J. Membrane Biol. 144, 81–91 (1995)

The Journal of
Membrane
Biology

Apical Membrane Sodium and Chloride Entry During Osmotic Swelling of Renal (A6) Epithelial Cells

W.E. Crowe,* J. Ehrenfeld,[2] E. Brochiero,[2] N.K. Wills[1]

[1]Department of Physiology and Biophysics, University of Texas Medical Br., Galveston, TX 77555
[2]Département de Cellular et Molécular Biology, Laboratoire Jean Maetz, Villefranche-sur-Mer, France 06230

Received: 13 April 1994/Revised: 3 November 1994

Abstract. To assess the role of chloride in cell volume and sodium transport regulation, we measured cell height changes (CH), transepithelial chloride and sodium fluxes, and intracellular chloride content during challenge with hyposmotic solutions under open circuit (OC) conditions. CH maximally increased following hyposmotic challenge within ~5 minutes. The change in CH was smaller under short circuit (SC) conditions or following replacement of chloride in the mucosal solution by gluconate or cyclamate (Cl^--free$_m$). When corrected for the osmotically inactive cell volume ($30 \pm 2\%$), ΔCH for controls (OC) were greater than predicted for an ideal osmometer. In contrast, ΔCH for Cl^--free$_m$ or SC conditions were similar to that predicted for an ideal osmometer.

Na^+ and Cl^- mucosa-to-serosa fluxes increased following hyposmotic challenge. Chloride fluxes increased maximally within 5 min, then decreased. In contrast, the Na^+ flux increased slowly and reached a steady state after ~25 min. Under isosmotic conditions, exposure to Cl^--free$_m$ solutions led to decreases in the transepithelial conductance, Na^+ flux, and CH. Chloride permeabilities in the apical and basolateral membranes were detected using the fluorescent intracellular chloride indicator MQAE.

The results indicate that during osmotic swelling, the entry of both sodium and chloride is increased. The time courses of these increases differ, suggesting distinct mechanisms for the osmotic regulation of these apical membrane transport processes.

* *Present address:* Department of Physiology, Medical College of Pennsylvania, PA 19129

Correspondence to: N.K. Wills

Key Words: A6 cells — Chloride — Sodium — Cell volume — Epithelium — MQAE

Introduction

The cultured renal cell line A6 has become a popular model for studies of sodium transport regulation across the distal renal tubule. A primary role of the distal nephron in vivo is the absorption of sodium and chloride from the lumen of the kidney tubule into the blood, thereby "fine tuning" the plasma concentrations of these ions. Recently, Wills, Millinoff and Crowe (1991) demonstrated that sodium channel activity in A6 epithelia is sensitive to small changes in serosal solution osmolality. Since plasma osmolality of aquatic amphibians can vary according to the environmental conditions (Mayer, 1969), this response may be physiologically important for the regulation of plasma sodium content.

In previous studies (Crowe & Wills, 1991; Ehrenfeld, Raschi & Brochiero, 1994), we showed that the hyposmotic solution-induced stimulation of Na^+ transport occurred more slowly than the onset of cell swelling. In addition, Crowe and Wills (1991) reported that the increase in cell volume was greater than expected for an ideal osmometer. This result suggested a paradoxical accumulation of intracellular solutes during cell swelling.

We now investigated the possible role of chloride ions in the osmotic response of A6 cells and Na^+ transport regulation. Specifically, we determined the chloride permeability of the apical membrane using intracellular fluorescent probes, cell-volume measurements, and transepithelial chloride flux measurements. In addition, we assessed how changes in the driving force for apical chloride or sodium movement affect the cell-volume response to hyposmotic solutions.

Materials and Methods

Cell Culture

A6 cells were grown on transparent permeable filter supports according to previously described methods (Wills & Millinoff, 1990; Ehrenfeld et al. 1994). For cell height measurements, the filter supports (Anocell 25: Wheaton, NJ) were coated with microbeads by placing a 0.5 ml of a suspension of microbeads (0.03% stock solution in 100% ethanol) on the top surface of the filter. The microbeads consisted of 0.6 μm diameter latex spheres which were fluorescent at red wavelengths (L-5363, Molecular Probes, OR). The filters were left in a sterile hood until the ethanol evaporated, leaving the microbeads adhered to the filter surface. Cells were then seeded on the filters and grown as usual.

Access of microbeads to the apical surface can be hindered by the presence of extracellular debris on top of the apical membrane. For this reason, we removed the serum from the mucosal medium after eight days and completely replaced the mucosal solution each time the cells were fed. In addition, the apical surface was washed several times to remove as much of this material as possible. Cells were grown to confluence and studied 14–30 days after plating.

Solutions

Unless otherwise noted, the composition of the Ringer's solution was (in mM): 74.4 NaCl, 5.4 KCl, 8.0 $NaHCO_3$, 1.4 $CaCl_2$, 1.7 $MgSO_4$, 0.9 NaH_2PO_4, 5.5 glucose, 1.0 sodium pyruvate, 1.0 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) and 30 mannitol. The osmolality of the solution was 200 mosmol/kgH_2O and was measured with a vapor pressure osmometer (model 5500; Wescor, Logan, Utah). Solutions were maintained at a pH of 7.4 by gassing with air containing 1% CO_2. For experiments investigating the osmotic responses of A6 cells, the mannitol was removed from the solution. Alternatively, the solution osmolality was reduced to 100 mosmol/kgH_2O by reducing the solution NaCl concentration to 39.4 mM and omitting mannitol. To obtain the correct osmolality for other solutions, appropriate concentrations of mannitol were added. For the transepithelial flux or intracellular chloride measurements, changes on the serosal side of the monolayer from isosmotic solutions to hyposmotic (76% of the isosmotic osmolality) solutions were assessed using two protocols: (1) mannitol omission from the isosmotic solution or (2) a 24% water dilution of the isosmotic solution (*see* Ehrenfeld et al., 1994). Chloride-free solutions were obtained by substituting chloride salts with either gluconate or cyclamate salts. Amiloride (a gift of Merck, Sharp and Dohme) was prepared as a concentrated stock in distilled water and used at a final concentration of 20 μM.

Cell Height Measurements

Before each experiment, the apical surface of the epithelium was labeled with microbeads by exposure to a Ringer's solution that contained 2% of the microbead stock. After a 15-min exposure, the microbead solution was removed and the apical surface washed several times to remove loosely attached beads. The epithelium and filter support were then mounted in a specially designed Ussing-type chamber that allowed simultaneous optical and electrical studies (Crowe & Wills, 1991), and placed on the stage of an inverted microscope (model IM: Zeiss, Oberkochen, FRG). Cell height was determined by alternately focusing between fluorescent beads located at the apical and basal surfaces. The distance between the two planes was measured electrically by friction coupling the shaft of a potentiometer to a large wheel (diameter, 130 mm) attached to the coarse focus knob of the microscope, to reduce hysteresis in the system. Calibration was achieved by focusing between two surfaces of a glass coverslip of known thickness and measuring the voltage difference as a result of rotating the potentiometer. This calibration factor was then multiplied by the ratio of the refractive index of the cell to that of the glass coverslip (1.37/1.52) to compensate for the elongation of the depth scale in the glass (Persson & Spring 1982; Carlsson, 1991). Without this correction, cell heights are overestimated by ~10% (Crowe & Wills, 1991).

It was possible to make a cell height reading every 3 sec. To reduce scatter in the measurements, seven consecutive readings were averaged. The typical standard error of the averaged measurements was ~0.5 μm and the time resolution was ~30 sec.

Electrical Measurements

The method for measuring transepithelial voltage (V_T) and current (I) were similar to those described previously (Wills & Millinoff, 1990). In brief, V_T was monitored with 3M KCl half cells (connected to the chamber by agar bridges) and attached to an automatic current/voltage clamp. Transepithelial resistance (R_T) was calculated from Ohm's law using the measured deflection in V_T to an applied current pulse passed through Ag-AgCl wires located on either side of the epithelium. The short-circuit current (I_{SC}), a measure of active sodium transport across the A6 epithelium, was calculated as V_T/R_T.

Radioisotopic Flux Measurements

Unidirectional Na^+ and Cl^+ fluxes were measured with the isotopes ^{22}Na (15μCi/ml; Amersham, France) and ^{36}Cl (15μCi/ml; Amersham, France) added to one side of the epithelium and 2 ml samples were collected from the opposite bathing solution at 5-min intervals, after a 20-min equilibration period. The radioactivity of the samples was measured after addition of 6 ml of liquid scintillation fluid (ACS, Amersham; France) in a scintillation counter (Intertechnique, France). The fluxes were calculated from the quantity of radioactivity transferred per unit time and the initial specific radioactivity of the labelled compartment.

Fluorescent Dye Measurements

To monitor intracellular chloride concentrations, the fluorescent probe MQAE (*N*-[6-methoxyquinoly]acetoethyl ester, Molecular Probes, Eugene, OR) was used. Due to low membrane permeability to this dye, A6 monolayers were loaded overnight in amphibian cell culture medium containing 7 mM MQAE at 28°C, in a CO_2 incubator. After several rapid washes, the filter and the monolayer were cut off the support, mounted in an adapted "Ussing chamber," and placed in the sample compartment of a spectrophotometer system (PTI Deltascan, N.J.). The excitation light was set at 360 nm and the emission was monitored at 450 nm (6 nm band pass). The fluorescence intensity was measured every second and plotted graphically. Intracellular chloride activity (a_iCl) was estimated using a technique derived from that used by Issley and Verkman (1987). At the end of the experiment, the monolayer was perfused with a KSCN(120 mM) solution (buffered with 10 mM HEPES/KOH, pH 7.2) which quenched MQAE fluorescence by >90%. For data analysis, this value was subtracted from the fluorescence measured experimentally. To calculate a_iCl, a calibration curve was obtained by bathing the epithelium on both sides in a high K^+ (105 mM) medium containing tributyltin-chloride (10 μM, Fluka; Buchs, Switzerland), nigericin (2.5 μM Sigma, St. Louis, MO) and valinomy-

Table 1. Effects of hyposmotic challenge (15%) under different conditions

	I_{sc}[a] (% change)	G_T (% change)	Cell height (% change)
Open circuit			
5 min (n = 9)	12 ± 3	3 ± 3	19 ± 2
20 min (n = 6)	130 ± 20	40 ± 10	8 ± 6
Short circuit			
5 min (n = 6)	16 ± 8	2 ± 3	8 ± 3[c]
20 min (n = 3)	130 ± 30	30 ± 20	7 ± 6
Chloride free			
5 min (n = 5)	19 ± 6	13 ± 8	11 ± 1[b]
20 min (n = 4)	120 ± 20	50 ± 10	1 ± 7

[a] I_{sx} is calculated as V_T/R_T and is an estimate of the rate of net Na^+ transport (*see* Materials and Methods)
[b] $P < 0.05$, unpaired t-test, open circuit *vs.* chloride free
[c] $P < 0.01$, unpaired t-test, open circuit *vs.* short circuit
Means ± SEM

cin (5 μM Sigma, St. Louis, MO) and by changing the extracellular chloride concentration (by equimolar substitution of gluconate for chloride). The MQAE fluorescence was found to be linear over a range of cell chloride concentrations of 0–60 mM, values within the physiological range (*see below*). Dye leakage was low and was estimated as <5% over a period of 20 min.

Results

The Effects of Hyposmotic Challenge under Open Circuit Conditions

Table 1 illustrates the mean responses (n = 9) for cell height (CH), I_{SC} and G_T to a 15% decrease in solution osmolality under open circuit conditions. As shown in Fig. 1, swelling began within 1 min following the solution change and reached its peak within 5 min, followed by a regulatory volume decrease (RVD) to a new steady-state value (in 20 min) that was ~8% higher than the control level. In contrast, I_{SC} and G_T began to increase after the onset of cell swelling and continuously increased for ~25 min, reaching new plateau values within 30 min following the solution change (*plateau data not shown* in Fig. 1).

In agreement with our previous preliminary findings, the maximum observed increase in cell height (ΔCH) was 19 ± 2% (n = 9). To further assess cell volume responses to osmotic challenge, we next measured cell height following changes in the solution osmolality over a range of values from 100 to 300 mosmol/kgH_2O.

Relationship between Cell Height and Solution Osmolality

Figure 2 illustrates cell height measurements obtained for a representative experiment during step changes in

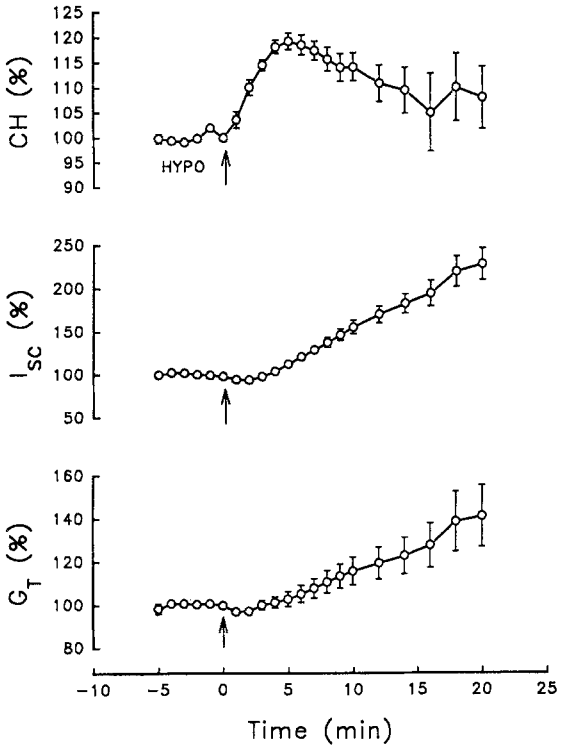

Fig. 1. Hyposmotic challenge under open circuit conditions. Mean values from nine experiments showing cell height (CH), short circuit current (I_{SC}) and transepithelial conductance (G_T) as percentages of their control values in isosmotic solutions. At time = 0 the serosal bathing solution was changed to a 15% hyposmotic solution. I_{SC} and G_T reached a new maximum steady state value at t = ~25 min (*data not shown*). Note that the maximum increase in CH occurred earlier, at t = ~5 min.

solution osmolality. The small dots represent individual measurements, the solid line is a running average of the data, and the large filled circles represent the mean values of ten sequential measurements. (The standard errors of the sequential measurements averaged 0.3 ± 0.01μm; n = 66). The epithelium was first incubated in an isosmotic low NaCl Ringer's solution (200 mosmol/kgH_2O) containing low NaCl (39 mM, *see* Materials and Methods). As expected, a hyposomotic challenge resulted in cell swelling whereas hyperosmotic challenge resulted in a decrease in cell height.

In preliminary experiments, we noted that hyposmotic solutions evoked nonlinear increases in cell height. For this reason, we next assessed the osmotic responses of the cells in more detail by avoiding the possible complications of transepithelial sodium transport. In these experiments, epithelia were first exposed to 20 μM amiloride in the mucosal bath. Under these conditions, V_T was −8.5 ± 2.3 mV, I_{SC} was 0.5 ± 0.1 μA/cm^2, R_T was 17 ± 5 kΩ · cm^2 and cell height averaged 15 ± 1 μm (n = 5). In these experiments, bathing solutions were changed to hyperosmotic solutions (300 mosmol/kgH_2O), followed by symmetrical step decreases in os-

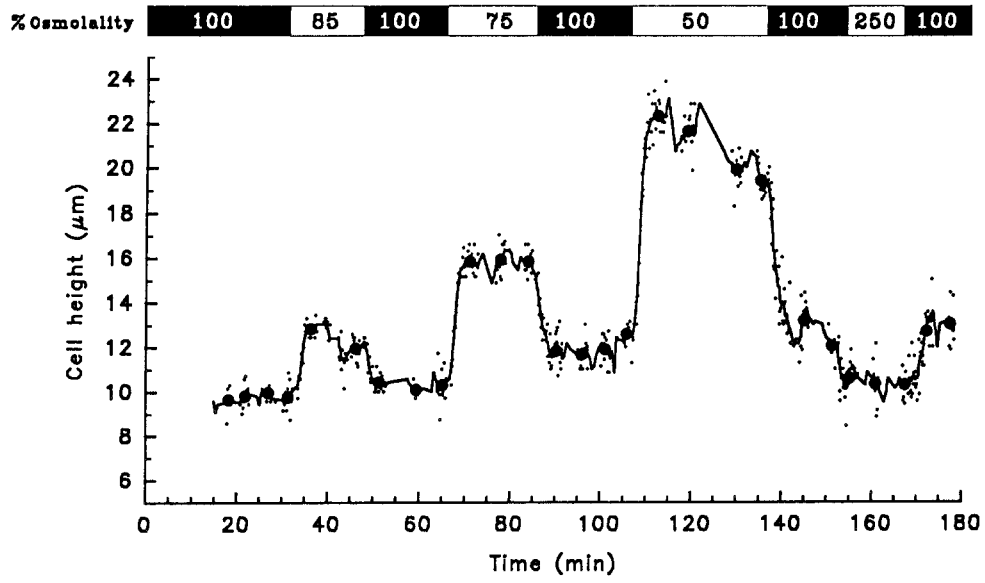

Fig. 2. The effect of stepped changes in osmolality on cell height. In this experiment, the epithelium was bathed on both sides in an isosmotic (200 mosmol/kgH_2O) Ringer's solution (39 mM NaCl, 100 mM mannitol, *see* "100% osmolality" above). Osmolalities were varied by changing the mannitol concentration and are indicated as the percentage of the isosmotic osmolality.

molality (200, 150, 120, 100 mosmol/kgH_2O), and finally isosmotic solutions. Figure 3*A* shows the maximum cell height increase (at time ≤5 min) normalized to the cell height in isosmotic solutions shown as a function of the inverse normalized solution osmolarity for a typical epithelium. Longer exposure times led to regulatory volume decreases as indicated in Fig. 3*B*, where 10-min exposures were used. For this reason, data for maximum cell height change (i.e., 5-min data points) were used in subsequent calculations.

The data in Fig. 3*A* are described by the Boyle-van't Hoff equation (*cf.* Dick, 1966; Hallows & Knauf, 1993):

$$\frac{CH_T}{CH_o} = \frac{\Pi_o}{\Pi_T}\left(1 - \frac{b}{CH_o}\right) + \frac{b}{CH_o} \qquad (1)$$

where Π_o and CH_o are the original (isosmotic) external solution osmotic pressure and cell height, Π_T and CH_T are the same parameters for the anisosmotic condition and b is the nonsolute or osmotically inactive volume (OIV) of the cell. A linear regression of averaged data from four experiments in which osmolality was varied from 150–300 mosmol/kgH_2O yielded a regression coefficient of 0.998 and a slope of 0.7 ± 0.02 and a y intercept of 0.30 ± 0.01. As described above, the later parameter is the percentage of the cell volume that is osmotically inactive.

From Eq. 1 above, it is evident that when $b = 0$ (i.e., an ideal osmometer), $\Pi_o/\Pi_T = CH_T/CH_o$. Thus, given a

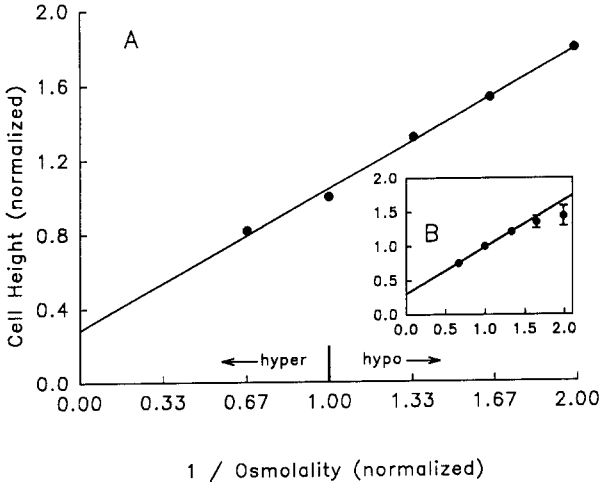

Fig. 3. The relationship between cell height of amiloride-treated A6 cells and the reciprocal of osmolality. (*A*) Mean cell heights (filled circles) were normalized to isosmotic conditions and plotted against normalized 1/osmolality. (Sampling time ~5 min after solution change). The linear regression coefficient of the fitted line was 0.999. The osmotically inactive volume can be estimated by extrapolation to infinite osmolarity (i.e., y intercept = 0.30) as 30% of the cell volume. The slope of the regression was 0.7 and is less than the predicted value for an ideal osmometer (slope = 1) because of the osmotically inactive volume. (*B*, inset). In four experiments, a longer sampling interval was used (≥10 min) and RVD was observed at lower osmolalities. If these points are deleted from the analysis, a regression line fitted to the remaining data is in good agreement with Fig. 3*A* (r^2 = 0.997 and y intercept = 0.29).

change in osmolality from 200 to 170 mosmol/kgH_2O, an ideal osmometer should show an increase in volume of ~18%. If 30% of the cell volume is osmotically inactive, then only a 12% increase should occur. Therefore, the observed ~19% change in cell height for Na^+ transporting monolayers exposed to this hyposmotic challenge (*see* Fig. 1) represents an increase in the osmotically active volume.

A6 cells are generally cuboidal in shape and the average cell density of the monolayer is 2×10^6 cells/cm^2 (Jovov, Wills & Lewis, 1991). Thus, the estimated apical surface of the cell is approximately 7 μm × 7 μm square. Since the average cell height is 14.5 ± 0.6 μm (n = 30), the total cell volume is ~710 μm^3. Using the above value for osmotically inactive volume (30%), we calculate that the osmotically active volume is ~500 μm^3. Thus, the observed 19% increase in cell height corresponds to a 27% increase in the osmotically active volume (OAV). The increase in OAV is approximately 50% higher than predicted for the behavior of an ideal osmometer, suggesting a net gain in intracellular solute composition during swelling.

The Role of the Apical Membrane Chloride Electrochemical Gradient in the Hyposmotic Response

It is possible that movement of chloride into the cell across the apical membrane of open-circuited epithelia may partly contribute to this net gain of intracellular solutes. To test this hypothesis, we first replaced chloride with an impermeant anion to create a chemical gradient for chloride efflux. Second, the electrical gradient was altered by short circuiting the monolayer such that the apical membrane was hyperpolarized and the electrical gradient for chloride entry was reduced.

Chloride Replacement

Replacement of mucosal chloride with either gluconate or cyclamate under isosmotic conditions resulted in a decrease in cell height (9 ± 5%, n = 9) over 5 min and a decline in mean I_{SC} and G_T (22 ± 7% and 30 ± 7%, respectively; Fig. 4 and Table 2). However, we note that in some experiments there was no apparent decrease in cell height and in others, a partial recovery of I_{SC} occurred, indicating an inherent variability in this permeability under isosmotic conditions.

Although the observation of a decrease in cell height is consistent with an apical membrane chloride conductance, the decrease in G_T could reflect either a decrease in other apical or basolateral membrane conductances or an alteration in the paracellular pathway. Data from amiloride experiments show the transepithelial conductance was 31 ± 1% (n = 6) lower following mucosal chloride replacement by gluconate. This reduction appears to be related to a decrease in sodium absorption (*see below*).

When A6 epithelia were challenged with hyposmotic solutions following similar replacement of mucosal chloride (Fig. 5*A* and Table 1), the maximum change in mean cell height was 11 ± 1% (n = 5). When corrected for the osmotically inactive volume, this increase in cell height represents a 17% increase in the osmotically active volume, significantly lower than the

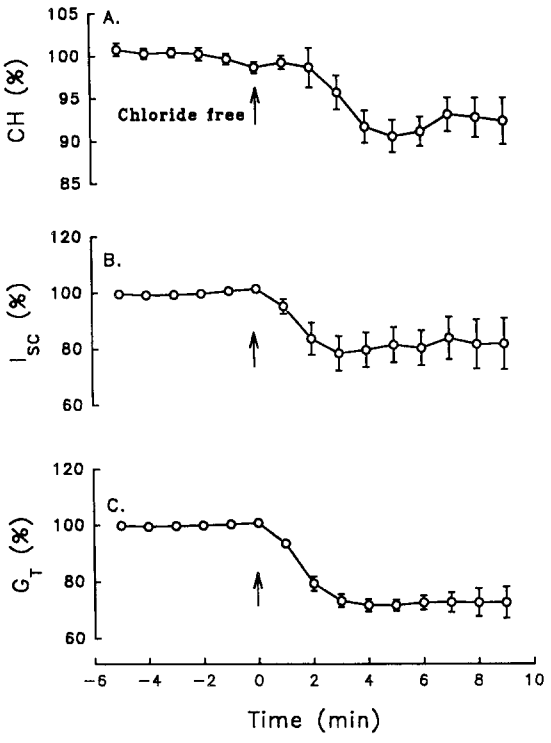

Fig. 4. Replacement of mucosal chloride under isosmotic conditions. At time = 0, mucosal chloride was replaced with an impermanent anion (cyclamate or gluconate). I_{SC} and G_T rapidly declined and the decrease in cell height was delayed (n = 9).

Table 2. Effects of mucosal Cl^- replacement on cell height and electrical parameters

	I_{sc} (μA/cm^2)	G_T (μScm^2)	Cell height (μm)
Control	9.4 ± 0.7	207 ± 12	12.1 ± 0.8
Cl^- free	7.5 ± 1.0	146 ± 29	11.0 ± 0.8
% change	−22 ± 17	−30 ± 7	−9 ± 5
P^*	<0.01	<0.0001	<0.001

* Paired t-test; (n = 9)

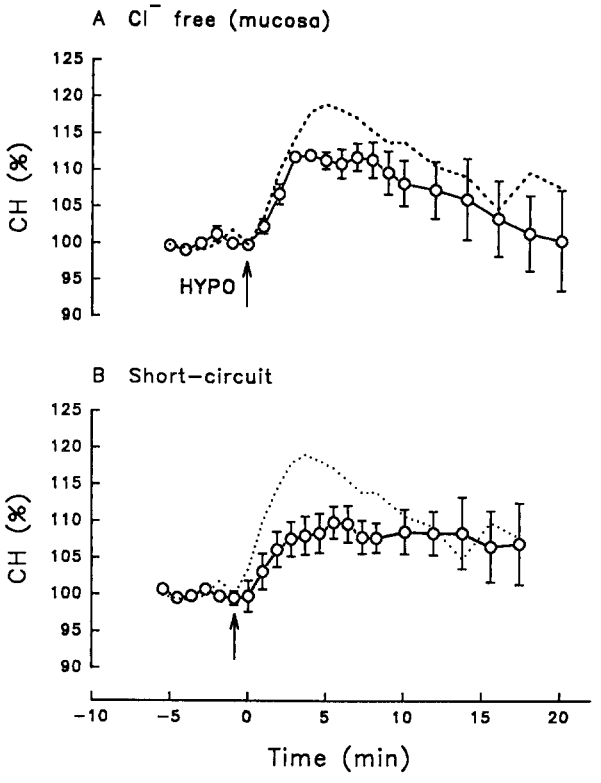

Fig. 5. Cell height responses to hyposmotic solutions following mucosal chloride replacement (*A*) or short circuit conditions (*B*). (*A*) Mucosal chloride was replaced with cyclamate or gluconate ($n = 5$). Tissues were initially bathed with isosmotic solutions and changed to a hyposmotic solution at t = 0. The broken line represents cell height changes in the presence of chloride under normal open circuit conditions (data from Fig. 1). Note that at t = 5 min, the increase in cell height was lower in the absence of chloride. (*B*) Data from similar experiments in which epithelia were voltage clamped to 0 mV. Under these conditions, the hyposmotic-induced cell swelling at t = 5 min was also reduced ($n = 6$; broken line indicates CH results for control experiments as described above.)

27% increase in OAV seen in the presence of mucosal chloride ($P < 0.01$; *see above*).

Short Circuit Conditions

As described above, A6 cells behave as simple osmometers in the presence of mucosal amiloride (*see* Fig. 3). Amiloride blocks the apical membrane sodium conductance, leading to decreased sodium entry and hyperpolarization of the apical membrane potential. To investigate this effect of apical membrane hyperpolarization, experiments were conducted under short circuit conditions. This manipulation allows the apical membrane to be hyperpolarized without blocking sodium channels. Short circuiting A6 epithelia did not have a significant effect on cell height (Table 3), suggesting a lack of change in net intracellular solute content.

Table 3. Effects of short circuiting on cell height and conductance

	G_T (μS/cm²)	Cell height (μm)
Control	300 ± 40	14.5 ± 0.8
Short circuit	320 ± 50	14.8 ± 1.0
% change	7.4 ± 3.6	1.5 ± 2.0
*P**	NS	NS

* Paired *t*-test; ($n = 8$)

Figure 5*B* shows the response of cell height to hyposmotic challenge (15%) for short-circuited epithelia. Note that the increase in mean cell height of 10 ± 2% ($n = 6$) was less than that seen under open circuit conditions ($P < 0.05$). The cell height change corresponds to a 14% increase in the osmotically active volume, less than the 18% change in CH predicted for an ideal osmometer exposed to a 15% decrease in solution osmolarity (*see above*).

Short circuiting the epithelium or replacing mucosal chloride under open circuit conditions did not significantly alter the normal open circuit response of I_{SC} and G_T to hyposmotic challenge (Table 1). This suggests that the osmotic stimulation of sodium transport does not require mucosal chloride.

Transepithelial Flux Measurements

Under open circuit, isosmotic conditions, mucosa-to-serosa Cl^- fluxes were approximately seven times larger than the serosa-to-mucosa fluxes (137 ± 9 nEq/cm² hr and 21 ± 2 nEq/cm² hr, respectively; $n = 3$). Therefore, there was a net absorption, i.e., mucosa-to-serosa movement, of chloride ($J_{net}Cl = 116 \pm 7$ nEq/cm² hr; $n = 3$). Short circuiting the tissue significantly reduced the mucosa-to-serosa Cl^- flux by 64 ± 8% ($n = 4$; $P < 0.003$), consistent with the elimination of the lumen negative transepithelial potential.

Figure 6*A* and Table 4 compare the time course of changes in mucosa to serosa fluxes of chloride and sodium under open circuit conditions before and during exposure to a 24% hyposmotic serosal solution (mannitol omission, *see* Protocol 1 in Materials and Methods). Following hyposmotic challenge, $J_{ms}Cl$ increased rapidly, by 192 ± 31% ($n = 3$) within 5 min, and then declined to a new steady state value that was 108 ± 27% higher than control levels. In contrast to the immediate change in $J_{ms}Cl$, $J_{ms}Na$ increased slowly and was not significantly increased during the first 5 min of serosal hyposmotic solution application. $J_{ms}Na$ reached a new steady state within 25 min ($\Delta J_{ms}Na = 120 \pm 14\%$; $n = 3$). As shown in Fig. 6*B*, similar results were found when the serosal solution was made hyposmotic by a 24% water dilution: (*see* Protocol 2 in Materials and Methods; $n = 9$).

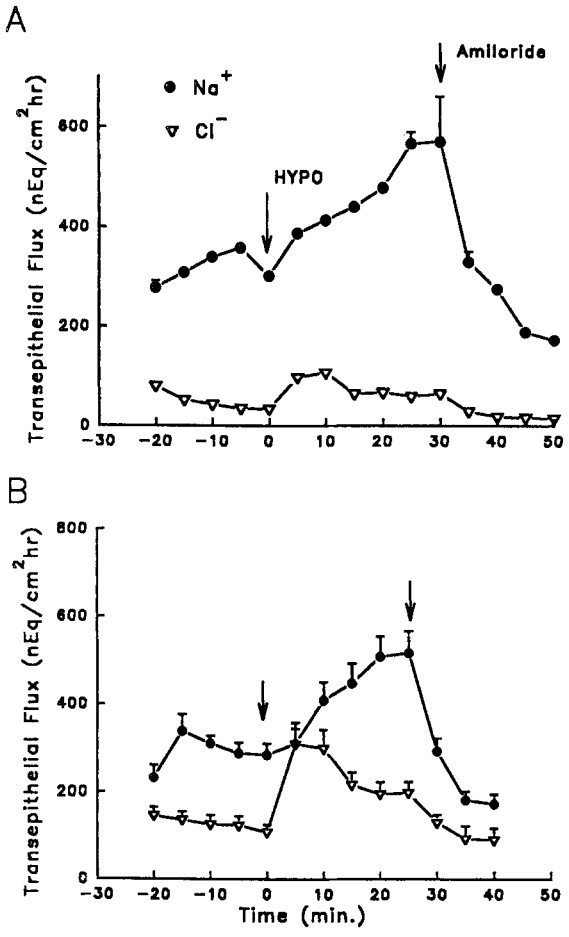

Fig. 6. Transepithelial absorptive fluxes of sodium and chloride. (*A*) Epithelia were initially bathed in isosmotic solutions and switched to hypotonic serosal solution (protocol 1, *see* Materials and Methods) at t = 0. Note that chloride fluxes transiently increase then decrease to a new steady state value whereas sodium fluxes increase and plateau at t = ~25 minutes. Both fluxes were reduced by mucosal amiloride at t = 30 min. ($n = 3$) the Na^+ and Cl^- concentrations in the bathing solutions were 80.2 mM and 56.5 mM, respectively. (*B*) Mean results from similar experiments in which the serosal solution was made hyposmotic by water dilution (protocol 2; $n = 9$). Note the similar time course of the sodium and chloride fluxes in *A*. (Note: the initial Na^+ and Cl^- concentrations in the bathing solutions were 115 mM and 92 mM, respectively.)

As also shown in Table 4 and Fig. 6*A*, addition of mucosal amiloride (5×10^{-5} M) under open circuit conditions reduced $J_{ms}Na$ and $J_{ms}Cl$ by 74 ± 2% and 76 ± 2%, respectively. Similar reductions are evident in Fig. 6*B*. This finding is expected following blockage of the apical membrane Na^+ conductance and is consistent with a reduction in the electrical driving force favoring mucosa-to-serosa chloride movement. It must be noted that for both protocols, $J_{ms}Na$ was larger than $J_{ms}Cl$. Although these are unidirectional fluxes, this large difference makes it likely that anions other than chloride might also balance the entry of Na^+.

In isosmotic open circuit conditions, mucosal-to-serosa Na^+ fluxes were significantly reduced when chloride in the mucosal bathing solution was replaced with gluconate (J_{ms} = 249 ± 23 nEq/cm^2 hr and 118 ± 18 nEq/cm^2 hr for control and Cl^--free, respectively; $n = 5$). The replacement of chloride by gluconate did not prevent the osmotic stimulation of $J_{ms}Na$ seen in Cl^- containing solutions. Following a 24% hyposmotic challenge, fluxes in the same tissues were increased similarly (ΔJ_{ms} = 98 ± 12%, control and 124 ± 13%, gluconate, NS).

Intracellular Chloride Measurements Using MQAE

Transepithelial chloride fluxes do not distinguish between cellular and paracellular transport pathways. To assess whether a chloride permeability was present in the apical or basolateral membranes, we depleted cells of chloride by replacing this ion in both bathing solutions with gluconate. The chloride permeability of each membrane was assessed by readdition of chloride to either side of the epithelium.

The mean value of intracellular chloride activity was 27 ± 2 mM ($n = 23$) with a 89.5 mM chloride concentration in the Ringer's solution. As shown Fig. 7, removal of chloride caused an increase in the fluorescence signal which reached a new steady state within 7.0 ± 0.8 min, indicating a decrease in the intracellular chloride activity ($\Delta a_iCl = -23 \pm 2$ mM; $n = 23$). Re-addition of chloride to the serosal side of the epithelium, resulted in a rapid increase (within 7.4 ± 0.7 min) in intracellular chloride content to control values ($\Delta a_iCl = -23 \pm 2$ mM; $n = 23$). This finding indicates the existence of a large basolateral membrane chloride permeability.

As also shown in Fig. 7, restoration of mucosal chloride-to-chloride-depleted cells led to a small increase in a_iCl (6 ± 2 mM; $n = 9$) within 2.9 ± 0.6 min which could be reversed by subsequent removal of mucosal chloride. Therefore, an apical membrane chloride permeability is present. However, this permeability is smaller than that of the basolateral membrane and is insufficient to fully restore the intracellular chloride activity in the absence of serosal chloride.

Discussion

The results of this paper confirm and extend our previous observation that A6 cells paradoxically gain intracellular solutions following osmotic challenge (Crowe & Wills, 1991). The present data indicate that this response involves sodium and chloride entry across the apical membrane. In addition, the time course of the sodium and chloride fluxes differed, suggesting distinct mechanisms for the osmotic regulation of these apical membrane transport processes.

Table 4. Effects of hyposmotic solutions on mucosal to serosal fluxes

Time	0 (min)	5 (min)	25 (min)	Amiloride
Protocol 1	(Mannitol)			
J_{Na} (nEq/cm^{2}hr)	302 ± 10	389 ± 13	568 ± 23[a]	173 ± 7[b]
J_{Cl} (nEq/cm^{2}hr)	34 ± 3	97 ± 4[a]	59 ± 4[a]	15 ± 1
$n = 3$				
Protocol 2	Dilution			
J_{Na} (nEq/cm^{2}hr)	284 ± 24	310 ± 48	517 ± 50[a]	173 ± 21[a]
J_{Cl} (nEq/cm^{2}hr)	108 ± 14	309 ± 35[a]	130 ± 17	90 ± 27
$n = 9$				

[a] $P < 0.05$ compared to $t = 0$, paired t-test
[b] $P < 0.05$ compared to $t = 25$, paired t-test

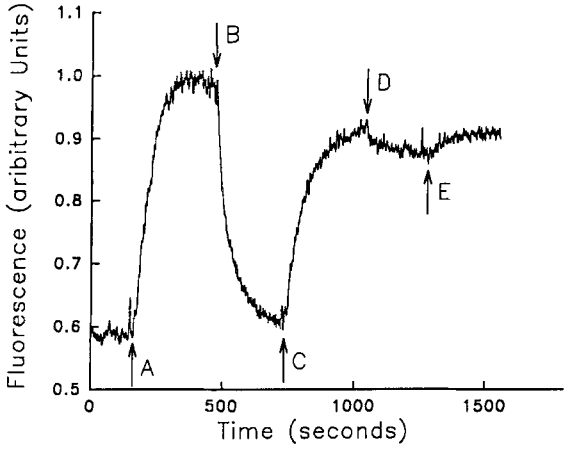

Fig. 7. Quenching of the intracellular chloride dye MQAE. Results from a typical epithelium showing the effects of: (*A*) bilateral chloride removal (replacement with gluconate), (*B*) restoration of serosal chloride, (*C*) removal of serosal chloride, (*D*) restoration of mucosal chloride, and (*E*) removal of mucosal chloride. Note that signal is quenched in the presence of chloride (*B* and *D*), indicating entry of this ion into the cell. Entry across the basolateral membrane was greater than across the apical membrane.

Osmotic Response of A6 Cells: Osmotically Active and Osmotically Inactive Volumes

In experiments in which a range of hyposmotic solutions were used, the cell height increased more than expected from the change in solution osmolarity. In contrast, when cells were treated with mucosal amiloride, the cells behaved as simple osmometer, i.e., there was a linear relationship between cell height and inverse solution osmolality (*see* Fig. 2). At infinite osmolarity, the estimated normalized cell height was 0.3, indicating that ~30% of the cell volume is osmotically inactive.

The above 30% value for the osmotically inactive cell volume is comparable to values found for frog anterior tibial muscles (33%, Blinks, 1965) and MDCK cells (30%, Roy and Sauvé, 1987) but is larger than that obtained for mitochondria-rich cells of isolated epithelium of toad skin (21%, Larsen et al., 1987). Our value is presumably an upper estimate since regulatory volume decrease in the hyposmotic solution may reduce the magnitude of the cell height change and since the microbeads lie on top of the apical surface, cell height may be overestimated.

We have previously reported that changes in mucosal solution osmolality did not significantly alter cell volume or Na^+ transport (Crowe & Wills, 1991; Wills et al. 1991; Ehrenfeld, *unpublished observations*). Thus the water permeability of the apical membrane is low compared to the basolateral membrane.

Intracellular Solutes During Cell Swelling

As stated above, intracellular solute content in open-circuited A6 cells is apparently increased during cell swelling. This conclusion is supported by two aspects of the present studies. First, when amiloride was used to block the sodium entry across the apical membrane, the increase in cell height in response to hyposmotic solutions was decreased and the cells behaved as simple osmometers. Second, in experiments performed in the absence of mucosal chloride or under short circuit conditions, the cell swelling was similarly reduced, and again, was consistent with simple osmotic swelling. These findings are in contrast to the large, nonlinear increases in osmotically active volume that occurred under open circuit conditions (*see* Fig. 2). The most simple explanation of this finding is that cell swelling under this condition causes an increase in intracellular solute content.

Evidence for Apical Membrane Chloride Entry

As implied above, at least part of the above increase in intracellular solute content is related directly or indirectly to the presence of an apical membrane chloride permeability. Additional evidence for a role of an apical

membrane permeability in the intracellular solute content increase includes the following: (i) replacement of chloride with impermeant anions in the mucosal bathing solution resulted in a decrease in cell height, (ii) measurements with the fluorescent intracellular chloride indicator dye MQAE demonstrated chloride entry across the apical membrane in chloride-depleted cells, and (iii) transepithelial mucosa-to-serosa chloride fluxes increased under open circuit conditions during hyposmotic challenge.

The most straightforward explanation for the above results is that the increase in intracellular solute content during cell swelling is due to the entry of chloride and a cation. Alternatively, mucosal chloride could regulate the entry of sodium into the cell either as a co-anion or by some allosteric regulation of the apical membrane sodium channel. As expected from considerations of macroscopic electroneutrality, sodium fluxes under the open circuit condition were significantly reduced when the mucosal chloride was replaced by an impermeant anion. Thus the rate of sodium entry into the cell and the intracellular solute content should decrease under these conditions, consistent with the observed reduction in cell height.

Electrochemical Considerations

The direction of conductive movements of chloride across the apical membrane will depend on the sum of the chemical and electrical driving forces. From the estimated intracellular chloride activity (~27 mM), the calculated chemical driving force (E_{Cl}) is ~30 mV, favoring chloride entry. These values are comparable to those previously reported for some other sodium absorptive tight epithelia (Wills, 1985).

The apical to basolateral membrane resistance ratio (R_A/R_B) of A6 epithelia is high (R_A/R_B ~6–8; Granitzer et al., 1991, Wills, Purcell & Clausen, 1992), therefore, most of the voltage drop occurs across the apical membrane. Presently, no measurements of apical membrane potential are available for A6 epithelia with sodium transport rates comparable to the present study. Nonetheless, under the open circuit conditions, the apical membrane potential (V_a) should be approximately equal or greater than the transepithelial potential (~47 mV), resulting in a small cell interior negative or positive apical membrane potential. Thus, under open circuit conditions of our study, the net electrochemical gradient for chloride ($\Delta\mu_{Cl} = E_c + V_a$) would favor mucosal chloride entry.

As mentioned above, cell swelling was reduced when the electrical gradient for chloride entry across the apical membrane was made unfavorable by short circuiting the epithelium. Under these conditions or during conditions of low Na^+ transport (Thomas & Mintz, 1987), V_a is hyperpolarized (i.e., cell interior becomes more negative) and the electrical driving force for chloride entry becomes unfavorable. For example, Thomas and Mintz reported V_a measurements of −52 mV for A6 epithelia with low sodium transport rates (~4 $\mu A/cm^2$). Similarly, Wills and Millinoff (1990) estimated that the intracellular potential was ~45 mV under short circuit conditions, comparable to values obtained by Granitzer et al. (1992*a*; −46 mV). Assuming that a_iCl is unaltered, under these conditions $\Delta\mu_{Cl}$ would be ~−12 mV, favoring chloride exit from the cell.

Although the mechanism of the chloride permeability was not identified in the present study, it is possible that the entry of chloride or some other anion occurs by a conductive mechanism. Patch clamp studies have demonstrated the presence of two types of chloride channels in the apical membrane of A6 cells (Marunaka & Eaton, 1990). Marunaka and Eaton (1990) speculated that an apical membrane chloride conductance would facilitate sodium transport across the A6 epithelium at high transport rates. An increase in anion conductance would help offset the changes in apical membrane potential induced by the increased flux of sodium. However, we cannot rule out the possibility that an anion exchange mechanism could contribute to the apical membrane chloride permeability. We note that Kersting, Napathron and Spring (1993) reported that exposure of gallbladder epithelial cells to hyposmotic solutions led to an electroneutral influx of Na^+ across the basolateral membrane and Cl^- exchange across the apical membrane. It is conceivable that a similar mechanism could account for intracellular solute increases noted in the present study.

Chloride and sodium absorption across high resistance (tight) sodium transporting epithelia are generally believed to follow separate pathways. In toad urinary bladder (Macknight, DiBona & Leaf, 1980), chloride is believed to move paracellularly, whereas in the toad skin, chloride movements are through the mitochondria-rich cells (Larsen, Ussing & Spring, 1987). A6 epithelia consist of one cell type and the selectivity of the paracellular pathway appears to be cationic (Jovov et al., 1991). Moreover, A6 cells have a high junctional resistance (~17 $k\Omega \cdot cm^2$ when grown on Anocell filters). Therefore, it would be advantageous for chloride to move passively through the cell, instead of paracellularly. However, we cannot exclude the possibility of some paracellular chloride movement.

Implications for the Mechanism(s) of Osmotic Regulation of Sodium and Chloride Absorption

We have previously shown that sodium channel activity is activated by serosal hyposmotic solutions. A striking finding of the present results is that the time course of

cell volume change was distinct from the time course of sodium channel activation. After an initial increase which was maximum after 5 min, the cell volume began to recover and reached a new steady state that was ~8% higher that the control level. In contrast, the amiloride-sensitive sodium current and conductance increased more slowly and reached maximum, steady-state values after ~25 min. Because the maximum cell volume and sodium channel activity occurred at different times (i.e., 5 and 25 min after the onset of hyposmotic challenge), cell stretch or mechanoactivation cannot be the only signal for channel activation. Investigations are presently underway to determine whether putative intracellular second messenger systems are responsible for this stimulation of sodium transport. Recent studies by Ehrenfeld et al. (1994) suggest that RVD mechanisms in A6 cells are located in the basolateral membrane. They observed an increase in the K^+ permeability of the basolateral membrane which occurred with the opening of an anionic pathway within minutes of hyposmotic challenge. In isolated A6 cells, they found that cell volume increases were similar to those predicted for an ideal osmometer. Thus the present data for short-circuited epithelia are in agreement with their findings.

In contrast to sodium and similar to the cell volume changes, chloride fluxes were maximal 5 min following hyposmotic challenge. It is possible that this difference may reflect a transient increase in the chloride permeability, the electrochemical driving force, or conductance. Ehrenfeld et al. (1994) suggested that an initial depolarization (due to Cl^- exit or Na^+ entry) on the basolateral membrane was responsible for the increased K^+ exit across this membrane. Such a depolarization would be reflected on the apical membrane and thus could increase the electrochemical driving force for chloride across this membrane, explaining the increased $J_{ms}Cl$. Electrophysiological experiments are currently underway to measure these potentials.

Summary

In conclusion, A6 cells under open circuit conditions show a complex response to hyposmotic challenge. Initially (within 5 min) the cells swell and gain intracellular solutes in a process that requires mucosal chloride. In addition, the transepithelial sodium absorption gradually increases, reaching a new steady state after the period of maximum cell volume change (~25 min). Fluorescent intracellular chloride indicator dye and cell height measurements suggest the presence of a chloride permeability in the apical membranes of A6 cells. These results are consistent with a paradoxical increase in chloride and sodium entry across the apical membrane during anisosmotic cell swelling.

It is a pleasure to thank Drs. K. Spring and J. Alvarez-Leefmans for helpful discussions concerning cell volume measurements. Thanks also to Drs. L. Reuss, S. Lewis, and J. Russell for reading a preliminary version of this manuscript. Excellent technical assistance was provided by C. Raschi and L. Mo. This work was supported by National Institutes of Health grant DK-29962 (N. Wills), John Sealy Foundation grant CRG 2549 (N. Wills), the Wingfield and Smith Research Grant of the Texas Affiliate of the American Heart Association and NATO grant CRG 921221 (J. Ehrenfeld). W.E. Crowe was partially supported by a Fogarty International Fellowship (#TWO4428).

References

Blinks, J.R. 1965. Influence of osmotic strength on cross-section and volume of isolated single muscle fibers. *J. Physiol.* **177:**42–57

Carlsson, K. 1992. the influence of specimen refractive index, detector signal integratior., and nonuniform scan speed on the imaging properties in confocal microscopy. *J. Microscopy* **163:**176–178

Crowe, W.E., Wills, N.K. 1991. A simple method for monitoring changes in cell height using fluorescent microbeads and an Ussing-type chamber for the inverted microscope. *Pfluegers Arch.* **419:**349–357

Dick, D.A.T. 1966. Cell Water. Butterworths, London

Ehrenfeld, J., Raschi, C., Brochiero, E. 1994. Basolateral potassium membrane permeability of A6 cells and cell volume regulation. *J. Membrane Biol.* **138:**181–195

Granitzer, M., Bakos, P., Nagel, W., Crabbé, J. 1992a. Osmotic swelling and membrane conductances in A6 cells. *Biochim. Biophys. Acta* **1110:**239–242

Granitzer, M., Leal, T., Nagel, W., Crabbé, J. 1991. Apical and basolateral conductance in cultured A6 cells. *Pfluegers Arch.* **417:**463–468

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

Hallows, K.R., Knauf, P.A. 1993. Principles of cell volume regulation. *In:* Cellular and Molecular Physiology of Cell Volume Regulation. K. Strange, editor. pp. 3–47. CRC, Boca Raton, FL

Jovov, B., Wills, N.K., Lewis, S.A. 1991. A spectroscopic method for assessing confluence of epithelial cell cultures. *Am. J. Physiol.* **261:**C1196–C1203

Kersting, U., Napathron, S., Spring, K.R. 1993. *Necturus* gallbladder epithelial cell volume regulation and inhibitors of arachidonic acid metabolism. *J. Membrane Biol.* **135:**11–18

Larsen, E.H., Ussing, H.H., Spring, K.R. 1987. Ion transport by mitochondria rich cells in toad skin. *J. Membrane Biol.* **99:**25–40

Macknight, A.D.C., DiBona, D.R., Leaf, A. 1980. Sodium transport across toad urinary bladder: A model tight epithelium. *Physiol. Rev.* **60:**615–715

Marunaka, Y., Eaton, D.C. 1990. Chloride channels in the apical membrane of a distal nephron A6 cell line. *Am. J. Physiol.* **258:**C352–C368

Mayer, N. 1969. Adaptation de *Rana esculenta* a des milieux varies. Etude speciale de L'excretion renale de l'eau et des electrolytes au cours des changements de milieux. *Comp. Biochem. Physiol.* **29:**27–50

Persson B.-E., Spring, K.R. 1982. Gallbladder epithelial cell hydraulic water permeability and volume regulation. *J. Gen. Physiol.* **79:**481–505

Roy, G., Sauvé, R. Effect of Anisotonic media on volume, ion and amino acid content and membrane potential of kidney cells (MDCK) in culture. *J. Membrane Biol.* **100:**83–96

Thomas, S.R., Mintz, E. 1987. Time-dependent apical membrane K^+ and Na^+ selectivity in cultured kidney cells. *Am. J. Physiol.* **253:**C1–C6

Wills, N.K. 1985. Apical membrane potassium and chloride permeabilities in surface cells of rabbit descending colon. *J. Physiol.* **358:**433–455

Wills, N.K., Millinoff, 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