J. Membrane Biol. 133, 17–27 (1993)

The Journal of
Membrane
Biology

Effects of Aldosterone on the Impedance Properties of Cultured Renal Amphibian Epithelia

N.K. Wills, R.K. Purcell, C. Clausen†, and L.P. Millinoff

Dept. of Physiology and Biophysics, University of Texas Medical Branch, Galveston, Texas 77555-0641, and †SUNY Stony Brook, Stony Brook, New York 11704-8861

Summary. The cultured renal amphibian cell line A6 has proven advantageous for studies of Na^+ transport regulation. In the present study, the effects of aldosterone action on the transepithelial electrical properties of this epithelium were assessed. Specifically, the time course of aldosterone action was determined and the effects of chronic (10–18 day) aldosterone elevation were assessed using transepithelial equivalent circuit methods and impedance analysis techniques.

Short-term (<4 hr) exposure to aldosterone (0.1 μM) stimulated the amiloride-sensitive short-circuit current (I_{sc}) by over twofold and increased the transepithelial conductance (G_T) by approximately 12%. The increases in I_{sc} and G_T were maintained in epithelia subjected to chronic aldosterone exposure. In contrast to previous reports, paracellular resistance (R_j) was not altered by aldosterone. This difference may be related to the longer time of exposure or different basal Na^+ transport rates in the present study.

The apical membrane conductance was significantly increased for aldosterone-treated epithelia compared to aldosterone-depleted (i.e., serum-deprived) controls. Apical membrane area (capacitance) was not significantly affected. This finding is consistent with a higher density (number of channels per membrane area) of conducting Na^+ channels in this membrane following aldosterone stimulation. Basolateral membrane properties were not significantly altered for aldosterone- treated tissues compared to serum-treated control tissues. In contrast, basolateral membrane-specific conductance (i.e., basolateral membrane conductance normalized to basolateral membrane capacitance) was significantly lower for serum-deprived epithelia than for serum-treated controls or aldosterone-treated tissues.

The effects of chronic aldosterone exposure were also evaluated for the A6 subclonal cell line, 2F3. Similar to A6 epithelia, I_{sc} was essentially doubled following aldosterone stimulation while R_j and cellular driving force (E_c) were not affected. Apical membrane conductances under control conditions for 2F3 epithelia were higher than those for A6, but were not significantly different from A6 following aldosterone exposure or serum deprivation. These findings suggest possible differences in the regulation of apical membrane Na^+ channels for 2F3 and A6 epithelia.

Key Words A6 · epithelium · cultured renal epithelia · aldosterone · sodium transport · impedance analysis

Introduction

Mineralocorticoids play an important role in the regulation of plasma Na^+ composition by so-called tight epithelia such as the renal distal tubule and the descending colon. Aldosterone is generally believed to initially stimulate Na^+ entry into the transporting cells of these epithelia by activation of apical membrane Na^+ channels (*cf.* Palmer et al., 1991). Chronic exposure to this hormone results in increased production of basolateral membrane Na-K ATPase (*cf.* Garty, 1986) and elaboration of basolateral membrane area (Wade et al., 1979; Kashgarian et al., 1980).

Advances in tissue culture techniques over the past decade have made it possible to assess the effects of chronic aldosterone stimulation in vitro. The A6 cell line has been a particularly useful model for such studies. This continuous cell line was derived from the kidney of *Xenopus laevis* and possesses two different receptors for aldosterone (Watlington et al., 1982; Claire et al., 1989). One of these receptors is similar to a mineralocorticoid receptor that is restricted to the renal distal straight segment of this animal (Claire et al., 1989; Gnionsahe et al., 1989), implicating this region as the site of origin of this cell line.

Na^+ transport across A6 epithelium (measured as amiloride-sensitive short-circuit current; I_{sc}) is stimulated by aldosterone (*see* Perkins & Handler, 1981). In addition, aldosterone has recently been shown to stimulate the rate of ouabain-binding to the Na-K ATPase within 3 hr (Pellanda et al., 1992). A small aldosterone-regulated pool of Na-K ATPase and a larger constitutive aldosterone-independent pool have also been reported for these cells (*cf.* Rossier, Pellands & Jaisser, 1991). Verrey et al. (1987) reported a subclonal cell line, 2F3, which

showed increased responsiveness to aldosterone and induction of Na-K ATPase subunits within 6 hr.

We have recently used impedance analysis techniques as a tool for studying the membrane properties of A6 and 2F3 epithelial cells (Wills, Purcell & Clausen, 1992). This method allowed resolution of apical and basolateral membrane conductances and capacitances (*cf.* Clausen, Lewis & Diamond, 1979; Kottra & Fromter, 1984). Since capacitance is proportional to membrane area (Cole, 1972), this method is particularly well suited for measuring changes in membrane areas during ion transport regulation.

In the present study, we have employed impedance methods together with transepithelial DC equivalent circuit analysis techniques to assess the effects of chronic aldosterone stimulation on the membrane properties of A6 and 2F3 epithelia. We asked the following questions: (i) What is the time course of the effects of aldosterone stimulation on I_{sc} and G_T in A6 epithelium?, (ii) What are the effects of chronic aldosterone stimulation on cellular and paracellular resistances and membrane driving forces? and (iii) How are apical and basolateral membrane properties of the A6 and 2F3 epithelia affected by this hormone?

Materials and Methods

Cell Culture

A6 renal epithelial cells derived from *Xenopus laevis* were obtained from American Type Culture Collection (Rockville, MD) at passage 69. Cells from passages 74–79 were grown as confluent monolayers at a density of 2.4×10^5 cells/cm^2 on permeable support systems (Millicell-HA: 0.45 μm pore, 4.2 cm^2, Millipore, Bedford, MA) according to previously described methods (Wills & Millinoff, 1990). Cells were fed three times weekly with a growth medium consisting of Dulbecco's Modified Eagle medium (amphibian formula: GIBCO, Grand Island, NY, catalogue no. 84-5022) supplemented with 40 mU/ml penicillin, 40 μg/liter streptomycin and 10% fetal bovine serum (FBS; Hyclone, Logan, UT; final solution osmolarity, 200 mOsm). After one week, some tissues were fed with culture media that did not contain serum. Mannitol (20 mM) was added to maintain the osmolarity of the medium at 200 mOsm. Epithelia that were chronically exposed to aldosterone were grown in 0.1 μM concentrations of hormone from the time of initial cell plating. Cells were maintained at 28 °C in a humidified incubator gassed with 1% CO_2 in air and experiments were performed after 10–21 day of incubation.

Experimental Chambers

The epithelium and underlying filter paper were cut away from the culture plate insert and mounted between the chamber halves of a modified Ussing chamber (aperture area = 2 cm^2). The filter paper side was supported against a nylon mesh by a slight excess of mucosal solution. Water jackets maintained the solutions in the chambers at a temperature of 28 °C. In addition, tissues were continuously bubbled with 1% CO_2 in air to maintain the solution pH at 7.4 and stirred by magnetic spin bars in the bottom of each chamber.

Solutions

Sodium chloride Ringer solution was designed to match the electrolyte composition of the culture medium and contained in mM: 74.4 NaCl, 5.4 KCl, 8 $NaHCO_3$, 1.4 $CaCl_2$, 1.7 $MgSO_4$, 0.9 NaH_2PO_4, 5.5 glucose, 1 Na pyruvate, and 1 N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES). The solution pH was maintained at 7.4 and the osmolarity was 170 mOsm. Potassium chloride and gluconate Ringer solutions (used in nystatin experiments) were also prepared in the same manner, except that sodium salts were replaced by potassium salts at equal concentrations, and chloride was replaced by gluconate, respectively. Amiloride (a gift from Merck, Sharp, and Dohme) and nystatin (Sigma, St. Louis, MO) were prepared as stock solutions by previously described methods (Wills & Millinoff, 1990) and microliter quantities of the stock solutions were added to the bathing solutions to achieve concentrations of 70 μM and 20 units/ml, respectively. A stock solution of aldosterone (GIBCO) was prepared in ethanol diluted with culture medium and microliter quantities of the stock solution were added to the feeding medium to achieve concentrations of 0.1 μM.

Electrical Measurements

Transepithelial DC Measurements

Methods for measuring transepithelial current and voltage were similar to those described previously (Wills & Millinoff, 1990). The transepithelial voltage (V_T) and current (I) were monitored using pairs of Ag-AgCl wires (for chloride solutions) or 1M NaCl agar bridges connected to Ag-AgCl wires (for gluconate solutions) leading to an automatic voltage clamp. A digital meter and computer A/D system (Labmaster, Axon Instruments, Foster City, CA) were used to read currents to within 0.01 μA accuracy. Ohm's Law was used to calculate the transepithelial resistance (R_T) from the measured deflection in V_T to an applied current pulse and the short-circuit current (I_{sc}) as V_T/R_T. We have previously shown that the current-voltage relationship for A6 and 2F3 epithelia are essentially linear under these conditions over a range of ±100 mV (Wills & Millinoff, 1990).

Determination of Paracellular Resistance

Paracellular resistance (R_j) and cellular electromotive force (E_c) were assessed using a V_T-R_T plot method previously described (Wills & Millinoff, 1990; Wills et al., 1992). For amiloride experiments, a plot of V_T *vs.* R_T yielded a linear double intercept plot as follows (Wills et al., 1979):

$$1 = V_T/E_c + R_T/R_j \quad (1)$$

When nystatin was used, E_c represented the basolateral driving

force (E_{bl}) as nystatin essentially eliminated the Na^+ and Cl^- gradients across the apical membrane.

Transepithelial Impedance Analysis

Transepithelial impedance was measured using previously described methods (Wills & Clausen, 1987; Wills et al., 1992). A wide-band pseudorandom binary noise signal was generated digitally and converted to a constant current signal of 1.4 $\mu A/cm^2$ (peak-to-peak). Good resolution at low and high frequencies was attained by employing two signal bands of 2.2 to 860 Hz and 22 Hz to 8.6 kHz. The transepithelial voltage produced in response to this signal was measured using a low noise amplifier and filtered through a 120 dB/octave low-pass anti-aliasing filter (Unigon). A laboratory computer with a 12-bit A-D converter (LSI 11/73 Indec Systems; Sunnydale, CA) controlled digitalization of current and voltage signals and data acquisition.

Transepithelial impedance was calculated by dividing the cross-spectral density of the voltage and current by the power spectral density of the applied current yielding two sets of 400 linearly spaced data points, one set for the low and one for the high bandwidth. These points were merged and reduced to 100 data points spaced logarithmically in frequency from 2.2 Hz to 8.6 kHz. Each data point consisted of two numbers: a phase angle and magnitude measurement.

The data were fitted by a nonlinear, least-squares curve-fitting algorithm using a morphologically based equivalent circuit model. In this model, resistors represent the membrane ionic conductances and capacitors represent the membrane capacitances, where 1 $\mu F \simeq 1$ cm^2 membrane area (Cole, 1972). The model employed was specifically derived for A6 and 2F3 epithelia in a previous study (Wills et al., 1992) and takes into account an additional series RC component that arises from basal membrane protrusions in the underlying filter support (*see* Fig. 4 insert). The quality of the fit to the data was evaluated by the Hamilton R-factor and the parameter standard deviations. Fits yielding R-factor values of more than 3.5% or standard deviations of more than 10% of the fitted parameter value were defined as poor fits and the data excluded from further analysis.

Statistical Analysis

All results are reported as means ± SEM. Data were analyzed using either a one-way analysis of variance (ANOVA) and conservative post-tests, paired *t*-tests, or the Mann Whitney U test where appropriate. Statistical significance was defined as $P < 0.05$.

Results

The effects of aldosterone on cultured renal epithelial cells were characterized using two approaches. First, we examined the effects of short-term (≤4 hr) elevations of aldosterone on transepithelial electrical properties. In particular, the time courses of increases in the transepithelial short-circuit current (I_{sc}) and conductance (G_T) were determined. Second, the effects of chronic (10–18 day) aldosterone exposure were assessed. Specifically, equivalent circuit analysis techniques were used to study the effects of aldosterone on the transepithelial electrical properties. In addition, impedance analysis methods were used to resolve the effects of aldosterone on apical and basolateral membrane conductances and areas (measured as membrane capacitance). Lastly, the effects of aldosterone on the subclonal cell line, 2F3 were evaluated and the results compared to those for A6 cells.

Effects of Aldosterone on A6 Epithelium

As a first step in evaluating the effects of aldosterone on A6 epithelia, we examined the effects of short-term exposure on transepithelial electrical properties.

Short-Term Effects

Short-term effects were assessed in two studies. In the first set of experiments, eight tissues were incubated in serum-free culture media for 72–98 hr in order to minimize cellular aldosterone levels. Since solution osmolarity has a potent effect on apical membrane Na^+ channel activity in A6 epithelia (Wills, Millinoff & Crowe, 1991), mannitol was added to the serum-free culture media to maintain constant solution osmolarity (200 mOsm; defined as isosmotic). The epithelia were then grouped into four pairs, mounted in Ussing-type chambers, and bathed with the serum-free culture media. Aldosterone was added to the serosal bathing solution of one member of each pair and transepithelial electrical measurements were then monitored for both epithelia over the next four hours.

The initial I_{sc} values (i.e., at 0 hr) were not significantly different for the control and aldosterone-treated groups (10 ± 1 $\mu A/cm^2$ for untreated and 8 ± 2 $\mu A/cm^2$ for aldosterone-treated). These values agree well with our previously published I_{sc} values measured in 200 mOsm "isosmotic solutions" (Wills et al., 1991). Figure 1 summarizes the mean short-circuit current values for control and aldosterone treated tissues at 4 hr. I_{sc} for the aldosterone-treated tissues was increased by approximately twofold (I_{sc} = 22 ± 2 $\mu A/cm^2$; $P < 0.002$), but no significant change was observed for control tissues (13 ± 2 $\mu A/cm^2$; NS).

Paccolat et al. (1987) reported that the response of A6 epithelia to aldosterone was influenced by fetal bovine serum (FBS). For this reason, in an additional set of experiments, tissues were bathed in culture media containing FBS, treated with aldosterone, and measured as described above. As

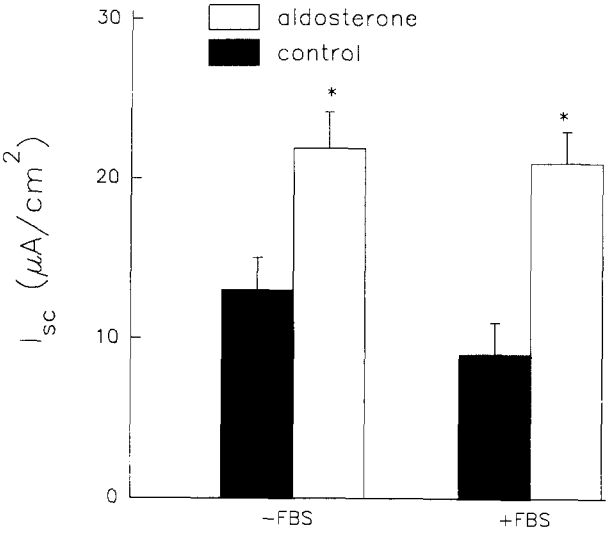

Fig. 1. Effects of aldosterone on short-circuit currents across A6 epithelium. Electrical measurements were monitored continuously for aldosterone-treated (open bars) and untreated controls (filled bars). Data represent means and SEM measured 4 hr after hormone addition. Asterisks indicate a statistically significant increase ($P < 0.05$) for aldosterone-treated tissues compared to untreated controls. Tissues were preincubated in culture media with or without serum (+FBS and −FBS, respectively; for further details, *see text*).

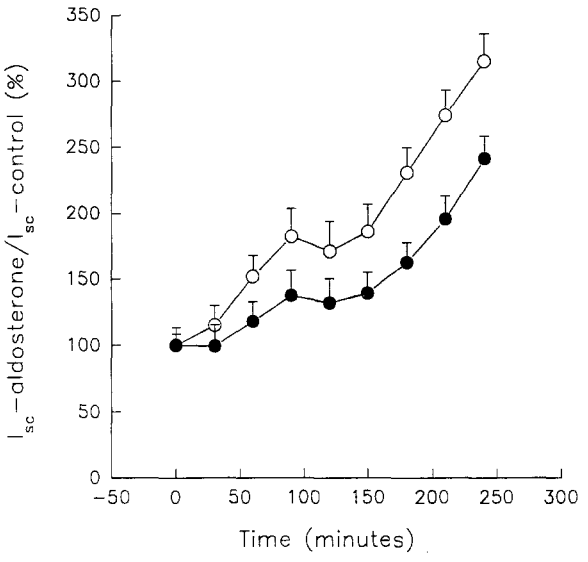

Fig. 2. Time course of aldosterone effects on short-circuit currents. Results are expressed as the ratio of I_{sc} for aldosterone-treated tissues over paired control tissues. The effects of two aldosterone concentrations were compared (0.1 μM = filled circles; $n = 7$, and 1 μM = open circles; $n = 3$). As in Fig. 1, serum removal did not significantly affect the results.

shown on the right side of Fig. 1, the results were essentially the same as the previous experiment: I_{sc} was increased by approximately twofold (control $I_{sc} = 9 \pm 2$ compared to aldosterone-treated $I_{sc} = 21 \pm 2; n = 7, P < 0.05$). Consequently, the presence of FBS apparently had little effect on the action of aldosterone on I_{sc}.

Time course. The time course of aldosterone effects on the short-circuit current is shown in Fig. 2. Two different concentrations of aldosterone were used, 0.1 μM (filled circles) and 1 μM (open circles). The increase in I_{sc} was significantly larger for the higher dose of aldosterone (i.e., at $t = 240$, $\Delta I_{sc} = 215 \pm 21\%$ for 1 μM aldosterone compared to $142 \pm 17\%$ for 0.1 μM aldosterone, unpaired t-test, $P < 0.001$). Figure 3 presents transepithelial conductance data for the above experiments. A similar increase in G_T was observed following aldosterone stimulation for both concentrations used. The average increase in G_T after 4 hr was $22 \pm 18\%$ for 1 μM aldosterone ($n = 3$) and $10 \pm 4\%$ for 0.1 μM aldosterone ($n = 7$).

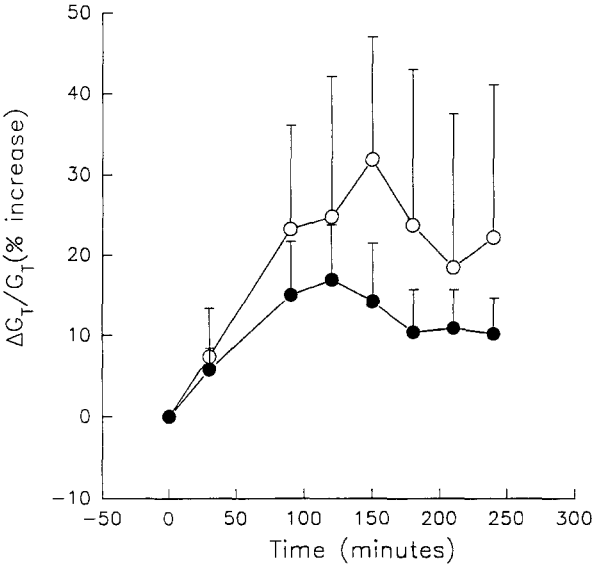

Fig. 3. Time course of aldosterone effects on transepithelial conductance (G_T) for the same experiments shown in Fig. 2.

Long-Term Effects of Aldosterone

In this series of experiments, transepithelial electrical properties were measured for tissues grown under conditions of chronic aldosterone exposure. Three levels of aldosterone treatment were assessed: (1) aldosterone-free control tissues ("−FBS") which were grown under normal conditions for one week following cell plating (*see* Materials and Methods), and subsequently placed in serum-free media, (2) low-aldosterone control tissues ("+FBS") that were continuously grown in

Table 1. Effects of long-term aldosterone exposure on A6 transepithelial electrical properties

	V_T (mV)	I_{sc} (μA/cm^2)	G_T (mS/cm^2)
Control (−FBS)	−38 ± 7	19 ± 2	0.53 ± 0.05
+ Amiloride (n = 5)	−3 ± 1[c]	1 ± 0.5[c]	0.38 ± 0.05[c]
Control (+FBS)	−60 ± 9	28 ± 2	0.53 ± 0.07
+ Amiloride (n = 8)	−2 ± 1[c]	0 ± 0.3[c]	0.31 ± 0.09[c]
Aldosterone	−72 ± 8[a]	44 ± 4[a,b]	0.65 ± 0.06
+ Amiloride (n = 8)	−7 ± 2[c]	1 ± 0.3[c,d]	0.29 ± 0.06[c]

[a] $P < 0.05$ compared to control (−FBS).
[b] $P < 0.05$ compared to control (+FBS).
[c] $P < 0.05$ compared to amiloride-free.
[d] $P < 0.05$ compared to zero.

FBS-supplemented culture media, and (3) aldosterone-treated epithelia ("aldosterone") that were grown in the continuous presence of FBS and 0.1 μM aldosterone. All tissues were studied at the same age (2–3 week following initial cell plating). Earlier investigations (Wills & Millinoff, 1990) indicated that transepithelial electrical properties are stable during this period.

As summarized in Table 1, transepithelial electrical parameters tended to be higher for A6 epithelia grown with serum-containing medium than for tissues that were incubated in serum-free medium. However, none of these values were significantly different. In contrast, aldosterone-treated epithelia demonstrated significantly larger I_{sc} values compared to control groups, averaging ~57% larger than +FBS controls and ~132% larger than −FBS controls (*see* Table 1). Of interest is that G_T was slightly increased by aldosterone (G_T = 0.65 ± 0.06 mS/cm^2). However, this difference was not significantly different from controls (0.53 ± 0.05 for control−FBS and 0.53 ± 0.07 for control+FBS).

Following mucosal addition of amiloride, V_T, I_{sc}, and G_T were significantly reduced for all treatment conditions (*see* Table 1). A small but statistically significant current remained in the presence of amiloride for the aldosterone-treated condition (I_{sc} = 1 ± 0.3; n = 8).

Equivalent Circuit Properties. Table 2 summarizes the effects of chronic aldosterone exposure on cellular driving forces (E_c and E_{bl}), cellular conductance (G_c), and paracellular conductance (G_j). E_c, defined as the net cellular driving force for net transepithelial Na^+ transport (Yonath & Civan, 1971; Wills & Millinoff, 1990), was similar for control (−FBS and +FBS, 103 ± 7 and 118 ± 6 mV, respectively; NS) and aldosterone-treated tissues (104 ± 5 mV). The basolateral membrane driving force, E_{bl}, and paracellular conductances also did not significantly differ across different treatment conditions. In contrast, cellular conductance (G_c) was significantly increased for aldosterone-treated A6 epithelia groups compared to both −FBS and +FBS control groups.

Impedance Properties. Figure 4 shows a Bode plot of the impedance data for a typical aldosterone-treated epithelium. As in our previous study of untreated A6 epithelia, excellent fits to the data were obtained using the equivalent circuit model shown in the inset of Fig. 4. The mean residual error of the fits for aldosterone-treated tissues was 0.5 ± 0.03% (n = 8). Similar residual errors were obtained for other treatment conditions.

Table 3 summarizes the effects of long-term aldosterone exposure on impedance properties of A6 epithelia. Aldosterone tended to increase the apical membrane conductance (G_a) and was significantly higher for aldosterone-treated epithelia (G_a = 0.59 ± 0.12 mS/cm^2) compared to control (−FBS) tissues (G_a = 0.22 ± 0.02; n = 5; $P < 0.01$). Other membrane impedance parameters (apical membrane capacitance, and basolateral membrane conductance and capacitance were not significantly affected by aldosterone).

Mean apical and basolateral membrane conductances normalized for area (G_{a-n} and G_{b-n}, respectively) are presented in Fig. 5. G_{a-n} averaged 0.18 ± 0.020 mS/μF for control (−FBS, n = 5), 0.30 ± 0.06 mS/μF for control (+FBS; n = 8) and 0.42 ± 0.07 mS/μF for aldosterone treated tissues ($P <$ 0.005 compared to control-FBS). The mean G_{b-n} for the control+FBS and aldosterone conditions were similar and averaged 0.66 ± 0.10 and 0.64 ± 0.14 mS/μF, respectively. The mean value for the control-FBS group was 0.43 ± 0.03 and was significantly lower than for the aldosterone treated tissues ($P <$ 0.05) or the +FBS controls ($P < 0.01$).

Effects of Amiloride on Impedance. Table 3 summarizes the effects of amiloride on epithelial impedance properties. In agreement with our previous findings (Wills et al., 1992), G_a values were significantly decreased by amiloride for all treatment conditions. No significant changes were detected in other membrane parameters.

Effect of Aldosterone on the Subclone 2F3

In a previous study (Wills et al., 1991), we noted that 2F3 epithelia, a subclonal cell of A6 cells, had a significantly higher basal Na^+ transport rate (mea-

Table 2. A6 equivalent circuit properties

	E_c (mV)	E_{bl} (mV)	G_c (mS/cm^2)	G_j (mS/cm^2)
Control (−FBS) (n = 5)	103 ± 7	68 ± 3	0.17 ± 0.02	0.31 ± 0.06
Control (+FBS) (n = 6)	118 ± 6	67 ± 1	0.26 ± 0.03	0.26 ± 0.08
Aldosterone (n = 8)	104 ± 5	65 ± 3	0.43 ± 0.04[a,b]	0.22 ± 0.03

[a] $P < 0.05$ compared to control (−FBS).
[b] $P < 0.05$ compared to control (+FBS).

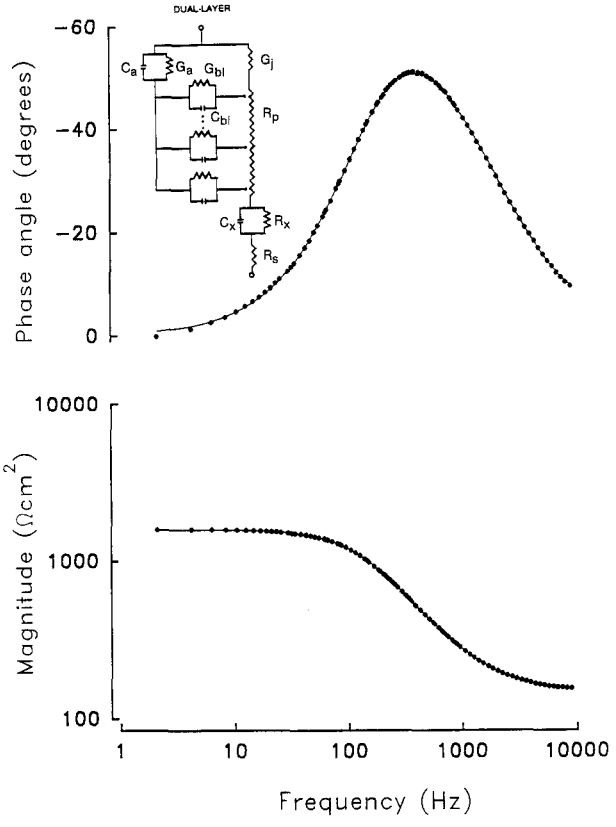

Fig. 4. Representative results from an aldosterone experiment showing a Bode plot of impedance phase angle and magnitude data. The smooth curves are the results of fitting the data using a morphologically based equivalent circuit model and an independent measurement of G_j (0.23 mS/cm^2). The estimated membrane parameters where: G_a = 0.77 mS/cm^2, C_a = 1.2 μF/cm^2, G_{bl} = 1.4 mS/cm^2, C_{bl} = 4.3 μF/cm^2, R_s = 147 Ωcm^2, R_p = 24.8 Ωcm^2, C_x = 3.1 μF/cm^2, R_x = 45.2 Ωcm^2, R_f = 0.3%.

sured as I_{sc}) and different equivalent circuit properties than A6 epithelia. For this reason, the effects of long-term aldosterone exposure were also evaluated for this cell line. Preliminary experiments indicated that 2F3 electrical properties were extremely variable following serum-deprivation. Therefore, all 2F3 experiments were performed in the presence of FBS. Transepithelial properties for 2F3 epithelia are shown in Table 4. V_T for 2F3 epithelia did not differ from values for A6 epithelia and was not significantly altered by aldosterone. (V_T = −77 ± 8 mV for control and −75 ± 10 mV for aldosterone-treated tissues).

I_{sc} values for 2F3 epithelia were significantly larger than for A6 epithelia for both control and aldosterone-treated conditions. As in the case of A6 epithelia, aldosterone-treated 2F3 tissues showed a significantly higher mean I_{sc} value (84 ± 11 μA/cm^2; n = 11) than controls (49 ± 3; n = 5; $P < 0.05$). In order to confirm that I_{sc} values reflected Na^+ transport, amiloride was applied to the mucosal bathing solution. Table 6 summarizes the results of the amiloride experiments. In agreement with our previous findings (Wills & Millinoff, 1990; Wills et al., 1992), G_T, V_T and I_{sc} were greatly decreased by amiloride for all groups. However, a small but statistically significant residual I_{sc} was detected for most conditions.

In the presence of amiloride, the transepithelial conductances did not significantly differ for 2F3 and A6 epithelia (*see* Tables 1 and 4). In the absence of amiloride, G_T was significantly larger for aldosterone-treated tissues (1.21 ± 0.23 mS/cm^2), than the control group (G_T = 0.66 ± 0.07 mS/cm^2; $P < 0.05$, Mann Whitney U test). In agreement with our previous findings (Wills et al., 1992), G_T values for 2F3 epithelia and A6 epithelia did not significantly differ for the control conditions. In contrast, aldosterone-treated epithelia showed significantly higher G_T values for 2F3 than A6 epithelia.

Table 5 summarizes the equivalent circuit properties of 2F3 epithelia. No significant differences were observed for the control and aldosterone-treated conditions. The cellular conductance for 2F3 epithelia under control conditions was significantly

Table 3. A6 impedance parameters

	G_a (mS/cm^2)	C_a (μF/cm^2)	G_{bl} (mS/cm^2)	C_{bl} (μF/cm^2)
Control (−FBS)	0.22 ± 0.02	1.26 ± 0.08	2.96 ± 0.53	6.7 ± 1.0
+ Amiloride	0.06 ± 0.02[b]	1.20 ± 0.04	2.35 ± 0.66	10.2 ± 1.3
(n = 5)				
Control (+FBS)	0.37 ± 0.08	1.27 ± 0.07	2.95 ± 0.40	4.7 ± 0.6
+ Amiloride	0.11 ± 0.07[b]	1.13 ± 0.09	4.00 ± 1.27	8.8 ± 2.0
(n = 8)				
Aldosterone	0.59 ± 0.12[a]	1.36 ± 0.08	2.98 ± 0.68	6.2 ± 1.7
+ Amiloride	0.11 ± 0.03[b]	1.17 ± 0.07	4.19 ± 0.41	10.2 ± 1.3
(n = 8)				

[a] $P < 0.05$ compared to control (−FBS).
[b] $P < 0.05$ compared to amiloride-free condition.

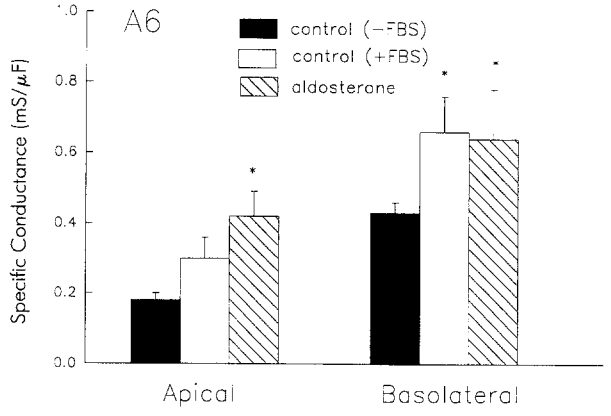

Fig. 5. Comparison of apical and basolateral membrane conductances for A6 epithelia under different treatment conditions. Asterisks indicate a statistically significant increase ($P < 0.05$) compared to the control (−FBS) condition. For further details, *see text*.

Table 4. Effects of long-term aldosterone exposure on 2F3 transepithelial electrical properties

	V_T (mV)	I_{sc} (μA/cm^2)	G_T (mS/cm^2)
Control	−77 ± 8	49 ± 3[c]	0.66 ± 0.07
+ Amiloride	−8 ± 3[a]	2 ± 0.5[a]	0.31 ± 0.09[a]
(n = 5)			
Aldosterone	−75 ± 10	84 ± 11[b,c]	1.21 ± 0.23
+ Amiloride	−9 ± 3[a]	2 ± 0.4[a]	0.26 ± 0.08[a]
(n = 11)			

[a] $P < 0.05$ compared to amiloride free condition.
[b] $P < 0.05$ compared to control (no aldosterone).
[c] $P < 0.05$ compared to A6 epithelium.

higher than for A6 epithelia, in agreement with our previous findings (Wills et al., 1992).

The mean G_a values for 2F3 epithelia are presented in Table 6 and were 0.79 ± 0.23 for control (n = 4) and 1.04 ± 0.16 mS/cm^2 for aldosterone-treated tissues (n = 9). G_a was significantly higher than values for A6 epithelia in the control (+FBS) condition ($P < 0.05$), but not for aldosterone-treated tissues. Other membrane parameters did not significantly differ from those for A6 epithelia.

The mean G_{a-n} for aldosterone-treated 2F3 tissues was 0.78 ± 0.11 mS/μF and was significantly higher than G_{a-n} for control 2F3 tissues (0.44 ± 0.11; $P < 0.03$). G_{a-n} for aldosterone-treated 2F3 epithelia was also significantly higher than G_{a-n} for aldosterone-treated A6 epithelia ($P < 0.02$). G_{bl-n} was not significantly affected by aldosterone and averaged 1.06 ± 0.26 for control compared to 0.89 ± 0.20 for aldosterone-treated tissues.

Discussion

The present study confirms previous reports that aldosterone stimulates Na^+ transport across cultured renal A6 epithelium and represents the first use of impedance analysis techniques to resolve the effects of long-term aldosterone stimulation on apical and basolateral membrane conductances and areas. In agreement with previous studies (Perkins & Handler, 1981; Fidelman & Watlington, 1987; Verrey et al., 1987; Bindels, Schafer & Reif, 1988), aldosterone was a potent stimulator of amiloride-sensitive I_{sc}. A6 epithelia that were chronically treated with this hormone demonstrated larger apical and basolateral membrane specific conductances than aldosterone-depleted controls. The effects of

Table 5. 2F3 equivalent circuit properties

	E_c (mV)	E_{bl} (mV)	G_c (mS/cm^2)	G_j (mS/cm^2)
Control (n = 4)	111 ± 5	62 ± 3	0.41 ± 0.02[a]	0.13 ± 0.03
Aldosterone (n = 5)	125 ± 10	69 ± 3	0.65 ± 0.14	0.22 ± 0.07

[a] $P < 0.05$ compared to A6.

Table 6. 2F3 impedance parameters

	G_a (mS/cm^2)	C_a (μF/cm^2)	G_{bl} (mS/cm^2)	C_{bl} (μF/cm^2)
Control	0.79 ± 0.23[b]	1.43 ± 0.04	3.48 ± 1.67	4.2 ± 2.0
+ Amiloride (n = 4)	0.13 ± 0.08[a]	1.24 ± 0.18	2.85 ± 0.47	6.3 ± 4.2
Aldosterone	1.04 ± 0.16	1.26 ± 0.24	6.19 ± 1.15	9.5 ± 2.5
+ Amiloride (n = 10)	0.17 ± 0.13[a]	1.33 ± 0.19	3.22 ± 0.42	9.4 ± 1.9

[a] $P < 0.05$ compared to amiloride-free (condition).
[b] $P < 0.05$ compared to A6 epithelium (Table 4).

aldosterone on apical membrane properties were larger for 2F3 epithelia compared to A6 epithelium. These findings raise the possibility of a different regulation of Na^+ transport in the 2F3 subclonal cell line.

Onset of Aldosterone Action

Spooner and Edelman (1975) divided the effects of aldosterone into three phases: (1) the "latent period," which comprised the first hour following aldosterone addition, and showed no change in G_T or I_{sc}, (2) the "early period", which occurred from 1–4 hr following aldosterone addition and was characterized by a sharp increase in I_{sc} and G_T, and (3) a "late response" after 4 hr which was characterized by a decrease in the rate of rise, and eventually, new steady-state values for I_{sc} and G_T. In contrast to this scheme, as indicated in Figs. 2 and 3, the action of aldosterone on G_T and I_{sc} in the present study was clearly detectable within 30–60 min, and thus was more rapid than previous reports for toad urinary bladder epithelium (Spooner & Edelman, 1975) or for A6 epithelium (Fidelman & Watlington, 1984).

Transepithelial electrical parameters in the present study for both aldosterone-stimulated and unstimulated tissues were higher than those reported in previous studies (Perkins & Handler, 1981; Fidelman & Watlington, 1984; Verrey et al., 1987). However, they are in excellent agreement with those of Bindels et al. (1988). The latter investigators reported average I_{sc} and G_T values of 16 μAcm^2 and 0.26 mS/cm^2 that increased to 28 μAcm^2 and 0.42 mS/cm^2 following aldosterone exposure. One possible reason for the close agreement between our study and that of Bindels et al. (1988) is that these investigators used a culture medium and measurement solutions that had osmolarities (190 mOsm) that were similar to those used in the present study (200 mOsm), whereas other investigators tended to use more hypertonic solutions (*cf.* Wills & Millinoff, 1990). We recently observed that hyposmotic serosal solutions markedly stimulate apical membrane Na^+ channel activity and that hyperosmotic solutions inhibit this activity (Wills et al., 1991). Interestingly, Bindels et al. (1988) reported that baseline Na^+ current levels had a significant effect on the synergistic actions of arginine vasopressin and aldosterone on I_{sc}. Experiments are now underway to

determine the interaction of solution osmolarity effects on basal Na^+ transport and aldosterone regulation of the Na^+ channel.

Late Effects of Aldosterone

Comparison of A6 and 2F3 Epithelia

In previous studies, we have reported that 2F3 epithelium has a higher basal I_{sc} than A6 epithelium (Wills & Millinoff, 1990; Wills et al., 1991) and that this difference is due in part to a larger apical membrane conductance in 2F3 epithelium. In this present study, these differences in I_{sc} and G_a were maintained following chronic aldosterone stimulation. In both tissues, I_{sc} was increased by a similar percentage, approximately 200%. Therefore, the difference in basal Na^+ transport between the two epithelia is not due to an inherent difference in sensitivity to aldosterone and may reflect a difference in the regulation or expression of the apical membrane Na^+ channel proteins. In preliminary experiments, we observed large values of amiloride sensitive I_{sc} (53 ± 7 $\mu A/cm^2$) and G_a (1.24 ± 0.21 mS/cm^2) in serum-depleted 2F3 tissues ($n = 4$). This finding was unexpected and is consistent with the view that Na^+ channel regulation in 2F3 may be fundamentally different than in A6 epithelium.

Paracellular Pathway and Cellular Driving Forces. Fidelman and Watlington (1987) previously demonstrated that tissues exposed to aldosterone for 24 hr showed increases in the ion permeability of the paracellular pathway. They postulated that this increase was necessary to support the increase in transepithelial Na^+ transport. Nonetheless, the present study did not detect significant changes in G_j for A6 or 2F3 epithelia following long-term aldosterone stimulation. We note that basal Na^+ transport rates in Fidelman and Watlington (1987) were approximately an order of magnitude lower than in the present study. Therefore, it is conceivable aldosterone might not affect G_j in tissues with high basal Na^+ transport rates. Additional experiments examining the effects of aldosterone on G_j in tissues with low basal Na^+ transport rates (e.g., tissues exposed to hyperosmotic solutions) are necessary to resolve this issue.

Membrane Effects of Aldosterone

Membrane Resistance Ratios. The average ratio of apical to basolateral membrane resistance (Ra/Rb) was approximately 13 for control conditions and approximately 5 for aldosterone-treated tissues. As yet, no microelectrode measurements during chronic (2–3 week) aldosterone stimulation are available for A6 epithelia. However, Kemendy, Kleyman and Eaton (1992) have demonstrated that aldosterone and glucocorticoids have similar effects on apical membrane Na^+ channels. Recently, Granitzer, Nagle and Crabbe (1992) have performed microelectrode measurements on A6 epithelia that were treated with dexamethasone for 24 hr. They reported a slightly lower average membrane resistance ratio ($Ra/Rb = 2$) and an apical membrane conductance of 0.60 mS/cm^2 for dexamethasone-treated tissues. Their G_a value is in excellent agreement with the present results for aldosterone (0.59 mS/cm^2, *see* Table 3), although G_{bl} was lower (1.3 mS/cm^2, compared to 3.0 mS/cm^2 for the present study; *see below*).

Apical Membrane. During chronic treatment with aldosterone, Na^+-transporting cells of the mammalian renal cortical collecting duct and urinary bladder show sustained increases in apical and basolateral membrane conductances (Wills & Lewis, 1980; Samson and O'Neil, 1986; Sansom, Muto & Giebisch, 1987). In agreement with these findings, G_a in A6 epithelia was significantly increased by ~170% for aldosterone-treated epithelia compared to serum-depleted controls. This increase was not due to a net increase in membrane area, since apical membrane capacitances were similar for the two groups. Therefore, as indicated in Fig. 5, the increase in apical membrane conductance was due to a significant increase in the specific conductance of the membrane, i.e., a greater density of conducting Na^+ transporters.

The above finding is consistent with the notion that aldosterone leads to an increase in the number of conducting Na^+ channels, possibly by the activation of quiescent channels (*cf.* Palmer et al., 1991). Recently, Kemendy et al. (1992) reported that the probability of opening for highly Na^+-selective channels in A6 epithelia was increased by aldosterone and that I_{sc} was abolished in the absence of this hormone. Single channel properties were not evaluated in the present study; however, a sizable amiloride-sensitive Na^+ current was present for aldosterone-depleted conditions. In serum-deprived tissues, I_{sc} averaged 19 $\mu A/cm^2$ (*see* Table 1). Therefore, Na^+ channel opening in the present study cannot be solely dependent on hormonal activation.

One possible explanation for the above discrepancy could be the presence of more than one type of amiloride-sensitive Na^+ channel in A6 epithelium. Hamilton and Eaton (1985) reported two populations of amiloride-sensitive apical channels in A6 cells: a

highly Na^+-selective channel and a poorly Na:K selective channel. This raises the possibility that poorly selective channels could be activated in the absence of aldosterone. However, the amiloride-sensitive conductance in aldosterone-depleted epithelia was highly selective for sodium, similar to normal controls. We observed that I_{sc} was abolished and G_a was reduced in serum-deprived tissues, following replacement of mucosal Na^+ by K^+ (Wills & Millinoff, 1990 and *unpublished observations*). Subsequent addition of mucosal amiloride had little effect. Therefore, G_a was highly selective for Na^+ over K^+ in these epithelia, similar to the highly selective Na^+ channels reported by Hamilton and Eaton (1985) and studied by Kemendy et al. (1992).Therefore, our finding of a large amiloride-sensitive current in serum-deprived tissues is not due to the activation of poorly Na^+-selective channels.

A more likely explanation is that aldosterone-stimulated Na^+ channel activity is modulated by an osmotic-activated mechanism. In preliminary experiments, we were able to completely inhibit I_{sc} in aldosterone or dexamethasone-stimulated epithelia by exposing these tissues to hyperosmotic serosal bathing solutions. Although it was not possible to activate corticosteroid-sensitive Na^+ channels osmotically, it was possible to inhibit their activity (once activated) by using hyperosmotic solutions. Therefore, it is likely that the mechanism(s) for hormonal regulation of these channels interacts with an independent system for regulation of the channel by other factors.[1]

Basolateral Membrane Properties. Basolateral membrane conductances for control and aldosterone-treated A6 and 2F3 epithelia were variable and significant differences were not detected. Several factors could account for this result. First, other conductances may be present that are unrelated to Na^+ transport and these may vary considerably among tissues. Because of the large background variability, impedance methods may not be able to resolve aldosterone-induced changes in basolateral membrane conductances or areas. As noted by Kottra and Fromter (1982) in their impedance studies of the amphibian gallbladder, basolateral membrane properties are more difficult to resolve than apical membrane properties using this technique.

Nonetheless, previous studies of the mammalian colon and cortical collecting duct have demonstrated large increases in basolateral membrane area following chronic hyperaldosteronism (Wade et al., 1979; Kashgarian et al., 1980). Although basolateral membrane areas tended to be higher in aldosterone-treated tissues than for serum-treated tissues, this effect did not reach statistical significance. Interestingly, the specific conductance of the basolateral membrane (i.e., conductance normalized to capacitance) was similar for these two conditions. This implies that if any membrane is inserted into the basolateral membrane, the inserted membrane has a similar number of conducting units per unit area as the original membrane. A striking contrast to this finding was the basolateral membrane-specific conductance of serum-deprived controls (*see* Fig. 4). This parameter was significantly lower than for other treatment conditions. Therefore, basolateral membrane properties were apparently altered in the absence of serum.

Granitzer et al. (1992) reported that basolateral membrane conductance in dexamethasone-treated A6 epithelium was ~1.3 mS/cm^2 and was significantly lower (~0.3 mS/cm^2) in the presence of amiloride (4 μM) in the mucosal bathing solution. G_{bl} in the present study was also large (~3 mS/cm^2) but was not significantly affected by amiloride (*see* Table 3). Given the numerous methodological differences between the studies, the disagreement in these findings is difficult to interpret. Granitzer et al. (1992) concluded that G_{bl} included a basolateral membrane chloride conductance. It is not known whether the larger value of G_{bl} in the present study reflects a chloride conductance.

In summary, the present study has confirmed that aldosterone is a potent modulator of Na^+ transport in cultured renal epithelia. Aldosterone increased I_{sc} in A6 epithelia within 30 min. Both A6 and 2F3 epithelia exhibited approximately twofold increases in I_{sc} and G_a increased by approximately 300–400 μS/cm^2 following chronic exposure to hormone. The increases in I_{sc} in both epithelia were accompanied by increases in apical membrane-specific conductances, i.e., membrane conductances increased without an increase in apical membrane area. Following aldosterone depletion (serum-deprivation), the basolateral membrane-specific conductance was significantly reduced. Apical membrane conductances in 2F3 epithelia showed a smaller percentage increase in response to aldosterone than A6 epithelium. Therefore, apical membrane Na^+ channel activity might be regulated differently for A6 and 2F3 epithelia.

[1] The present experiments were started before we found that hyposmotic solutions stimulate Na^+ transport. The measurement solution was slightly hyposmotic (170 mOsm) compared to the growth medium (200 mOsm). Preliminary investigations indicate that this hyposmotic solution does not qualitatively change the effects of aldosterone on membrane impedance properties, although I_{sc} values and apical and basolateral membrane conductances were larger in 170 mOsm solutions compared to 200 mOsm solutions. Membrane capacitances were unaltered.

We wish to thank Dr. Simon Lewis for his comments on this manuscript, Dr. B. Rossier for his generous donation of the 2F3 cell line, and Dr. E. Fulep for her technical support. This work was supported by NIH grant DK-29962 (to N.K.W.).

References

Bindels, R.J.M., Schafer, J.A., Reif, M.C. 1988. Stimulation of sodium transport by aldosterone and arginine vasotocin in A6 cells. *Biochim. Biophys. Acta* **972:**320–330

Claire, M., Machard, B., Lombes, M., Oblin, M.E., Bonvalet, J.P., Farman, N. 1989. Aldosterone receptors in A6 cells: physicochemical characterization and autoradiographic study. *Am. J. Physiol.* **257(26):**C665–C677

Clausen, C., Lewis, S.A., Diamond, J.M. 1979. Impedance analysis of a tight epithelium using a distributed resistance model. *Biophys. J.* **26:**291–318

Cole, K.S. 1972. Membranes, Ions and Impulses. University of California, Berkeley, CA

Fidelman, M.L., Watlington, C.O. 1984. Insulin and aldosterone interaction on Na^+ and K^+ transport in cultured kidney cells (A6). *Endocrinology* **115(3):**1171–1178

Fidelman, M.L., Watlington, C.O. 1987. Effect of aldosterone and insulin on mannitol Na^+ and Cl^- Fluxes in cultured epithelia of renal origin (A6): Evidence for increased permeability in the paracellular pathway. *Biochim. Biophys. Acta* **931:**205–214

Garty, H. 1986. Mechanisms of aldosterone action in tight epithelia. *J. Membrane Biol.* **90:**193–205

Gnionsahe, A., Claire, M. Koechlin, N., Bonvalet, J.P., Farman, N. 1989. Aldosterone binding sites along nephron of *Xenopus* and rabbit. *Am. J. Physiol.* **257:**R87–R95

Granitzer M., Nagel, W., Crabbe J. 1992. Basolateral membrane conductance in A6 cells: effect of high sodium transport rate. *Pfluegers Arch.* **420:**559–565

Hamilton, K.L., Eaton, D.C. 1985. Single-channel recordings from two types of amiloride-sensitive epithelial Na^+ channels. *Membr. Biochem.* **6(2):**149–171

Kashgarian, M., Taylor, C.R., Binder, H.J., Hayslett, J.P. 1980. Amplification of cell membrane surface in potassium adaptation. *Lab. Invest.* **42:**581–588

Kemendy, A.E., Kleyman, T., Eaton, D.C. 1992. The effects of aldosterone on single amiloride-blockable channels in A6 epithelia. *Am. J. Physiol.* **263:**C825–C837

Kottra, G., Fromter, E. 1984. Rapid determination of intraepithelial resistance barriers by alternating current spectroscopy. II. Test of model circuits and quantification of results. *Pfluegers Arch.* **402:**421–432

Paccolat, M.P., Geering, K., Gaeggeler, H.P., Rossier, B.C. 1987. Aldosterone regulation of Na^+-K^+-ATPase in A6 cells: role of growth conditions. *Am. J. Physiol.* **252(c21):** C468–C476

Palmer, L.R., Pacha, J., Frindt, G. 1991. Regulation of apical sodium channels in the rat cortical collecting tubule by aldosterone. *In:* Aldosterone Fundamental Aspects J.P. Bonvalet, N. Farman, M. Lombes, and Rafestin-Oblin, editors. pp. 285–294. John Libber Eurotext and INSERM, Paris

Pellanda, A.M., Gaeggeler, H.P., Horisberger, J.D., Rossier, B.C. 1992. Sodium-independent effect of aldosterone on initial rate of ouabain binding in A6 cells. *Am. J. Physiol.* **262:**C899–C906

Perkins, F.M., Handler, J.S. 1981. Transport properties of toad kidney epithelia in culture. *Am. J. Physiol.* **241:**C154–C159

Rossier, B.C., Pellands, A., Jaisser, F. 1991. Dual sodium independent effects of aldosterone on Na,K ATPase in amphibian cell lines. *In:* Aldosterone Fundamental Aspects (J.P. Bonvalet, N. Farman, M. Lombes, and Rafestin-Oblin, editors. pp. 123–130.) John Libber Eurotext and INSERM, Paris

Sansom, S.C., Muto, S., Giebisch, G. 1987. Na-dependent effects of DOCA on cellular transport properties of CCDs from ADX rabbits. *Am. J. Physiol.* **253:**F753–F759

Sansom, S.C., O'Neil, R.G. 1986. Effects of mineralocorticoids on transport properties of cortical collecting duct basolateral membrane. *Am. J. Physiol.* **251(20):**F743–F757

Spooner, P.M., Edelman, I.S. 1975. Further studies on the effect of aldosterone on electrical resistance of toad bladder. *Biochim. Biophys. Acta* **406:**304–314

Verrey, F., Schaerer, E., Zoerkler, P., Paccolat, M.P., Geering, K., Kraehenbuhl, J.P., Rossier, B.C. 1987. Regulation by aldosterone of Na^+, K^+-ATPase mRNAs, protein synthesis, and sodium transport in cultured kidney cells. *J. Cell Biol.* **104:**1231–1237

Wade, J.B., O'Neil, R.G., Pryor, J.L., Boulpaep, E.L. 1979. Modulation of cell membrane area in renal collecting tubules by corticosteroid hormones. *J. Cell Biol.* **81:**439–445

Watlington, C.O., Perkins, F.M., Munson, P.J., Handler, J.S. 1982. Aldosterone and corticosterone binding and effects on Na^+ transport in cultured kidney cells. *Am. J. Physiol.* **242:**F610–F619

Wills, N.K., Clausen, C. 1987. Transport-dependent alterations of membrane properties of mammalian colon measured using impedance analysis. *J. Membrane Biol.* **95:**21–35

Wills, N.K., Eaton, D.C., Lewis, S.A., Ifshin, M.S. 1979. Current-voltage relationship of the basolateral membrane of a tight epithelium. *Biochim. Biophys. Acta* **555:**519–523

Wills, N.K., Lewis, S.A. 1980. Intracellular Na^+ activity as a function of Na^+ transport rate across a tight epithelium. *Biophys. J.* **30:**181–186

Wills, N.K., Milinoff, L.P. 1990. Amiloride-sensitive Na^+ transport across cultured renal (A6) epithelium: Evidence for large currents and high Na:K selectivity. *Pfluegers Arch.* **416:** 481–492

Wills, N.K., Millinoff, L.P., Crowe, W.E. 1991. Na^+ channel activity in cultured renal (A6) epithelium: regulation by solution osmolarity. *J. Membrane Biol.* **121:**79–90

Wills, N.K., Purcell, R.K., Clausen, C. 1992. Na^+ transport and impedance properties of cultured renal (A6 and 2F3) epithelia. *J. Membrane Biol.* **125:**273–285

Yonath, J., Civan, M.M. 1971. Determination of the driving force of the Na^+ pump in toad bladder by means of vasopressin. *J. Membrane Biol.* **5:**366–385

Received 16 July 1992; revised 10 November 1992