Cell Swelling Activates the K^+ Conductance and Inhibits the Cl^- Conductance of the Basolateral Membrane of Cells from a Leaky Epithelium

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ABSTRACT *Necturus* gallbladder epithelial cells bathed in 10 mM HCO_3/1% CO_2 display sizable basolateral membrane conductances for Cl^- (G^b_{Cl}) and K^+ (G^b_K). Lowering the osmolality of the apical bathing solution hyperpolarized both apical and basolateral membranes and increased the K^+/Cl^- selectivity of the basolateral membrane. Hyperosmotic solutions had the opposite effects. Intracellular free-calcium concentration ($[Ca^{2+}]_i$) increased transiently during hyposmotic swelling (peak at ~30 s, return to baseline within ~90 s), but chelation of cell Ca^{2+} did not prevent the membrane hyperpolarization elicited by the hyposmotic solution. Cable analysis experiments showed that the electrical resistance of the basolateral membrane decreased during hyposmotic swelling and increased during hyperosmotic shrinkage, whereas the apical membrane resistance was unchanged in hyposmotic solution and decreased in hyperosmotic solution. We assessed changes in cell volume in the epithelium by measuring changes in the intracellular concentration of an impermeant cation (tetramethylammonium), and in isolated polarized cells measuring changes in intracellular calcein fluorescence, and observed that these epithelial cells do not undergo measurable volume regulation over 10–12 min after osmotic swelling. Depolarization of the basolateral membrane voltage (V_{cs}) produced a significant increase in the change in V_{cs} elicited by lowering basolateral solution [Cl^-], whereas hyperpolarization of V_{cs} had the opposite effect. These results suggest that: (*a*) Hyposmotic swelling increases G^b_K and decreases G^b_{Cl}. These two effects appear to be linked, i.e., the increase in G^b_K produces membrane hyperpolarization, which in turn reduces G^b_{Cl}. (*b*) Hyperosmotic shrinkage has the opposite effects on G^b_K and G^b_{Cl}. (*c*) Cell swelling causes a transient increase in $[Ca^{2+}]_i$, but this response may not be necessary for the increase in G^b_K during cell swelling.

KEY WORDS: transport • channel • gallbladder • cross talk • calcium

INTRODUCTION

The *Necturus* gallbladder (NGB)[1] epithelium is a useful model system for functional studies of a family of epithelia that perform near-isosmotic fluid absorption. The mechanisms accounting for ion and water transport in NGB epithelium under control conditions have been characterized in detail and considerable information is also available on regulation of transport (for reviews, see Reuss, 1988; Reuss and Altenberg, 1995). An issue requiring further study is the regulation of basolateral membrane Cl^- and K^+ channels, especially its relationship to the mechanism of cross talk between apical and basolateral membranes, in which cell volume changes could play a major role (Schultz and Hudson, 1991). For instance, we have proposed that elevation of cAMP causes a reduction in Cl^- efflux across the basolateral membrane. This is based on the effects of cAMP on fluid transport (Petersen and Reuss, 1983), intracellular ion contents, and cell volume (reviewed in Reuss and Altenberg, 1995; Reuss et al., 1991). Further, exposure to cAMP reduces basolateral membrane conductance (Copello et al., 1993). We suspected that this may include a decrease in basolateral membrane G_{Cl} (G^b_{Cl}) brought about by the cell shrinkage produced by cAMP. In other words, the hypothesis is that the changes in apical membrane conductances elicited by cAMP result in a loss of cell KCl, which in turn causes cell shrinkage, and that the shrinkage results in a decrease in basolateral membrane G_{Cl}. Accordingly, we assessed the effects of cell volume and membrane voltage on the Cl^- and K^+ conductances of the basolateral membrane. Specifically, we investigated the rapid effects of cell swelling and shrinkage on these conductances, in order to test the hypothesis that cell volume changes are involved in cross talk between apical and basolateral membranes. Inasmuch as G^b_{Cl} is small relative to the basolateral K^+ conductance (G^b_K), in addi-

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[1] *Abbreviations used in this paper:* ΔV^{Cl}_{cs}, changes in V_{cs} elicited by lowering external [Cl^-]; ΔV^{K}_{cs}, changes in V_{cs} elicited by raising external [K^+]; NGB, *Necturus* gallbladder; V_{cs}, basolateral membrane voltage; V_{mc}, apical membrane voltage; V_{ms}, transepithelial voltage.

tion to osmotic-shrinkage we carried out osmotic-swelling experiments. If cell swelling increases G^b_{Cl}, then swelling experiments would yield relative changes in G^b_{Cl} easier to measure than those elicited by cell shrinkage. In contrast with the apical membrane pathway (Copello et al., 1993; Heming et al., 1994), the G^b_{Cl} appears to be cAMP independent (Copello et al., 1993) and is activated by HCO_3^-/CO_2 (Stoddard and Reuss, 1988*a*). The results of these studies demonstrate that G^b_{Cl} is increased by cell shrinkage and decreased by cell swelling, probably via changes in cell membrane voltage (depolarization during cell shrinkage, hyperpolarization during cell swelling). The basolateral K^+ conductance (G^b_K) is apparently voltage insensitive (Stoddard and Reuss, 1988*b*), and possibly activated by elevations in internal Ca^{2+} (Bello-Reuss et al., 1981). Here, we demonstrate that the K^+ conductance is stimulated by cell swelling and inhibited by cell shrinkage.

METHODS

General

Mudpuppies (*Necturus maculosus*) were purchased from Kon's Scientific (Germantown, WI) or Nasco Biologicals (Ft. Atkinson, WI) and maintained at 5–10°C. Anesthesia was accomplished by immersion in a 1 g/liter tricaine methanesulfonate solution. The gallbladder was excised, opened, washed, and mounted in a modified Ussing chamber (Altenberg et al., 1990). When the gallbladders were mounted serosal side up, a patch of connective tissue was removed by dissection, to allow for microelectrode impalements through the basolateral membrane (Altenberg et al., 1990). Some experiments were carried out with polarized epithelial cells isolated from the intact tissue ("figure-eight cells"), using a method previously described (Torres et al., 1996*b*). The control bathing solution (bPSS) contained, in mM: 90 NaCl, 10 $NaHCO_3$, 2.5 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, and 0.5 NaH_2PO_4, gassed with 1% CO_2/99% air, pH 7.66, at room temperature, with an average osmolality of 207 mosmol/kg. A low-NaCl but isosmotic solution was prepared by replacing 35 mM NaCl with 70 mM sucrose. This solution was made hyposmotic (by ~17 or 34%) by removing sucrose, or hyperosmotic (also by ~17 or 34%) by adding a further 35 or 70 mM sucrose. Changes in bathing solution osmolality were made only in the apical bathing medium, inasmuch as reductions in basolateral solution [NaCl], with or without changes in osmolality, elicited long-lasting oscillations in membrane voltage and conductance. To assess cell membrane ionic selectivity, Cl^- was replaced with gluconate, and Na^+ was replaced with K^+. These replacements were isomolar.

Electrophysiological Techniques

Transepithelial voltage (V_{ms}, referenced to the basolateral bathing solution; V_{sm}, referenced to the apical bathing solution) and cell membrane voltages (apical = V_{mc}, basolateral = V_{cs}, referenced to the respective adjacent solutions) were measured as previously described (Altenberg et al., 1990). The ground electrode was an Ag-AgCl pellet separated from the apical bathing solution by a short Ringer-agar bridge. The basolateral bathing solution electrode was a flowing, saturated-KCl bridge in series with a calomel half-cell. Hence, liquid junction potentials upon changes in the basolateral solution were minimized. The transepithelial resistance, R_t ($\Delta V_{ms}/I_t$, where ΔV_{ms} is the change in V_{ms} elicited by a constant current pulse of density I_t), and the apparent ratio of cell membrane resistances (R_a/R_b, where the subscripts a and b denote apical and basolateral membranes, respectively) were determined from the steady-state voltage deflections elicited by DC pulses of 50 μA/cm² and 1-s duration, applied across the tissue through Ag-AgCl electrodes. The voltage deflections were corrected for series resistances. The absolute values of R_a and R_b were estimated from two-point cable analysis, as previously described (Petersen and Reuss, 1985; Copello et al., 1993). Intracellular-microelectrode studies were also carried out on isolated polarized cells attached with Cell-Tak® (Collaborative Medical Products, Bedford, MA) to a dialysis membrane mounted in the microelectrode chamber (Torres et al., 1996*b*).

Intracellular Cl^- activity (aCl_i) and tetramethylammonium (TMA^+) activity ($aTMA_i$) were measured with double-barrel ion-sensitive microelectrodes constructed and calibrated as previously described (Altenberg et al., 1990). Validation of impalements was as described before (Altenberg et al., 1990). To measure changes in cell water volume, epithelia were loaded with TMA^+ using transient exposure to nystatin, and the intracellular activity of TMA^+ was determined with double-barrel, TMA^+-sensitive microelectrodes (Reuss, 1985; Altenberg et al., 1990).

Fluorescence Techniques

Changes in cell water volume were assessed in isolated polarized cells attached to a coverslip coated with Cell Tak®, loaded with calcein,AM (3 μM, for 20 min) and then superfused with isosmotic solution for 15 min before starting the experiment. Calcein is a good choice among fluorescent indicators to assess changes in cell water volume because it undergoes less photobleaching than other probes and is retained better in the cells (Altenberg et al., 1994; Alvarez-Leefmans et al., 1995; Crowe et al., 1995). The experiments were performed using a digital video confocal laser imaging system (Odyssey; Noran Instruments, Middleton, WI). Excitation light was 488 nm, and emitted light was measured at wavelengths longer than 515 nm. Pictures were obtained at 30-s intervals, and fluorescence of a 15–20 μm² area in the center of a cell was measured. The records were corrected for fluorescence decay independent of cell volume changes (primarily due to dye photobleaching), which was fit by a single exponential. The data are presented as F_o/F_t, where F_o = fluorescence in isosmotic solution, at t = 0, and F_t = corrected fluorescence at time = t. The ratio F_o/F_t is proportional to cell volume and was ~75% of the ideal ("osmometric") responses after exposure to hypo or hyperosmotic solutions.

Intracellular free $[Ca^{2+}]$ ($[Ca^{2+}]_i$) was estimated from the 340/380 nm Fura-2 fluorescence ratio ($F_{340/380}$) in isolated polarized cells. Isolated polarized cells (Torres et al., 1996*b*) were loaded at room temperature with Fura-2,AM (5 μM for 1 h). The cells were then attached to a coverslip coated with Cell-Tak®, mounted in a chamber and superfused with isosmotic solution for ~15 min before starting the experiment. Measurements were carried out essentially as described (Altenberg et al., 1994). Data were acquired at 1 Hz. At the end of each experiment, cells were exposed to 10–20 μM ionomycin to obtain saturating free $[Ca^{2+}]_i$, and then to 1–2 mM $MnCl_2$ in the continuous presence of ionomycin, to quench the dye and thus correct for background fluorescence. Data are presented as $F_{340/380}$ after background correction. In some experiments, cells were loaded with acetoxymethyl ester of 1,2-bis-(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid (BAPTA,AM), Half-BAPTA,AM, or *N,N,N′,N′*-tetrakis(2-pyridylmethyl) ethylenediamine (TPEN), a heavy-metal chelator (Kao, 1994). All of these chemicals were obtained from Molecular Probes (Eugene, OR).

Statistical Analysis

Results are given as means ± SEM. Statistical comparisons were done by *t* tests for paired or unpaired data, as appropriate. A value of $P < 0.05$ was considered significant.

RESULTS

Effects of Changes in Apical Bathing Solution Osmolality on Voltages and Resistances

The effects of lowering the apical solution osmolality (from 200 to 135 mosmol/kg) on the basic electrophysiological properties of the epithelium are illustrated in Fig. 1. In this experiment, a cell was impaled from the apical side with a conventional microelectrode. Voltages and resistances were recorded before, during, and after exposure to hyposmotic solution on the apical side. Reducing bathing solution osmolality caused a hyperpolarization of V_{cs} and an increase in R_a/R_b. Both effects were reversible. Table I summarizes the results of experiments such as the one shown in Fig. 1. The hyperpolarization at 3 min was 8 ± 1 mV, and the repolarization was complete after 4 min of perfusion with isosmotic solution.

The experiment shown in Fig. 2 illustrates the effects of exposure to a hyperosmotic solution (the apical solution osmolality was increased from 200 to 270 mosmol/kg), and Table II summarizes the results with this experimental protocol. The main effects of hyperosmotic solution were cell membrane depolarization and a decrease in R_a/R_b, i.e., opposite changes to those elicited by hyposmotic solution. As in the experiments with hyposmotic solution, the effects were reversible. The effect of anisosmotic solutions on V_{ms} was small, but significant, apical side negative with hyposmotic solution and opposite in polarity with hyperosmotic solution. These changes can be explained by paracellular pseudostreaming potentials caused by water flow from apical to basolateral solution with hyposmotic solution and from basolateral to apical solution with hyperosmotic solution. These voltage changes, because of their small magnitude, do not contribute significantly to the changes in cell membrane voltages (Reuss et al., 1992*a*, *b*). There

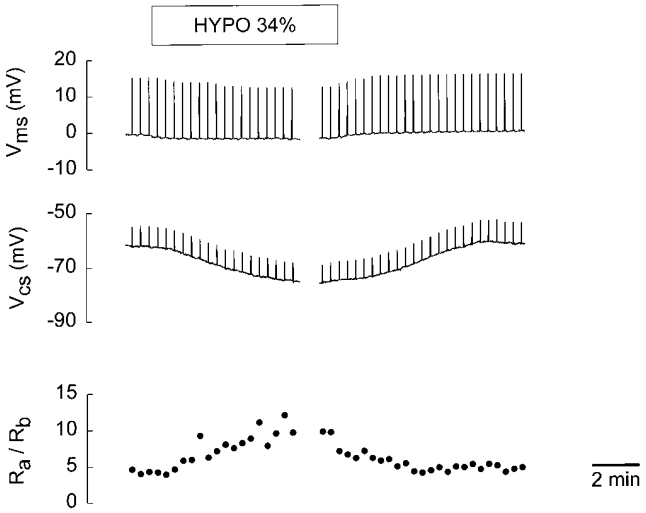

FIGURE 1. Effects of apical superfusion with hyposmotic solution on transepithelial (V_{ms}) and basolateral (V_{cs}) membrane voltages, and on apparent ratio of cell membrane resistances (R_a/R_b). Interruption of the records corresponds to an interval of ~2 min. Osmolality was decreased, on the apical side alone, by 34% (see METHODS). Voltage spikes were elicited by transepithelial current pulses (50 μA/cm², 1-s duration, 20-s interval) and were used to estimate R_a/R_b as explained in METHODS. The decrease in apical solution osmolality hyperpolarized V_{cs} and increased R_a/R_b. Both effects were reversible.

were also significant changes in R_t: a decrease with hyposmotic solution and an increase with hyperosmotic solution. These changes are in the directions expected for widening and narrowing of lateral-intercellular spaces, respectively (Reuss et al., 1992*a*), but changes in junctional resistance cannot be ruled out.

Effects of Changes in Apical Bathing Solution Osmolality on Relative K^+ and Cl^- Conductances of the Basolateral Membrane

The changes in V_{cs} elicited by increasing basolateral $[K^+]$ from 2.5 to 25 mM (ΔV_{cs}^K), or by lowering basolateral $[Cl^-]$ from 98.1 to 8.1 mM (ΔV_{cs}^{Cl}), were measured in the same cells, during exposure to isosmotic apical

TABLE I

Effects of Apical Superfusion with Hyposmotic Solution on Electrical Properties of Necturus Gallbladder Epithelium

	V_{ms}	V_{mc}	V_{cs}	R_a/R_b	R_t	ΔV_{cs}^K	ΔV_{cs}^{Cl}
		mV			*Ω · cm²*	*mV*	
Isosmotic	−0.2 ± 0.2	−65 ± 1	−65 ± 2	4.9 ± 0.4	245 ± 30	28 ± 4	5 ± 1
Hyposmotic	−0.9 ± 0.3*	−73 ± 2*	−74 ± 2*	9.0 ± 1.4*	222 ± 26*	45 ± 3*	1 ± 1*
Recovery	−0.2 ± 0.3	−67 ± 2	−67 ± 2	5.2 ± 0.5	245 ± 38	—	—

For voltages and R_a/R_b, values shown were obtained at 3 min of exposure to hyposmotic solution. Values during recovery ($n = 9$) were taken at 4 min. ΔV_{cs}^K and ΔV_{cs}^{Cl} were measured at ~20 min (see text and Fig. 3). Values are means ± SEM (for the first five columns, $n = 11$ paired experiments; for the last two columns, $n = 7$). *Significantly different from value in isosmotic solution ($P < 0.05$).

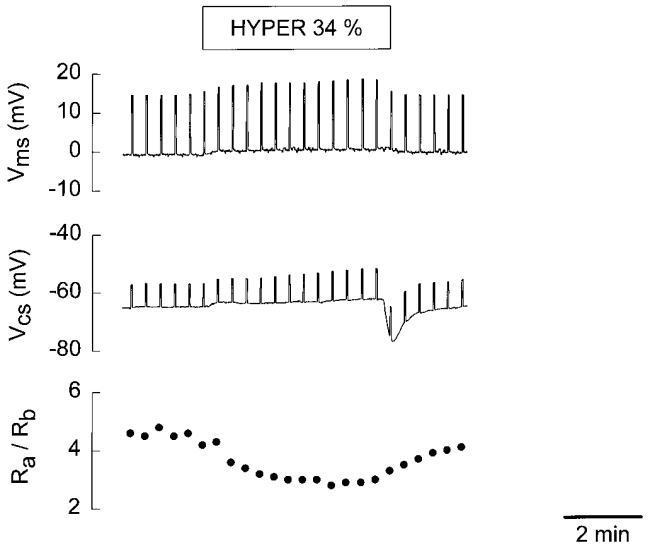

FIGURE 2. Effects of apical surface superfusion with hyperosmotic solution on V_{ms}, V_{cs}, and R_a/R_b. Osmolality was increased by 34%, on the apical side only. Protocol as in Fig. 1. The increase in apical solution osmolality caused depolarization of V_{cs} and decrease in R_a/R_b. Both effects were reversible. The transient hyperpolarization after returning to isosmotic solution was consistently observed, but variable in magnitude and duration.

bathing solution and at 20 min of exposure to hyposmotic solution. Similar results were obtained between 5 and 20 min in hyposmotic bathing medium (not shown). As illustrated in Fig. 3 and summarized in Table I, exposure to hyposmotic solution increased ΔV_{cs}^{K} and decreased ΔV_{cs}^{Cl}, relative to the respective values measured in isosmotic solution. Further, ΔV_{cs}^{Cl} in hyposmotic solution was not significantly different from zero.

Similar experiments were carried out to assess the changes in basolateral membrane ionic selectivity after a 5–10 min exposure to hyperosmotic solution. The results are illustrated in Fig. 4 and summarized in Table II. In hyperosmotic medium, ΔV_{cs}^{K} was reduced significantly, while ΔV_{cs}^{Cl} was increased. Both changes are opposite to those elicited by exposure to hyposmotic solution.

Because of the low paracellular electrical resistance of the NGB epithelium, the values of ΔV_{cs}^{K} and ΔV_{cs}^{Cl} are less than the corresponding changes in zero-current membrane voltages (the ionic substitutions produce changes in intraepithelial current flow). There are also concomitant paracellular diffusion potentials, but these are small and in both instances (high-K^+ and low-Cl^-) tend to compensate in part for the shunting effect of the paracellular pathway (see Reuss and Finn, 1975).

These results demonstrate that the relative G_K^b increases and relative G_{Cl}^b decreases during exposure to hyposmotic solution. Conversely, the relative G_K^b decreases, and the relative G_{Cl}^b increases during exposure to a hyperosmotic solution. To estimate the changes in absolute values of G_K^b and G_{Cl}^b in both sets of experiments, we performed the experiments described in the next section.

Effects of Exposure to Anisosmotic Solutions on Cell Membrane Resistances

Fig. 5, *A* and *B*, depicts representative two-point cable analysis experiments under control conditions and during transient exposure to hyposmotic and hyperosmotic solutions. Two cells were simultaneously impaled, and both transepithelial and intracellular current pulses were applied at intervals. Downward (hyperpolarizing) voltage spikes denote ΔV_x, i.e., the voltage changes elicited by current injection into another (electrically coupled) cell. Exposure to hyposmotic solution caused a decrease in ΔV_x, which reached a stable value in ~4 min and returned to control levels within 4–5 min of restoring superfusion with isosmotic solution (Fig. 5 *A*). In contrast, exposure to hyperosmotic solution increased ΔV_x. This effect was complete within 4 min and returned to control levels after a 4-min exposure to isosmotic solution (Fig. 5 *B*). Fig. 5 *C* depicts fits of the Bessel function K_0 to the two-point cable analysis values obtained with hyposmotic and hyperosmotic solutions, respectively. From these data, we calculated R_z (resistance for current flow out of the cell, i.e., R_a and R_b in parallel); from R_z and the concomitant values of R_a/R_b and R_t, we calculated the resistances of the cell membranes and the paracellular pathway. The method used requires normalization of the data to the values observed under control conditions. The bases,

TABLE II

Effects of Apical Superfusion with Hyperosmotic Solution on Electrical Properties of Necturus Gallbladder Epithelium

	V_{ms}	V_{mc}	V_{cs}	R_a/R_b	R_t	ΔV_{cs}^{K}	ΔV_{cs}^{Cl}
		mV			$\Omega \cdot cm^2$	*mV*	
Isosmotic	−0.4 ± 0.3	−65 ± 2	−65 ± 2	5.2 ± 1.3	184 ± 12	39 ± 3	4 ± 1
Hyperosmotic	0.6 ± 0.4*	−60 ± 2*	−60 ± 4*	1.3 ± 0.4*	301 ± 36*	31 ± 4*	6 ± 1*
Recovery	−0.9 ± 0.5	−65 ± 2	−66 ± 2	4.9 ± 0.6	173 ± 17	—	—

For voltages and R_a/R_b, values shown were obtained at 3 min of exposure to hyposmotic solution. Values during recovery ($n = 8$) were taken at 4 min. ΔV_{cs}^{K} and ΔV_{cs}^{Cl} were measured at 5–10 min, as illustrated in Fig. 4. Values are means ± SEM (for the first five columns, $n = 11$ paired experiments; for the last two columns, $n = 4$). *Significantly different from value in isosmotic solution ($P < 0.05$).

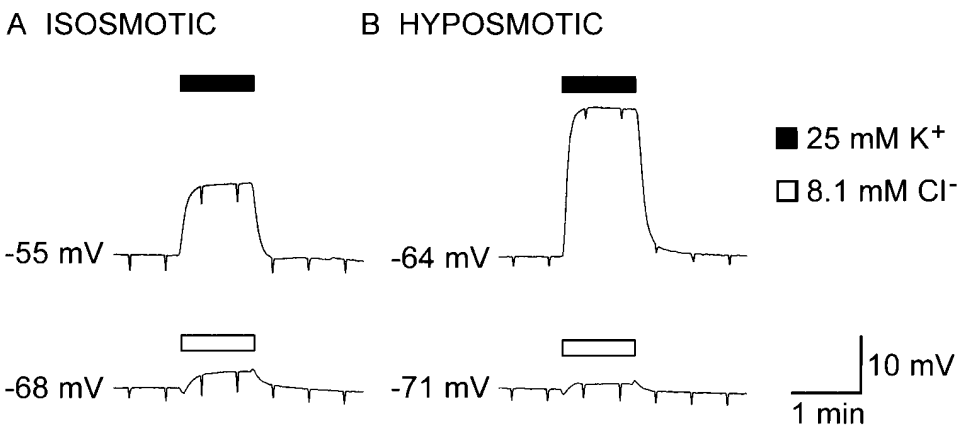

FIGURE 3. Effects of apical surface superfusion with isosmotic (*A*) or hyposmotic solution (*B*) on the ionic selectivity of the basolateral membrane. Changes in V_{cs} were elicited by increasing basolateral solution $[K^+]$ from 2.5 to 25 mM (ΔV_{cs}^K, *top*) or by lowering basolateral solution $[Cl^-]$ from 98.1 to 8.1 mM (ΔV_{cs}^{Cl}, *bottom*). All records were obtained in the same cell. During exposure to hyposmotic solution, ΔV_{cs}^K increased and ΔV_{cs}^{Cl} decreased, compared to the respective values in isosmotic solution.

assumptions, and limitations of this method have been discussed in detail elsewhere (Petersen and Reuss, 1985; Copello et al., 1993).

The results of these calculations are summarized in Table III. Exposure to anisosmotic solutions elicits mostly changes in basolateral membrane resistance: R_b decreases during superfusion with hyposmotic solution and increases during superfusion with hyperosmotic solution. Whereas hyposmotic solution causes exclusively a decrease in R_b, hyperosmotic solution both increases R_b and decreases R_a. The latter effect could be due to activation of apical membrane maxi-K^+ channels by depolarization (Segal and Reuss, 1990).

The ionic-substitution experiments illustrated in Figs. 3 and 4 and summarized in Tables I and II demonstrated an increase in relative G_K^b with hyposmotic solution and a decrease in relative G_K^b with hyperosmotic solution. These results, together with the resistance data, conclusively demonstrate that the changes in basolateral membrane conductance are principally due to changes in G_K^b in the same direction: increase with hyposmotic solution and decrease with hyperosmotic solution. Rather unexpectedly, we found that the changes in G_K^b and G_{Cl}^b were in opposite directions: when G_K^b increased (hyposmotic solution), G_{Cl}^b decreased; when G_K^b decreased (hyperosmotic solution), G_{Cl}^b increased. We next investigated the mechanism of the changes in G_K^b and G_{Cl}^b.

Cell Swelling Causes Elevation of $[Ca^{2+}]_i$, But the Latter Is Not Necessary for the Increase in G_K^b

Entry of Ca^{2+} has been shown to play a signaling role in cell volume regulation from hyposmotic swelling by activation of a Ca^{2+}-dependent G_K (Christensen, 1987). Therefore, the effects of hyposmotic solution on $[Ca^{2+}]_i$ in isolated polarized cells were estimated from the F_{340}/F_{380} Fura-2 fluorescence ratio. The results are shown in Fig. 6. The cells were exposed to a 34%-hyposmotic solution under control conditions or after loading with the Ca^{2+} chelator BAPTA,AM. After the onset of superfusion with hyposmotic solution, $[Ca^{2+}]_i$ rose rapidly and then (during continuous exposure to this solution) decreased monotonically to control levels in <2 min. In

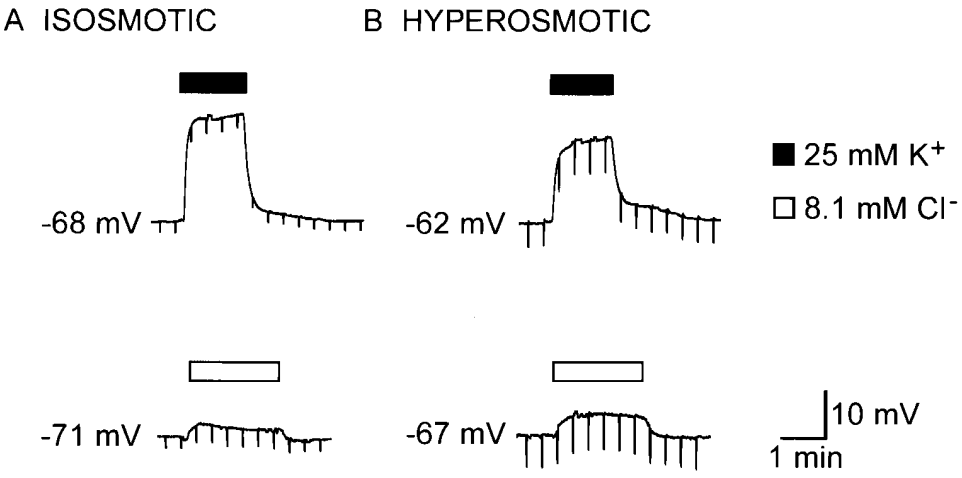

FIGURE 4. Effects of apical surface superfusion with isosmotic (*A*) or hyperosmotic solution (*B*) on ΔV_{cs}^K (*top*) and ΔV_{cs}^{Cl} (*bottom*). All records were obtained in the same cell. During exposure to hyperosmotic solution, ΔV_{cs}^K decreased and ΔV_{cs}^{Cl} increased.

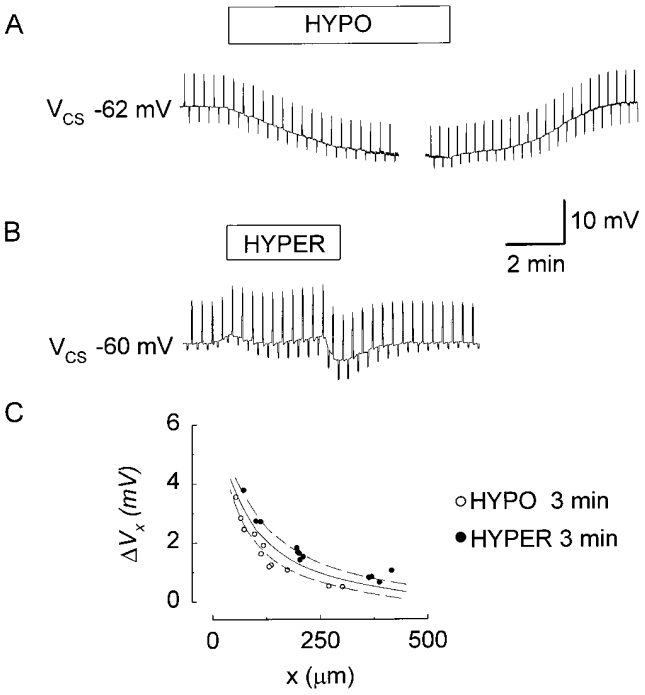

FIGURE 5. Two-point two-dimensional cable analysis under control conditions and during exposure to hyposmotic (−34%) and hyperosmotic (+34%) solutions. (*A*) Effect of exposure to hyposmotic solution. Cell-negative pulses of 40 nA and 1-s duration were applied at 20-s intervals. The changes in V_{cs} (ΔV_x) induced by the applied current were measured in another cell ~260 μm away. ΔV_x (downward spikes in the V_{cs} record) decreased in amplitude during superfusion with hyposmotic solution, returning to control levels after superfusion with isosmotic solution. The interruption in the record denotes a 2-min interval. (*B*) Effect of exposure to hyperosmotic solution (inter-electrode distance, 415 μm). ΔV_x increased during superfusion with hyperosmotic solution, returning to control levels in isosmotic solution. (*C*) Fits of Bessel function K_0 to cable analysis data obtained during continuous impalements across the apical membrane before, during, and after exposure to either hyposmotic (*open circles*, $n = 4$ tissues) or hyperosmotic solution (*filled circles*, $n = 3$ tissues) on the apical side. Control values of ΔV_x (in isosmotic solution) were superimposed on a curve [$\Delta V_x = AK_0(x/\lambda)$] characterized by previously measured average parameters in NGB epithelium under control conditions: $A = 2.15$ mV and $\lambda = 260$ μm (*solid line*). A normalized distance x was assigned to each point, and data obtained 180 s after exposure to apical hyposmotic or hyperosmotic solution were plotted for the same value of x. Best fits of the same Bessel function to these data (segmented line) yielded $A = 2.17$ mV and $\lambda = 200$ μm in hyposmotic solution and $A = 2.19$ mV and $\lambda = 327$ μm in hyperosmotic solution. From the values of A and λ, the values of R_z in hyper- and hyposmotic solution were calculated as previously described (Petersen and Reuss, 1985; Copello et al., 1993).

TABLE III

Effects of Apical Superfusion with Anisosmotic Solutions on Cell Membrane Resistances

	R_a/R_b	R_t	R_z	R_a	R_b	R_s
			$\Omega \cdot cm^2$			
Isosmotic	4.9	245	927	5469	1116	254
Hyposmotic	9.0	222	546	5460	607	230
Recovery	5.2	245	878	5444	1047	255
Isosmotic	5.2	184	913	5669	1089	189
Hyperosmotic	1.3	301	1498	3445	2650	317
Recovery	4.9	173	930	6538	1108	178

First two columns were taken from Tables I (hyposmotic) and II (hyperosmotic), respectively. Other data are averages calculated as described in METHODS. See also Fig. 5.

cells preloaded with BAPTA, the elevation in $[Ca^{2+}]_i$ by exposure to hyposmotic solution was abolished. Experiments were also carried out with 50 μM Half-BAPTA,AM and 50 μM TPEN, a heavy-metal chelator (Kao, 1994). Neither compound binds Ca^{2+} in the nanomolar range. In both instances, the change in $[Ca^{2+}]_i$ after exposure to hyposmotic solution was similar to that observed under control conditions (not shown). Hence, the effect of BAPTA cannot be attributed to nonspecific toxic effects or to heavy-metal chelation. Because of the effect of pH on Fura-2 fluorescence (Reers et al., 1989), we also measured pH_i with BCECF, using the same protocol as for the $[Ca^{2+}]_i$ measurements. Hyposmotic solution did not cause measurable changes in pH_i (data not shown). These control experiments validate the conclusion that $[Ca^{2+}]_i$ does in fact rise transiently during exposure of NGB epithelial cells to hyposmotic solutions.

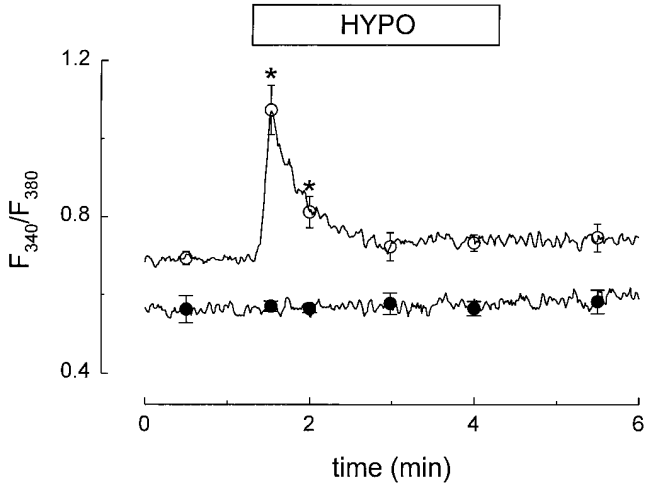

FIGURE 6. Changes in $[Ca^{2+}]_i$, assessed from changes in Fura-2 fluorescence ratio (F_{340}/F_{380}), in isolated polarized cells. Cells were exposed to a 34% hyposmotic solution under control conditions ($n = 10$, *open circles*) and after loading for 60 min with 50 μM BAPTA,AM ($n = 4$, *filled circles*). Continuous lines denote means; symbols and bars denote means ± SEM at specific time points. See METHODS for further details. The response consisted of a fast transient elevation followed by a decrease to control levels during continuous exposure to hyposmotic solution. BAPTA loading abolished the rise in $[Ca^{2+}]_i$.

To determine the role of $[Ca^{2+}]_i$ in the response of isolated polarized cells to hyposmotic swelling, we examined the effect of swelling (34% hyposmotic solution) on membrane voltage (V_m) with or without preloading the cells with 50 μM BAPTA (see METHODS). Without BAPTA, V_m hyperpolarized by 4 ± 1 mV; with BAPTA, the hyperpolarization was 3 ± 1 mV (not significantly different; $n = 4$ paired experiments).

G^b_{Cl} Is Modulated by Membrane Voltage

The changes in G^b_{Cl} described above could result from: (*a*) direct effect of changes in cell volume, (*b*) changes in membrane voltage produced by the activation or inhibition of G^b_K, or (*c*) other mechanisms. In other experiments, we have observed decreases in G^b_{Cl} associated with basolateral membrane hyperpolarization (Stoddard and Reuss, 1989; Altenberg et al., 1992; Altenberg et al., 1993). Cell swelling activates G^b_K; this in turn would hyperpolarize the membrane (because $E_K > V_{cs}$), and the hyperpolarization would cause a decrease in G^b_{Cl}. The opposite effects, i.e., decrease of G^b_K, depolarization, and activation of G^b_{Cl}, would be elicited by cell shrinkage. To test the hypothesis that the effects of cell volume changes on G^b_{Cl} are mediated by changes in membrane voltage, we studied the effects of baseline V_{cs} on ΔV^{Cl}_{cs}. Depolarization of V_{cs} was accomplished either by elevating apical solution $[K^+]$ from 2.5 to 25 mM (the change in V_{cs} results from a loop-current change, see Reuss and Finn, 1975); hyperpolarization of V_{cs} was accomplished by a transepithelial current clamp. The results are shown in Fig. 7. With depolarization of V_{cs}, ΔV^{Cl}_{cs} increased, and with hyperpolarization of V_{cs}, it decreased significantly. In the latter experiment, an extracellular electrode within 5 μm from the impaled cell was used as reference to avoid the effect of changes in voltage drops in the solutions (produced by the different conductivities of high-$Cl^-$and low-Cl^- solutions). We conclude that G^b_{Cl} is voltage dependent: decreased by hyperpolarization and increased by depolarization of V_{cs}.

In conclusion, these experiments show that after swelling of NGB epithelial cells G^b_K increases, causing basolateral membrane hyperpolarization, which may explain the parallel decrease in G^b_{Cl}. In previous studies, we found no increase of apical membrane Cl^- conductance after cell swelling by exposure to hyposmotic solutions (Heming et al., 1994). This is consistent with the lack of change in R_a (see above). Hence, our studies do not support the notion that NGB epithelial cells undergo volume-regulatory decrease by loss of K^+ and Cl^- via channels. However, cell volume measurements using an optical-sectioning technique have been reported to show virtually complete cell volume regulation within a few minutes of the onset of the osmotic perturbation (Persson and Spring, 1982; Larson and Spring, 1984; Furlong and Spring, 1990). Hence, it was of interest to test whether cell volume regulation takes place regardless of the decrease in G^b_{Cl}. This was done in the next series of experiments.

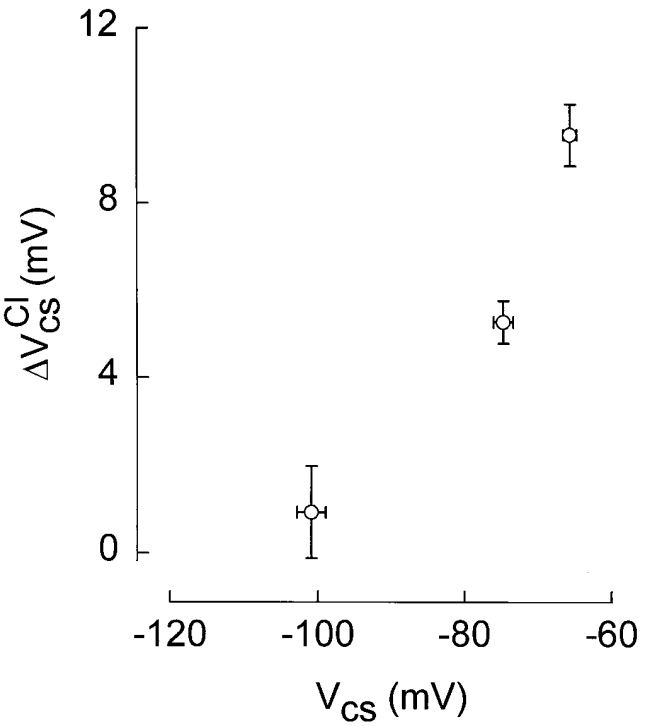

FIGURE 7. Effects of V_{cs} changes on the depolarization of V_{cs} elicited by lowering basolateral solution $[Cl^-]$ from 98.1 to 8.1 mM (ΔV^{Cl}_{cs}). V_{cs} was depolarized by elevating apical solution $[K^+]$ from 2.5 to 25 mM ($n = 8$) and hyperpolarized by a transepithelial current clamp ($n = 9$). V_{cs} depolarization caused an increase in ΔV^{Cl}_{cs}, whereas V_{cs} hyperpolarization caused a decrease. Both changes were significant ($P < 0.05$).

Effects of Exposure to Hyposmotic Solution on Cell Water Volume and Intracellular aCl_i

Cell water volume and aCl_i were measured before, during, and after exposure to hyposmotic solution (apical side alone). In Fig. 8 *A* we illustrate a cell water volume measurement in TMA^+-loaded cells. Exposure to hyposmotic bathing solution caused an increase in cell volume, evidenced by the decrease in $[TMA^+]_i$. However, there was no measurable cell volume regulation, although during this time G^b_K is clearly activated (see Figs. 1 and 3 and Tables I and III). One could argue that the experimental method, namely the TMA^+ loading by transient exposure to nystatin, caused a fall in G^b_K or otherwise changed the properties of the cells, abolishing the volume regulatory response. To test this possibility, we measured aCl_i before, during, and after exposure to hyposmotic solution, without TMA^+ loading. The expectations are that hyposmotic solution will initially cause a dilution of intracellular Cl^-; if there is

cell volume regulation by KCl efflux, then aCl_i would fall further during the regulatory response; if there is cell volume regulation by a different mechanism, without Cl^- loss, then aCl_i would rise during the regulatory response. Fig. 8 *B* illustrates the result of this experiment. Exposure to hyposmotic apical solution produced a monotonic change in aCl_i, i.e., there was no indication of more than one process (water influx) changing aCl_i. Further, Fig. 8 *C* depicts the intracellular TMA^+ and Cl^- activities during the first 5 min of exposure to the hyposmotic solution (records from Fig. 8, *A* and *B*). The linear relationship indicates that the rates of change in both ion activities are the same, i.e., that the time courses of aCl_i and $aTMA_i$ mirror each other. In addition, the maximal fractional changes in the two activities did not differ significantly (see Fig. 8). Inasmuch as TMA^+ is a cell water volume marker, i.e., effectively impermeant, this result indicates that during the 5-min period Cl^- was also effectively impermeant, i.e., its cell content did not change. If cell volume regulation were very fast, one could envision a monotonic "subosmometric" change in $aTMA_i$ after hyposmotic swelling (cell water volume tends to increase because of water influx and to decrease because of regulatory efflux of solute and water). However, the magnitude of the change in $aTMA_i$ is that expected for a cell behaving as an ideal osmometer, and the parallel measurements of aCl_i do not show the higher rate of decrease predicted if Cl^- were involved in the regulatory response, or the lower rate of decrease expected if the regulatory volume decrease did not involve Cl^-.

The possibility of cell volume regulation after exposure to hyposmotic solution was further explored in a preparation of isolated NGB epithelial cells that retain structural and functional polarity (Torres et al., 1996*a*, *b*). Changes in cell water volume were estimated from the changes in intracellular fluorescence in cells loaded with calcein, as illustrated in Fig. 9 *A*, *B*, and *C*. Exposure of isolated polarized cells to hyposmotic (Fig. 9 *D*) or hyperosmotic solutions (Fig. 9 *E*) elicited rapid changes in intracellular fluorescence, indicative of swelling and shrinkage, respectively, with no regulatory volume changes during the >10-min period of exposure to anisosmotic solution. These experiments confirm the results described in the preceding section, and argue against the possibility that the lack of volume regulatory responses results from artefacts related to microelectrode impalements.

To eliminate the possibility of an artifactual inability to measure a putative volume regulatory response, we exposed cAMP-stimulated cells to an isosmotic, high-K^+ external solution ($[K^+]$ was raised from 2.5 to 67.5 mM, replacing Na^+), at constant external $[Cl^-]$ (63.1 mM). The result, illustrated in Fig. 9 *F*, was rapid and reversible cell swelling, consistent with previous observations made with the TMA^+ technique in the assembled epithelium (Cotton and Reuss, 1991). These results further strengthen the conclusion that, in the experimental conditions of these studies, osmotically swollen NGB epithelial cells do not undergo short-term regulatory volume decrease.

DISCUSSION

Schultz (1981; see also Schultz and Hudson, 1991) developed the important notion that changes in transport rate at one of the membrane domains of an epithelium must result in "matching" changes in transport rate at

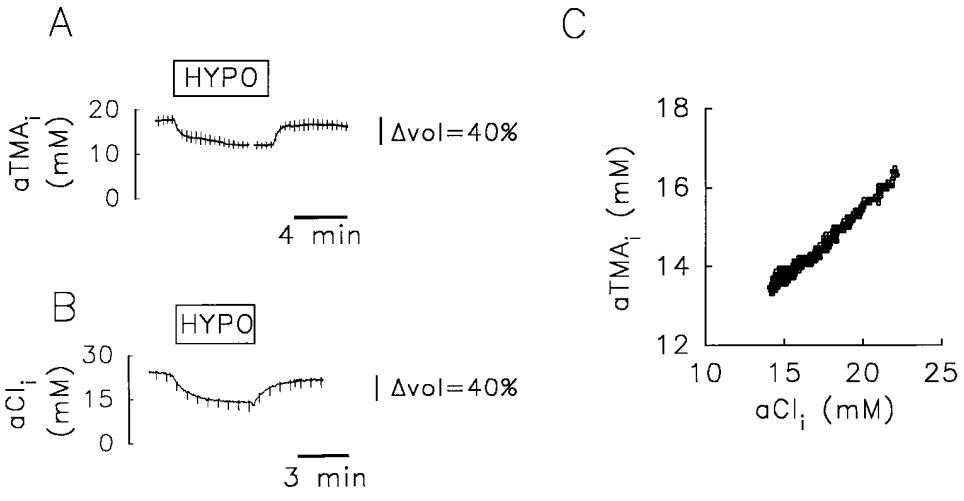

FIGURE 8. Effects of exposure to hyposmotic solution on: (*A*) Changes in cell water volume assessed from $[TMA^+]_i$ in TMA^+-loaded cells exposed to a hyposmotic bathing solution. The log of $\Delta(V_{TMA} - V_{cs})$ is directly proportional to $[TMA^+]_i$ and therefore inversely proportional to cell volume. There is no measurable cell volume regulation after osmotic swelling (interruption in the record denotes an ~2-min interval). (*B*) Changes in aCl_i after exposure to a hyposmotic bathing solution. The log of $\Delta(V_{Cl} - V_{cs})$ is inversely proportional to aCl_i. For 5 min during osmotic swelling, there is a monotonic decrease in aCl_i due to dilution. (*C*) Plot of aCl_i vs. $aTMA_i$ during the first 5 min of exposure to hyposmotic solution (records in *A* and *B*). The linear relationship between both ion activities indicates that the rates of change are the same. The decreases in aCl_i and $aTMA_i$ in response to a 34% reduction in osmolality (36 ± 2%, n = 8, and 31 ± 5%, n = 8, respectively) were not significantly different from each other.

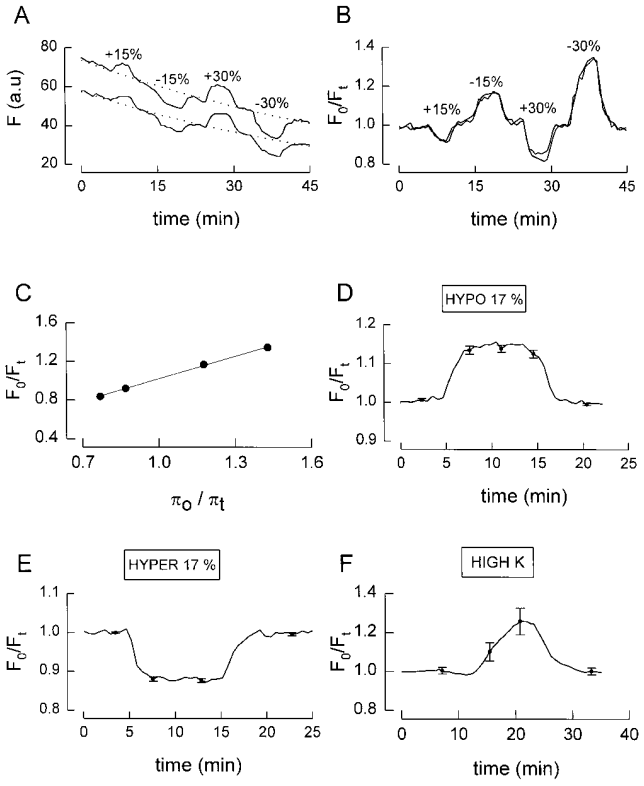

FIGURE 9. Cell volume changes in isolated polarized cells. Isolated polarized cells attached to a coverslip were loaded with calcein,AM and then superfused with PSS for 15 min. Pictures were taken every ~30 s and fluorescence from a 15–20 μm^2 area near the center of the cells was measured. (*A*) Fluorescence vs. time in two cells from the same preparation. Osmolality was changed by ±15% and ±30%, for 5-min periods, returning to isosmotic solution after each change. Fluorescence records were corrected for decay (single-exponential fit during superfusion with isosmotic solution). (*B*) F_o/F_t plot vs time (F_o = corrected fluorescence in isosmotic solution, F_t = corrected fluorescence at time = t, same experiment as in *A*). F_o/F_t is proportional to V_t/V_o (where V denotes volume and the subscripts have the above meanings), i.e., the plots show relative changes in cell water volume. (*C*) Calibration curve derived from the same experiment (π is solution osmolality and the subscripts have the above meanings). (*D*) Cell water volume change during 10-min exposures to hyposmotic solution (5 cells from 3 preparations; means are shown continuously, with SEM at selected times before, during, and after swelling). (*E*) Cell water volume change during 10-min exposures to 17% hyperosmotic solution (4 cells from 2 preparations). In *D* and *E* there was no cell volume regulation during the >10-min observation periods. Similar results were obtained changing osmolalities to ±34%, ±30%, and ±15% for 10 min (not shown). (*F*) Reversible cell swelling by isosmotic elevation of external $[K^+]$ from 2.5 to 86.5 mM in cells treated with 1 mM 8-Br-cAMP and 3 mM theophylline (6 cells from 2 preparations). These results validate the use of this technique to determine changes in cell volume and show that NGB epithelial cells do not regulate their volume after exposure to anisosmotic solutions.

the opposite membrane domain, so that in the steady state cell volume and composition remain near constant. This adaptive mechanism requires a signaling system between the cell membranes, i.e., intermembrane cross talk. The study of the mechanisms of cross talk between apical and basolateral membranes is of great importance to understand the regulation of salt and water transport in gallbladder and other epithelia. Parameters possibly involved in cross talk are cell volume, intracellular ionic activities, and membrane voltage.

One instance in which parallel changes in transport rates of apical and basolateral membranes occur in NGB epithelium is that resulting from the effect of cAMP. After an increase in cAMP levels, it has been demonstrated that an apical Cl^- conductance, not present under control conditions, develops and dominates the cell membrane conductances (Copello et al., 1993; Heming et al., 1994; Reuss and Altenberg, 1995). This results in net Cl^- loss across the apical membrane (Cotton and Reuss, 1991), because intracellular Cl^- is above the value predicted from electrochemical equilibrium as a consequence of the operation of the apical membrane Cl^-/HCO_3^- exchanger (Reuss, 1988). Garvin and Spring (1992) have also suggested that cAMP elevation results in a change in the dominant mechanism for Na^+ and Cl^- transport across the apical membrane: from Na^+/H^+ and Cl^-/HCO_3^- exchanges to Na^+-Cl^- cotransport. Further, Dausch and Spring (1994) have proposed that protein kinase C activation causes the opposite effect. Regardless of these possibilities, the increase in apical membrane G_{Cl} causes membrane depolarization and activation of voltage-sensitive apical membrane maxi-K^+ channels (Cotton and Reuss, 1991; Reuss, 1991). The parallel increases in apical membrane G_{Cl} and G_K cause net KCl efflux, intracellular Cl^- and K^+ contents fall, the cells shrink (Cotton and Reuss, 1991), and transepithelial salt and water transport decrease. The latter result indicates that net transport across the basolateral membrane must also be reduced. Our starting hypothesis was that cAMP causes a reduction in G_{Cl}^b. Hence, we assessed the effects of changes in cell water volume on basolateral membrane Cl^- and K^+ conductances.

Cell Swelling Stimulates and Cell Shrinkage Inhibits the Basolateral Membrane K^+ Conductance

Our results show that cell swelling causes a selective increase in basolateral membrane conductance, attributable to an increase in K^+ conductance. This effect is teleologically appropriate for cell volume regulation, inasmuch as it would facilitate K^+ loss from the swollen cells.

The opposite experimental perturbation, i.e., cell shrinkage by exposure to a hyperosmotic solution,

caused depolarization of both cell membranes, an increase in basolateral membrane resistance, and a decrease in the relative G_K^b. These effects are opposite to those elicited by exposure to hyposmotic solution and indicate that cell shrinkage decreases G_K^b. Again, this effect is teleologically appropriate, i.e., net K^+ efflux across the basolateral membrane would decrease in a cell that has undergone shrinkage.

The elevation in $[Ca^{2+}]_i$ after exposure to a hyposmotic solution could be solely or in part responsible for the increase of the G_K^b, inasmuch as this conductance is activated by maneuvers expected to elevate $[Ca^{2+}]_i$ (Bello-Reuss et al., 1981). Activation of nonselective cation channels, followed by Ca^{2+} entry and activation of Ca^{2+}-activated K^+ channels, has been demonstrated in other epithelia (Christensen, 1987). Our measurements demonstrated that $[Ca^{2+}]_i$ does rise during cell swelling; chelation with BAPTA prevents the elevation in $[Ca^{2+}]_i$ but not the membrane hyperpolarization. Although this result could be interpreted to indicate that $[Ca^{2+}]_i$ plays no role in the activation of G_K^b, we cannot rule out that, in the presence of BAPTA, $[Ca^{2+}]_i$ rose in a small compartment, undetectable by our method. An alternative to Ca^{2+} activation is that the basolateral K^+ channels are activated by stretch.

Cell Swelling Inhibits the Basolateral Membrane Cl^- Conductance

Cell swelling reduced the relative G_{Cl}^b to a value not different from zero, indicating that the basolateral membrane Cl^- conductance decreases. This effect is contrary to expectations, inasmuch as it is opposite to the effect of cell swelling on G_K^b and would, per se, tend to prevent KCl efflux via conductive pathways.

The effect of cell swelling on G_{Cl}^b could be indirect, since it occurs during membrane hyperpolarization. We found that membrane hyperpolarization per se reduces G_{Cl}^b, as indicated by basolateral solution Cl^--substitution experiments at three membrane voltages. Atypical osmosensitivity of Cl^- channels has been observed in other cell types (Chesnoy-Marchais and Fritsch, 1994). It is possible that cell swelling and membrane hyperpolarization have separate effects on G_{Cl}^b, but the membrane voltage changes appeared to dominate under the present experimental conditions.

Implications of the Results for Cell Volume Regulation

Osmotic swelling experiments yielded the expected increase in the total basolateral membrane conductance. The unexpected feature was that changes in G_{Cl}^b do not parallel the changes in G_K^b, because the basolateral membrane Cl^- conductance is voltage sensitive. Hence, it is possible that the effects of cell volume (e.g., via membrane stretch) are directly exerted on G_K^b and that the resulting change in membrane voltage is responsible for the change in G_{Cl}^b.

Our cell volume measurements suggest that NGB epithelial cells do not regulate their volume after exposure to anisosmotic solution. Our measurements of intracellular $[TMA^+]$ and aCl_i are in apparent contradiction with the results of Spring and associates (Persson and Spring, 1982; Larson and Spring, 1984; Furlong and Spring, 1990), which suggested that the epithelial cells from NGB undergo regulatory volume decrease from hyposmotic swelling by basolateral exit of K^+ and Cl^-, probably via basolateral membrane channels (Furlong and Spring, 1990). In contrast, our studies suggest that there is no measurable short-term cell volume regulation in NGB epithelial cells undergoing osmotic swelling. The experiments on isolated, polarized cells strengthen our conclusions in that they rule out artifacts resulting from TMA^+ loading and microelectrode impalement as possible explanations for the lack of cell volume regulation. Further, measurements of cell volume changes in isolated polarized cells in which apical membrane G_{Cl} was activated by cAMP showed that although these cells do not regulate rapidly their volume after osmotic swelling (data not shown), they undergo fast, sizable swelling after elevation of medium $[K^+]$, i.e., under isosmotic conditions. This again indicates that our results cannot be attributed to a methodological inability to detect cell swelling in isosmotic conditions. The explanation for the differences between our results and those of Spring and associates (e.g., Furlong and Spring, 1990) is not clear.

Implications of the Results for Cross Talk

Our results indicate that cell swelling causes rapid and reversible changes in basolateral membrane Cl^-- and K^+-channel activities. The demonstration that G_K^b is elevated by cell swelling supports the notion that cell volume is a signal responsible for cross talk between apical and basolateral membranes. However, under these circumstances G_{Cl}^b not only does not increase, but in fact decreases. This would not be the expected response if the cell had been swollen by increased solute entry across the apical membrane. Under these conditions, the expected cross talk response would be to increase solute exit across the basolateral membrane. It is in principle possible that net solute exit is activated by cell swelling, but not via conductive pathways. A pathway for basolateral membrane Cl^- efflux in NGB epithelium is KCl cotransport (Corcia and Armstrong, 1981; Reuss, 1981). Activation or inactivation of this mechanism during cell swelling and cell shrinkage, respectively, would fulfill the requirements of intermembrane cross talk, but would also result in cell volume regulation, and hence this possibility is not supported by our results.

The present observations suggest that G^b_{Cl} is regulated mainly or exclusively by V_m changes, although a minor role of cell volume is possible. The dominant role of membrane voltage suggests that this regulatory mechanism is designed to preserve intracellular Cl^- content instead of cell volume, i.e., there is an inverse relationship between electrical driving force and electrodiffusive permeability. Because of the hyperpolarization elicited by activation of G^b_K after cell swelling, G^b_{Cl} falls, and this prevents Cl^- loss and tends to maintain aCl_i closer to control levels. The need for this mechanism from the point of view of cell homeostasis is not obvious; cell Cl^- content ends up being preserved at the expense of cell volume. In other cell types, intracellular [Cl^-] has been shown or claimed to modulate membrane transporters (e.g., Robertson and Foskett, 1994) and regulators, such as protein kinases (Treharne et al., 1994). In NGB epithelium, in addition to effects derived from these "messenger" roles of intracellular Cl^-, a likely consequence of a decrease in intracellular [Cl^-] is a fall in intracellular pH. The apical membrane of this epithelium expresses a highly active anion (Cl^-/HCO_3^-) exchanger (Reuss, 1988). A fall in aCl_i at constant [Cl^-] in the apical bathing solution enhances Cl^- influx and HCO_3^- efflux across the apical membrane, which would cause cell acidification. In conclusion, osmotic swelling of NGB epithelial cells increases G^b_K, but reduces G^b_{Cl}, and hence there is no appreciable short-term regulatory volume decrease. If cell swelling is involved in cross talk between apical and basolateral membranes, then the effects of the increase in cell volume are not sufficient to provide an effective negative-feedback mechanism to restore cell volume to control levels, suggesting a more complex adaptive mechanism.

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