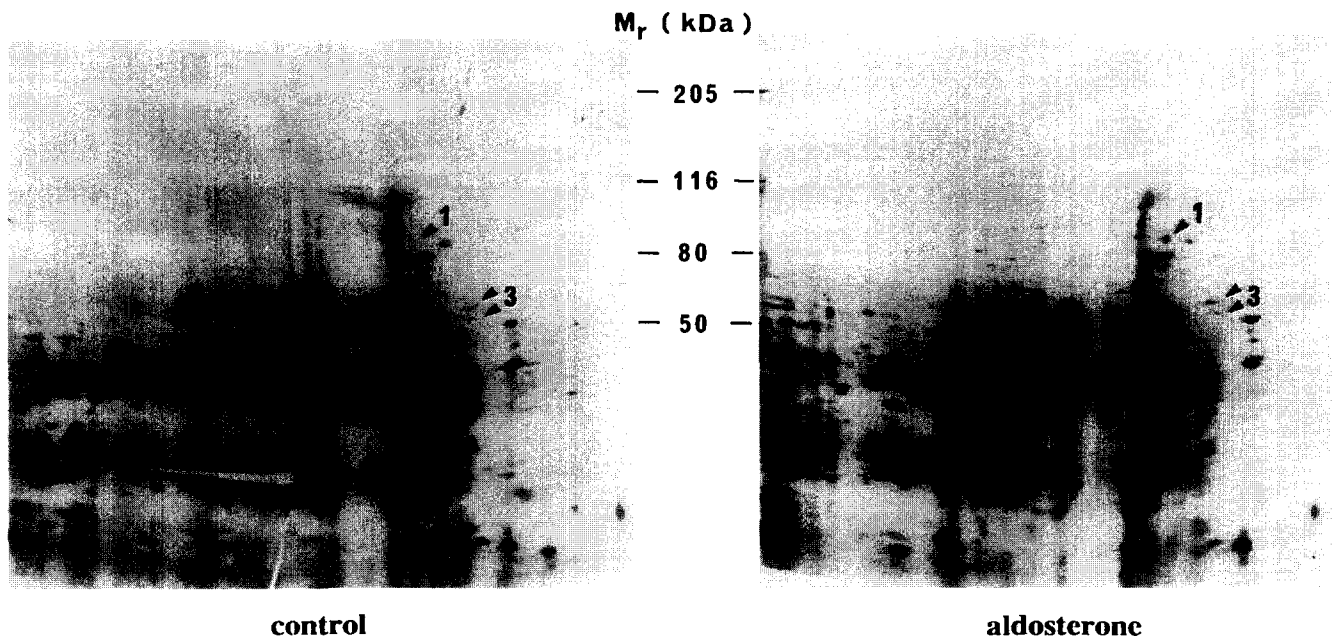
MEMBRANE FRACTION

Fig. 3. Autoradiographs of collecting system membrane proteins separated by 2D-PAGE. Cultured cells were incubated without and with aldosterone (10^{-7} M) in the presence of [^{35}S]methionine for 15 h. The arrowheads denote the same areas of each gel. The migration of the molecular weight standards are indicated. The isoelectric focusing gradient was a linear gradient from pH 7 (on the left) to pH 4 (on the right).

3. Results

The primary cultures of cells from rabbit cortical collecting system respond to aldosterone with an increase in transepithelial Na^+ transport measured as benzamil-sensitive short-circuit current (I_{SC}). After 2, 4, 6, and 16 h of incubation with aldosterone (10^{-7} M, both sides) the benzamil-sensitive I_{SC} was significantly ($P < 0.05$) in-

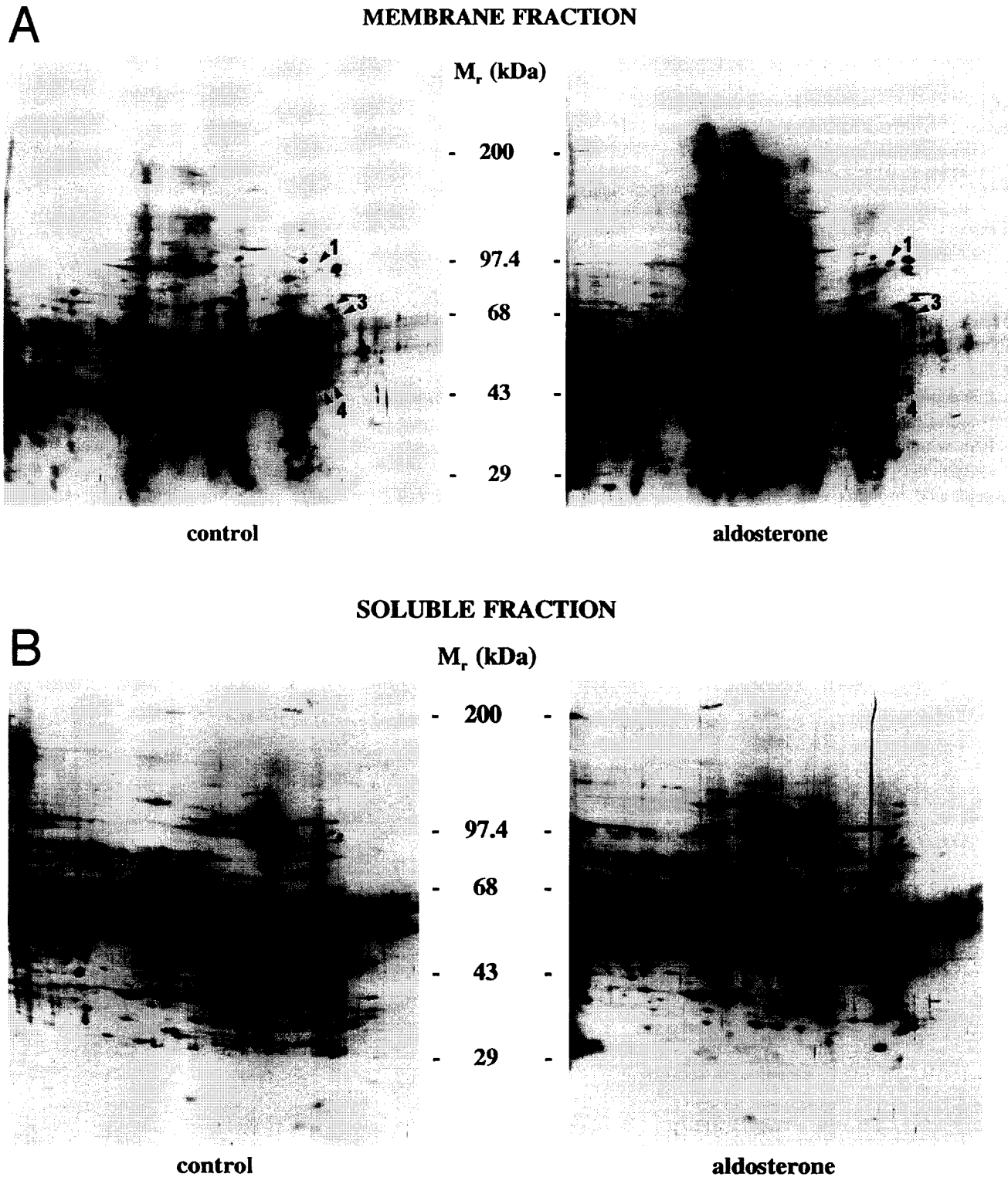## MEMBRANE FRACTION

SOLUBLE FRACTION

Fig. 4. Colloidal gold-stained blots of collecting system membrane (A) and soluble (B) proteins separated by 2D-PAGE. Cultured cells were incubated without and with aldosterone (10^{-7} M) for 15 h. The arrowheads denote the same areas of each gel. The migration of the molecular weight standards are indicated. The isoelectric focusing gradient was a linear gradient from pH 7 (on the left) to pH 4 (on the right).

creased compared to the non-stimulated monolayers (Fig. 1). The stimulatory response to aldosterone was consistently noted after 2 h of incubation and stabilized after 6 h.

We also studied the effect of benzamil on transcellular Na^+ transport in cultured cells pretreated for 15 h with aldosterone and in non-pretreated cells. Benzamil was added to the apical compartment and the minimal (2–5 min post addition) I_{SC} was subsequently determined, as shown in Fig. 2. In control and hormone-treated cells, I_{SC} was inhibited by benzamil in a dose-dependent way, with maximal inhibition occurring at 10^{-6} M. The IC_{50}'s estimated for each experimental condition were similar (45 ± 5 nM versus 52 ± 3 nM for control and aldosterone-treated cells, respectively ($n = 5$, $P > 0.1$)). Aldosterone (10^{-7} M, 15 h) significantly ($n = 6$, $P < 0.05$) decreased the transepithelial electrical resistance from 375 ± 36 $\Omega \cdot cm^2$ to 219 ± 26 $\Omega \cdot cm^2$.

Based on these electrophysiological data, subsequent studies to identify putative aldosterone-induced proteins (AIPs) were performed using an aldosterone incubation of 15 h. In some experiments, [^{35}S]methionine was added to the cultures at the time of hormone addition. Paired cultures of control and hormone-treated cells were fractionated into microsomal, membrane and soluble fractions. The protein component of the fractions was separated by 2D-PAGE and transferred to PVDF membranes. The separated proteins were visualized by autoradiography ([^{35}S]methionine-labelled samples) or colloidal gold protein staining. Colloidal gold will label all proteins in these complex mixtures while [^{35}S]methionine will label only those proteins synthesized during the incubation period. However, since the incubation period was 15 h, the pattern of radiolabelled proteins was not substantially different than that revealed by the protein staining reagent. In addition, the colloidal gold protein stain provided virtually the same sensitivity as the radiolabelled probe. Therefore, the majority (7 of 9) of the experiments were interpreted from protein-stained membranes.

Fig. 3 illustrates autoradiographs from a typical experimental pair of [^{35}S]methionine-labelled membrane fractions. Fig. 4 shows protein-stained membrane and soluble fractions derived from control and hormone treated cells. In both figures, hormone-induced proteins are indicated by arrowheads and are assigned numbers that are consistent in both figures. The numbered proteins are keyed to Table 1 which provides data on the relative incidence of the induction in various fractions.

The single protein labelled #1 is similar in molecular weight to both the Na^+ transporting subunit as well as the regulatory subunits of the mammalian Na^+ channel [8,9] but the exact identity is unknown.

The heterogenous set of proteins labelled as #2 represents the only protein induction in the soluble fractions. These peptides were detected only occasionally in the membrane and microsomal fractions. Interestingly, this series of proteins is very similar in both molecular weight

Table 1
Incidence of aldosterone-induced proteins in various cellular fractions

Protein	Membrane	Microsome	Soluble
(1) single high M_r protein			
$\sim$ 100 kDa pI $\sim$ 5.0	5/9	6/9	0/8
(2) Doublet/triplet			
$\sim$ 77 kDa pI $\sim$ 5.6–6.0	3/9	3/9	6/8
(3) Doublet			
$\sim$ 70–77 kDa pI $\sim$ 4.4–4.7	3/9	5/9	0/8
(4) Low M_r doublet			
$\sim$ 46–50 kDa pI $\sim$ 4.5–5.0	3/9	7/9	0/8

The number on the right of the backslash is the number of experimental pairs analyzed. The number on the left indicates the incidence of the aldosterone-induced protein identification in the particular cellular fraction.

and isoelectric point to a series of aldosterone-induced proteins previously identified in amphibian model systems [2–5].

The remaining AIPs, #3 and #4 are found only in the membrane and microsomal fractions. While these proteins have molecular weights that are the same as some reported Na^+ channel subunits of the large Na^+ channel complex [19], the exact identity of these AIPs remains unknown.

4. Discussion

In the present study, the action of aldosterone on Na^+ transport in primary cultures of immunodissected cells from rabbit cortical collecting system was characterized by electrophysiological and biochemical methods. In vivo aldosterone stimulates Na^+ reabsorption via a protein synthesis-dependent action on the epithelial cells lining the distal tubule and collecting duct [1–3].

The cultured cells respond to 10^{-7} M aldosterone with an increase in transepithelial Na^+ transport estimated by benzamil-sensitive I_{SC}. The stimulatory response to aldosterone was consistently noted after 2 h of incubation and stabilized after 6 h. The onset and magnitude of the effect of aldosterone is comparable to similar responses in the well-characterized amphibian high resistance models [2,5,20], and is consistent with a previously reported intermittent time course (measured in 3-h intervals) obtained from immunodissected cortical collecting duct cell cultures [21].

As predicted from the characteristics of the parent tissue, transcellular Na^+ transport of the cultured cells was sensitive to the apical addition of the conductive Na^+ channel blocker benzamil. Basal as well as hormone-stimulated I_{SC} were inhibited by benzamil in a dose-dependent way and similar IC_{50}'s of $\sim$ 50 nM were estimated. These data are consistent with a specific inhibition of the apical membrane Na^+ channel [22] and indicate that the majority of the I_{SC} is due to transcellular Na^+ flux.

Based on the electrophysiological data, subsequent stud-

ies were performed to identify putative aldosterone-induced proteins (AIPs). After an aldosterone incubation of 15 h, three AIPs (M_r = 100, 70–77 and 46–50 kDa) were consistently observed in the membrane and microsomal fractions, while a single heterogeneous AIP (M_r = 77 kDa) was detected in the soluble fraction.

The single protein of ~ 100 kDa is similar in molecular weight to both the Na^+ transporting subunit as well as the regulatory subunits of the mammalian Na^+ channel [8,9] but its exact identity is unknown.

The heterogenous set of proteins with a molecular weight of ~ 77 kDa represents the only protein induction in the soluble fractions. Interestingly, this series of proteins is very similar in both molecular weight and isoelectric point to a series of aldosterone-induced proteins previously identified in amphibian model systems [2–5]. In the amphibian models these proteins are linked to the heterogeneous, large molecular weight (~ 700 kDa) Na^+ channel complex and, as such, are found predominantly, though not exclusively, in the membrane fraction [2]. The reason for the finding that similar characteristics by 2D-PAGE occur predominantly in the soluble fraction of the mammalian cells is unknown. It is possible that the amphibian and mammalian AIPs are unrelated proteins or, alternatively, that they are similar proteins but slight differences in protein structure and/or membrane characteristics contribute to the apparent solubility of these peptides.

The remaining AIPs with molecular weights between 70–77 kDa and 46–50 kDa are found only in the membrane and microsomal fractions. While these proteins have molecular weights that are the same as some reported Na^+ channel subunits of the large Na^+ channel complex [19], the exact identity of these AIPs remains unknown.

Although much progress has been made in recent years, the exact nature of the conductive Na^+ channel(s) is unclear. Based on a plethora of data, the existence of different Na^+ channels with distinct biochemical structures has been hypothesized [19]. Some of these are likely to be multimeric complexes while others are likely to exist as individual transport proteins.

The mechanism of aldosterone modulation of channel activity has also not been clearly elucidated. While several AIPs have been identified in amphibian systems [1–5], the absence of mammalian model epithelia has hampered similar electrophysiological and biochemical characterization in higher organisms.

In this study we have elucidated the response of a mammalian renal model system to the steroid hormone aldosterone. A small number of AIPs which are synthesized concurrently with the physiological response have been characterized by 2D-PAGE. These studies form the groundwork for future analyses detailing the molecular mechanisms of aldosterone's action.

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