Actin-based Cytoskeleton Regulates a Chloride Channel and Cell Volume in a Renal Cortical Collecting Duct Cell Line*

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The regulatory volume decrease (RVD) of a renal cortical collecting duct cell line (RCCT-28A) exposed to a hypotonic solution was studied using electronic cell sizing to measure cell volume and the patch clamp technique to measure Cl- channel activity. Results demonstrate that RVD was mediated in part by KCl loss through separate K+ and Cl- channels. The Cl- channel had a conductance of 305 pS and was activated by cell swelling, membrane stretch, and disruption of F-actin by dihydrocytochalasin. In contrast, stabilizing F-actin with phalloidin prevented swelling and stretch activation of the Cl- channel and inhibited the RVD. Thus, the state of actin polymerization regulates the probability of the 305 pS Cl- channel being open. Short actin filaments activate whereas long actin filaments inactivate the channel. Taken together, our studies suggest that RVD in this renal collecting duct cell line is mediated in part by a 305 pS Cl- channel, which is activated, during cell swelling, by a signaling pathway that includes disruption of F-actin.

Most cells respond to a decrease in extracellular osmolality in two phases: 1) rapid swelling and 2) slow readjustment of the volume (5-7). This volume readjustment or regulatory volume decrease (RVD) results from the net loss of K+ and Cl- ions, amino acids and their metabolites, and water (5, 6, 8-11). In most mammalian cells, RVD is accomplished in part by efflux of K+ via KCl cotransport, the parallel operation of K+H+ and Cl-/HCO3- exchangers or K+ and Cl- channels. In renal epithelial cells, there is little information on the characteristics of K+ and Cl- channels responsible for RVD or on the signaling mechanisms underlying swelling-induced activation of these channels.

A variety of second messengers have been suggested as potential mediators of RVD including calcium, calmodulin-dependent protein kinase, protein kinase C, cAMP and protein kinase A, and arachidonic acid and its metabolites (16). Filamentous actin (F-actin) may also play a role in the RVD. For example, cell swelling induced by exposure to a hypotonic solution activates RVD and decreases F-actin content in promyelocytic leukemic cells (17). The decline in F-actin content correlates with the rate of RVD (17). In addition, cAMP activates Cl- secretion in Madin-Darby canine kidney cells while decreasing cell volume and depolymerizing F-actin (15). Finally, RVD in melanoma cells devoid of actin-binding protein is impaired; however, genetic rescue with actin-binding protein resulted in recovery of cell volume following exposure to a hypotonic solution, a process dependent on activation of a K+ channel (19). Presently, however, it is not known if the actin-based cytoskeleton contributes to RVD in renal epithelial cells.

Accordingly, the present study was conducted to determine if RVD in renal epithelial cells exposed to a hypotonic solution is mediated by Cl- channels and to determine if the actin-based cytoskeleton is an important element in the signal transduction pathway underlying RVD. In a previous study, we characterized Cl- channels in RCCT-28A cells. A continuous renal cell line derived from rabbit cortical collecting duct (20, 21). These cells express a large conductance (305 pS) Cl- channel in the apical membrane and an outwardly rectifying (13/96 pS) Cl- channel in the basolateral membrane (20, 22). Using this cell line as a model to study RVD in renal epithelial cells, we now report that hypotonic cell swelling activates the 305 pS Cl- channel in the apical membrane and that this channel contributes to RVD by a mechanism involving membrane stretch and disruption of F-actin.

MATERIALS AND METHODS

Cell Culture—As described previously, CCD cells were immunodissociated from rabbit kidney and infected with an adenovirus 12-simian virus 40 hybrid, resulting in a continuous cell line designated RCCT-28A (21). RCCT-28A cells were grown as described (20) and studied between passages 7 and 25 (20).

Patch Clamp Analysis of Single Cl- Channels—Single channel currents in the apical membrane were measured at 25 °C with a current-to-voltage converter (Warner Instrument Corp., model PC-501, Hamden, CT), low pass filtered at 300 Hz, and digitized at 1 kHz with an Everex AT computer as described in detail previously using PClamp Version 5.51 software (Axon Instruments, Foster City, CA) (20, 23). Briefly, the single channel current amplitude was calculated by constructing amplitude histograms of single channel currents (i). Channels were considered open when the current was larger than 12. Data were recorded for a minimum of one, 10-s trial every minute during control and experimental periods. In control and experimental periods, currents were recorded until a steady state was observed (steady state is defined here as a minimum of three consecutive 1-min periods in which the single channel open probability (Po) did not vary by more than 10%). The Po was defined as the total time the channel was open divided by the total time of data collection. Single channel currents were also displayed continuously on a strip chart recorder throughout the control and experimental periods. The patch pipettes were filled with (in mm): 140 NaCl; 5 KC1; 1 CaCl2; 1 MgCl2; 10 Hepes pH 7.4. The same solution was present in the bath during gigaseal formation. When membrane patches were excised to form the inside-out configuration, the bath solution contained (in mm): 5 NaCl; 140 KCl; 0.001 CaCl2, (buffered with 2 mM EGTA); 1 MgCl2, 10 Hepes pH 7.4.

Whole Cell Patch Clamp—Whole cell at Cl- currents were measured

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at 25 °C by the patch clamp technique as previously described (24). To generate I-V plots, the membrane was clamped to a holding voltage of 0 mV and then stepped in 20 mV increments between ±100 mV for 137.5 ms using PCampie 5.51 software. Three I-V plots were constructed every minute during control and experimental periods, and the plots were averaged to yield a mean I-V plot for each minute. In control periods, currents in I-V plots were recorded for a minimum of five consecutive minutes. During experimental maneuvers, currents for I-V plots were recorded until a steady state was observed (steady state is defined as three to five consecutive periods in which the slope of the I-V plot (i.e. conductance) did not vary by more than 10%). Whole cell conductance was calculated from the slope of the I-V plots. Currents were stored on the hard drive of an Everest AT computer and analyzed unfiltered using PCampie 5.51. Current magnitude was measured during the last 20 ms of each 137.5-ms voltage pulse. In some experiments, drugs were added to the pipette solution. Upon obtaining the whole cell configuration, the drugs diffused into the cell with a time constant of 30 s to 1 min (25).

We measured cell capacitance as previously described (24). The pipette solution contained (in mM): 140, N-methyl-D-glucamine Cl− (NMDCl); 5, Hesper; 1, MgCl2; 1, MgATP; 100, nM Ca2+ (buffered with 1 mM EGTA), pH 7.4. The bath solution contained (in mM): 140, NMDCl, 5, Hesper; 1, CaCl2; 60, sucrose; pH 7.4. In preliminary experiments, we found that addition of sucrose to the bath solution prevented swelling of Cl− currents and oscillations in Cl− currents in the absence of experimental maneuvers. Because these solutions do not contain K+ or Na+, the observed whole cell currents are referable primarily to Cl− (see below).

Measurement of Cell Volume—Cell volume was measured by electronic cell sizing as described in detail previously (26-28). Briefly, cells were grown on tissue culture-treated polystyrene flasks and were harvested by a mild trypsinization protocol (0.05% trypsin and 0.05 mM EDTA in a 0 Ca2+ and 0 Mg2+ phosphate-buffered saline solution). 8 x 106 cells were suspended in 4 mL of an isotonic cell solution (in mM: 70, NaCl; 2.4, KCl; 0.6, MgCl2; 1.2, CaCl2; 0.6, KH2PO4; 7.5, Hesper; 10, glucose; 160, sucrose; pH 7.4; osmolality, 325 mOsM/kg H2O) for 60 min. Thereafter, 500-µl aliquots of the cells in the isotonic solution were suspended in 20 mL of the isotonic solution, a hypotonic solution (isotonic) divided by the cells in the absence of experimental maneuvers. Because these solutions do not contain K+ or Na+, the observed whole cell currents are referable primarily to Cl− (see below).

Asterisks indicate significantly different from hypotonic-DIDS and hypotonic-Ba2+ data at same time point (p < 0.05).

RESULTS

Characterization of RVD—The first series of experiments were conducted to determine if RCCCT-28A cells volume regulate in response to a hypotonic solution and, if so, to determine if Cl− channels participate in RVD. Fig. 1 illustrates the effects of a hypotonic solution (i.e. reduction of the bath osmolality from 325 to 165 mOsM/kg H2O by removing sucrose from the isotonic solution) on cell volume. The volume response can be divided into three phases: 1) an initial and rapid, osmotic swelling reaching a peak 52% above control volume at 5 min; 2) a rapid shrinking in the following 10 min; 3) a more gradual decrease in cell volume reaching a steady state ~15% above control volume at 60 min.

To determine if the RVD involved activation of Cl− channels, the Cl− channel blocker, DIDS (10−4 M), was added to the hypotonic bath solution. DIDS increased the initial, rapid osmotic swelling, attenuated the shrinking phase, and inhibited the gradual, slower decrease in cell volume observed in cells not exposed to DIDS (Fig. 1). To determine if the RVD also involved K+ channels, the K+ channel blocker, barium (10−3 M), was added to the bath solution. The effects of barium were similar to those observed with DIDS (Fig. 1). Taken together, these observations suggest that RVD involves KCl loss through separate Cl− and K+ conductive pathways. However, because DIDS inhibits Cl− channels and the Cl−/HCO3− exchanger, it cannot be excluded that DIDS inhibited RVD by blocking a Cl−/HCO3− exchanger (see below).

Effects of Cell Swelling on Cl− Channels—To provide more definitive evidence that cell swelling activates a Cl− channel that contributes to RVD, we conducted cell-attached patch clamp recordings on the apical membrane. Fig. 2 illustrates the results of a representative experiment. Active Cl− channels were rarely observed (<1% of patches) in cell-attached patches of cells bathed in the isotonic solution. When the osmolality of the bath solution was reduced from 300 to 272 mOsM/kg H2O, a 10% dilution routinely experienced by renal cortical collecting duct cells in the deep cortex, Cl− channels activated after a variable delay of 2−5 min. The channels usually remained active for 5–10 min. The channels activated by the hypotonic solution were identified as 305 pS Cl− channels described previously by our laboratory (20). The channels had a Cl− to Na+ permeability ratio of 10 to 1 and a Cl− to HCO3− permeability ratio of 2 to 1. Furthermore, the single channel Po was inhibited.
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Fig. 2. Representative families of current records illustrating that cell swelling activates a 305 pS Cl⁻ channel in cell-attached patches. A, isotonic (300 mOsm/kg H₂O) bath solution. B, hypotonic (272 mOsm/kg H₂O) bath solution, 5 min after reduction of bath osmolality by addition of distilled water to the isotonic bath solution. C, hypotonic, 10 min after reduction of bath osmolality. D, hypotonic, 15 min after reduction of bath osmolality. O, the open current level; C, the closed current level. The command voltage was -10 mV.

by the Cl⁻ channel inhibitors DIDS (5 × 10⁻⁴ M), diphenylamine carboxylic acid (10⁻⁶ M), and 5-nitro-2-(3-phenylpropylamino) benzoic acid (3 × 10⁻⁶ M), but not by the K⁺ channel blocking compounds, barium (10⁻³ M) or tetraethylammonium (5 × 10⁻³ M) (n = 3–6/treatment). We also observed K⁺ channels in the apical membrane that were activated by cell swelling; however, K⁺ channels will be described in another report.

Fig. 3 summarizes the time course of Cl⁻ channel activation by the hypotonic bath solution. The Pₜ increased gradually in the first 5 min after exposure to the hypotonic solution, reached
the highest values between 6 and 10 min after exposure to the hypotonic solution, and declined slowly thereafter. Comparison of Figs. 1 and 3 reveals a good correlation between activation of the chloride channel and because DIDS inhibits both RVD and the C1- channel, it is reasonable to conclude that the 305 pS chloride channel contributes, at least in part, to the RVD.

Effect of Membrane Stretch on the 305 pS Cl- Channel—Some ion channels activated by cell swelling are also sensitive to membrane stretch (29-31). Accordingly, we examined the effects of membrane stretch on the 305 pS Cl- channel. As illustrated by the representative current records in Fig. 4, application of negative pressure to the patch pipette increased the Po of the channel in inside-out patches. Increasing negative pressure from 0 to 5, 10 and 15 cm of H2O progressively increased the P0. Removal of negative pressure partially reversed the increase in the P0. Fig. 5 summarizes the effects of negative pressure on channels with a P0 > 0 following patch excision to the inside-out configuration. In membrane patches that contained quiescent channels (n = 11), -2 cm of H2O pressure failed to increase the P0. However, -5 cm of H2O pressure increased the P0. Ten cm of H2O negative pressure maximally increased the P0. Application of -15 cm of H2O pressure failed to increase P0 further and often resulted in loss of the gigaohm seal. Two channels with an initial P0 = 0 were not activated by negative pressure (data included in analysis). Fig. 5B summarizes the effects of negative pressure on channels with a P0 > 0 following patch excision to the inside-out configuration (n = 8). Two cm of H2O negative pressure increase the P0 by ~100%. Five to ten cm of H2O negative pressure failed to increase P0 further. Sustained, ten cm of H2O negative pressure often resulted in the loss of the gigaohm seal. Thus, channels that were activated during conversion from the cell-attached to the inside-out configuration were more sensitive to small increases in negative pressure than channels that were not activated during formation of the inside-out configuration. In both subsets of channels, negative pressure increased the P0 within 1 min, and release of pressure reduced the P0 within seconds. Although the P0 decreased as negative pressure was released, the P0 returned to control values only in the subset of channels that were activated by patch excision (Fig. 5B).

Effects of F-actin Disruption on the 305 pS Cl- Channel—Structural integrity of the plasma membrane is maintained in part by the actin-based cytoskeleton (32). Many transport proteins including epithelial Na+ channels interact with the cytoskeleton (33) and disruption of F-actin activates Na+ channels in renal A6 epithelial cells (34). Because F-actin is disrupted by cell swelling (6, 17, 18, 35-38), we tested the hypothesis that the 305 pS Cl- channel is activated by disruption or fragmentation of F-actin. To this end, we used cytochalasins to disrupt and/or fragment F-actin (39-43) in inside-out patches of the apical membrane. A representative experiment illustrating the effects of dihydrocytochalasin B (DHCB) on a Cl- channel is shown in Fig. 6. DHCB increased the P0 of quiescent and excision-activated Cl- channels after a delay of 3-5 min. The
threshold for DHCB action was \(-10^{-7}\) M, and the EC_{50} was 0.8 \times 10^{-6} M (n = 4 paired experiments; 7 concentrations tested between 10^{-8} M and 10^{-5} M). Cytochalasins B and D also activated the Cl^- channel (Fig. 7). In contrast, chaetoglobosin C (10^{-6} M), a cytochalasin that does not depolymerize actin, failed to increase the P_{o} (n = 5).

Because disruption of F-actin activates the Cl^- channel, it follows that repolymerization of F-actin should inactivate the channel. Accordingly, we examined the effects of ATP-Mg^{2+}, a catalyst of actin polymerization, on the Cl^- channel after the P_{o} was increased by DHCB. ATP (10^{-3} M) reduced the P_{o} of channels activated by DHCB (Fig. 8). Washing DHCB from the bath in the absence of ATP did not reduce P_{o}. In previous experiments we showed that ATP-Mg^{2+} alone had no effect on the P_{o} of active 305 pS Cl^- channels (20). Thus, it is unlikely that the action of ATP involved the activation of protein kinases or ATPases. Taken together, these experiments are most consistent with the conclusion that disruption of F-actin activates the 305 pS Cl^- channel, whereas polymerization of actin inactivates the channel.

We also conducted experiments to determine if negative pressure applied to the membrane patch activates Cl^- channels by a mechanism involving the disruption of F-actin. We examined the effect of negative pressure on channels in membrane patches exposed either to DHCB or to phalloidin, which stabilizes F-actin (39, 40, 44, 45). As summarized in Table I, -10 cm of H_2O suction failed to increase the P_{o} of channels preactivated with DHCB. In contrast, DHCB added to membrane patches containing channels preactivated by negative pressure (-10 cm of H_2O pressure) increased the P_{o} (Table I). These observations suggest that DHCB is more effective than negative pressure in activating the Cl^- channel. In contrast, when membrane patches were treated first with phalloidin (2 \times 10^{-10} M), -10 cm of H_2O suction failed to activate the 305 pS Cl^- channel (Table I). These results suggest that the mechanism of activation of the 305 pS Cl^- channel by membrane stretch involves disruption of the cortical actin filament network.

**Whole Cell Cl^- Conductance**—Whole cell Cl^- currents were monitored to provide additional support for our single channel records indicating that cell swelling and disruption of F-actin activates Cl^- channels. Under the experimental conditions described under "Materials and Methods," whole cell RCCT-28A cells are referable exclusively to Cl^- (22). A 10% reduction in bath osmolality (350-315 mosm/kg H_2O; dilution with distilled H_2O) increased the whole cell Cl^- conductance from a steady-state value of 8.6 \pm 2.3 nS to a peak value of 11.9 \pm 2.8 nS at 1.5 min (Fig. 9; p < 0.05; n = 4). A 25% reduction in bath osmolality (350-262 mosm/kg H_2O; bath dilution with distilled H_2O) increased the Cl^- conductance from a steady-state value of 9.8 \pm 1.4 nS to a peak value of 18.7 \pm 1.1 nS (p < 0.05; n = 14). In addition, reducing the osmolality of the bath solution by removing sucrose from the isotonic solution (325-165 mosm/kg H_2O) also increased Cl^- conductance from a steady-state value of 15.8 \pm 1.3 nS to a peak value of 25.9 \pm 1.5 nS at 6 min (p < 0.05; n = 14). The characteristics of the whole cell Cl^- currents recorded from cells bathed in isotonic and hypotonic solutions are depicted in Fig. 9. Cl^- currents showed no appreciable time dependence, and the I-V plots were linear. The reversal potential of the I-V plots were near zero, in agreement with the value for the Cl^- reversal potential calculated by the Nernst equation. Additional evidence that RCCT-28A cells are conductive primarily if not exclusively to Cl^- in our experimental conditions comes from two experiments. First, reduction of the bath NaCl from 140 to 14 mM (mannitol added to the bath solution to maintain osmolality) shifted the reversal potential of the I-V plot from 0 mV to 54 \pm 4 mV (n = 8; p < 0.01), in close agreement with the value predicted by the Nernst equation for Cl^- (i.e. 58 mV). In addition, DIDS (10^{-4} M) reduced the increase in whole cell Cl^- conductance from 73 \pm 10% to 22 \pm 15% in response to a reduction in bath solution osmolality (325-165 mosm/kg H_2O; sucrose removed from the isotonic bath solution was p < 0.05; n = 3).

**F-Actin Regulates Swelling-activated Whole Cell Cl^- Currents**—Additional studies, using the whole cell patch clamp technique, were conducted to determine if the actin-based cytoskeleton participates in the swelling-induced stimulation of Cl^- channels (Table II). DHCB elicited a sustained increase in Cl^- conductance of cells bathed in the isotonic solution. In DHCB-treated cells, however, the hypotonic solution failed to elicit an additional increase in whole cell Cl^- conductance. Thus, when actin is disrupted by DHCB, cell swelling does not activate Cl^- channels. This observation is consistent with our single channel experiments demonstrating that cell swelling activates Cl^- channels by a mechanism involving the disruption of F-actin. To provide additional support for this conclusion, experiments were performed with phalloidin (Table II). Phalloidin reduced the Cl^- conductance of cells bathed in the isotonic solution and completely blocked the increase in whole cell Cl^- conductance elicited by the hypotonic solution. Phalloi-
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A. Control

B. DHCB, 1 μM

FIG. 6. Representative single channel patch clamp recordings illustrating activation of the 305 pS Cl⁻ channel by DHCB (1 μM). A, control. Five continuous, 2-s recordings immediately before addition of DHCB. B, DHCB. Five continuous, 2-s recordings 4 min after addition of DHCB. O, the open current level; C, the closed current level. The voltage across the membrane patch was -10 mV.

FIG. 7. Effect of cytochalasins on the P₀ of the 305 pS Cl⁻ channel in paired experiments. DHCB (10⁻⁸ M, n = 10), cytochalasin B (Cyto B; 10⁻⁴ M, n = 4), and cytochalasin D (Cyto D; 10⁻⁶ M, n = 4). Each cytochalasin increased the P₀ after a delay of 3-5 min. The voltage across the membrane patch was -20 mV. *, p < 0.05 versus control; **, p < 0.01 versus control.

Din also blocked the regulatory volume decrease in cells exposed to a hypotonic solution (i.e. reduction of the bath osmolality from 325 to 165 mOsm/kg H₂O by removing sucrose from the isotonic solution). As depicted in Fig. 10, cells treated with phalloidin and placed in the hypotonic solution exhibited an initial and rapid osmotic swelling reaching a peak 56% above control volume at 5 min. However, in contrast to control cells placed in the hypotonic solution, cells treated with phalloidin and placed in the hypotonic solution did not exhibit a regulatory volume decrease. Taken together, these results indicate that disruption of F-actin is required for swelling-induced activation of Cl⁻ channels and RVD.

Is Exocytosis Involved in RVD?—In many epithelia, stimulation of ion transport involves the exocytic insertion of transport proteins from a vesicular pool into the apical plasma membrane by a microtubule-dependent mechanism (46, 47). To determine if swelling-induced activation of Cl⁻ currents occurs by a mechanism involving exocytosis and the delivery of 305 pS Cl⁻ channels, and/or a regulatory protein, from an intracellular vesicular pool to the plasma membrane we monitored exocytosis by measuring capacitance using the whole cell patch clamp technique. Membrane capacitance is proportional to the cell surface area and can be monitored to detect increases in plasma membrane area as intracellular vesicles fuse with the plasma membrane. As illustrated in Fig. 11, a reduction in the osmolality of the bath solution (25% reduction in osmolality by distilled H₂O) increased Cl⁻ conductance; however, whole cell capacitance did not change. Similar observations were made in cells exposed to a 10% reduction in osmolality (n = 4, data not shown).
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Each inside out membrane patch served as its own control. The order of maneuvers in each experiment is listed sequentially (left to right). Depolarization (-30 mV) activation occurred within 1 min.

### TABLE I

| Exp. 1. Effect of negative pressure on DHCB-activated channels$^a$ | | **DHCB (10^{-6} M)** | **DHCB + 10 cm H$_2$O suction** |
|---|---|---|---|
| Control | 0.02 ± 0.02 | 0.65 ± 0.10$^b$ | 0.63 ± 0.15$^a$ |

### TABLE II

| Condition | Isotonic | Hypotonic | n | p value |
|---|---|---|---|---|
| Control | 15.0 ± 1.3 | 25.9 ± 1.5 | 14 | $p < 0.001$ |
| DHCB (10^{-6} M) | 34.8 ± 5.7$^a$ | 37.8 ± 5.4$^a$ | 7 | NS$^b$ |
| Phalloidin (2 × 10^{-10} M) | 8.1 ± 2.2$^a$ | 8.7 ± 2.2$^a$ | 7 | NS |
| Colchicine (10^{-6} M) | 12.9 ± 1.6 | 19.7 ± 2.4 | 4 | $p < 0.05$ |

$^a$ Significantly different from isotonic or hypotonic data in the same column by analysis of variance and Student-Neumann-Keuls test ($p < 0.05$).

### DISCUSSION

The major observation of this study is that RCCT-28A cells volume regulate in response to hypotonic cell swelling, at least in part, by activating 305 pS Cl$^-$ channels. Our data suggest that cell swelling activates the 305 pS Cl$^-$ channel by a signaling pathway involving the disruption of F-actin.

RCCT-28A cells also express outwardly rectifying Cl$^-$ channels (48). Accordingly, we cannot rule out the possibility that the outwardly rectifying Cl$^-$ channel may, in addition to the 305 pS Cl$^-$ channel, also contribute to RVD. However, it is unlikely that the outwardly rectifying Cl$^-$ channel plays a major role in cell volume regulation, because we found that cell swelling increases a whole cell Cl$^-$ conductance exhibiting a linear I-V relationship, not an outwardly rectifying I-V relationship.

Cl$^-$ Channels and Cell Volume Regulation—Cl$^-$ conductive pathways contribute to RVD in a variety of cell types (7, 9–12, 14, 15, 49, 50). However, only a few studies have characterized Cl$^-$ channels involved in RVD (51–54). In neuroblastoma cells, hypotonic cell swelling activates a 200–400 pS Cl$^-$ channel that is permeable to HCO$_3^-$ (P$_{Cl^-}$:P$_{HCO3^-}$ = 2.4:1) and rapidly inactivates when the voltage is changed to values more negative than −30 mV (51). The properties of this channel are similar to the 305 pS Cl$^-$/channel in RCCT-28A cells (20). Cell swelling activates a 3 pS Cl$^-$ channel in lymphocytes, a 23 pS Cl$^-$/channel in Ehrlich ascites tumor cells, and an outwardly rectifying

Colchicine (10^{-6} M), a drug that inhibits the polymerization of tubulin monomers and blocks microtubule-dependent exocytosis, had no effect on basal Cl$^-$ conductance (15.0 ± 1.3 nS in control) versus 12.9 ± 1.6 nS in the presence of colchicine) or on the increase in Cl$^-$ conductance induced by a reduction in bath osmolality from 350 to 290 mOsm/kg H$_2$O (osmolality reduced by removing sucrose from the isotonic solution). In colchicine-treated cells, the hypotonic bath solution increased the Cl$^-$ conductance from 12.9 ± 1.6 to 19.7 ± 2.4 nS ($n = 4, p < 0.05$). As reported above, a similar decrease in osmolality in the absence of colchicine increased Cl$^-$ conductance from 15.0 ± 1.3 nS to a peak value of 25.9 ± 1.5 nS ($p < 0.001; n = 14$). These experi-
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**Fig. 10.** Time course of cell volume changes following suspension of cells in isotonic (325 mOsm/kg H2O open circles) or hypotonic (185 mOsm/kg H2O filled circles) solutions (n = 7 experiments/group). Hypotonic-Phal. (Phalloidin, 1 nM), cells treated with phalloidin (1 nM filled triangles) and suspended in the hypotonic solution. The y axis depicts the ratio of cell volume at time 0 (volume in the isotonic solution) divided by the cell volume at the indicated times. Asterisks indicate significantly different from Hypotonic-Phal. at any time point (p < 0.05). Cells treated with phalloidin and placed in the hypotonic solution did not swell as much as cells in the hypotonic solution. As originally proposed by Kleinzeller et al. (75) we suggest that phalloidin, by stabilizing actin, may restrain the cell membrane from deforming in response to cell swelling. Thus, when actin is cross-linked the cells may not swell maximally as long as the cross-linked, cortical actin filament scaffold remains attached to the cell membrane.

(40–60 pS) Cl- channel in airway epithelial and T84 cells (53, 54).

**Does Membrane Stretch and Cell Swelling Activate the 305 pS Cl- Channel by Depolymerizing F-actin?**—Our studies suggest that membrane stretch activates the 305 pS Cl- channel by a mechanism involving disruption of F-actin. Several lines of evidence support a role for F-actin in regulating ion channels in other cell types. Fragmentation of F-actin activates Na+-selective channels in cell-attached and inside-out patches of the apical membrane of A6 cells (34). Phalloidin blocks CAMP-activated Cl- secretion and CAMP-induced rearrangement of F-actin organization in T84 cells (44). DHCB and cell swelling activate a Cl- conductance in cardiocytes (55). Because DHCB and swelling were not additive it was suggested that F-actin was involved in the swelling activation mechanism (55). Hypothesis cle cell swelling activates RVD in promyelocytic leukemic cells while simultaneously depolymerizing F-actin (17). Cytochalasins and CAMP depolymerize F-actin in Madin-Darby canine kidney cells and decrease cell volume by activating K+ and Cl- conductances (18). Finally, RVD in melanoma cells devoid of actin-binding protein is impaired; however, genetic rescue with actin-binding protein resulted in recovery of cell volume following exposure to a hypotonic solution, a process dependent on activation of a K+ channel (19).

**Do Cell Swelling and Negative Pressure Activate the 305 pS Cl- Channel by the Same Mechanism?**—The most parsimonious interpretation of our single channel and whole cell patch clamp data is that cell swelling activates Cl- channels by a mechanism involving membrane stretch and disruption of the actin-based cytoskeleton. Making a few reasonable assumptions, it can be calculated that –20 cm of H2O suction applied to an inside-out membrane patch produces a transmembrane pressure that is equivalent to the pressure generated by hypotonic cell swelling. According to Laplace's law, –20 cm of H2O suction applied to a 1.5 μm diameter membrane patch will produce a tension of 2 dynes/cm² (56–58). Using equation A4 from Sackin (57) and the lowest estimate of the plasma membrane area elasticity constant (130 dynes/cm²) (58), an increase in cell volume of only 1% should be sufficient to produce a membrane tension of 0.8 dynes/cm², a value comparable with a tension of 2 dynes/cm² created by suction (57). This calculation implies that cell swelling creates a pressure gradient across the cell membrane (direct measurements reveal an increase in intracellular pressure in Xenopus oocytes exposed to a hypotonic solution (59)). Although it is not yet possible to measure intracellular pressure in smaller cells normally used as models to study cell volume regulation, these calculations indicate that cell swelling is likely to increase membrane tension to levels equivalent to or even greater than those produced by application of negative pressure to membranes by patch clamp electrodes.

**Signaling Mechanism of Swelling-induced Activation of the 305 pS Cl- Channel**—Although volume-sensitive ion channels have been described in a variety of cell types, little information is available concerning the mechanisms by which cell swelling activates ion channels. Some evidence suggests that the actin-based cytoskeleton is involved. In particular, disruption of the filamentous actin network below the apical plasma membrane may increase channel currents by: 1) removing a physical barrier preventing the exocytic insertion of vesicles containing channels and/or a channel regulator from an intracellular pool into the plasma membrane; 2) liberating second messengers; 3) changing the conformation of the channel protein due to an alteration in the channel-actin-based cytoskeleton interaction. For example, in some cells, RVD involves the insertion of vesicles into the plasma membrane, from a cytoplasmic pool, by a mechanism that is sensitive to cytochalasins and microtubule-disrupting agents (60–62). However, in the present study the hypotonic bath solution had no measurable effect on whole cell capacitance (i.e. membrane area). Furthermore, colchicine, a drug that inhibits microtubule-dependent exocytosis, did not prevent swelling activation of the whole cell Cl- conductance. Accordingly, our data are most consistent with the conclusion that exocytosis, and in particular microtubule-dependent exocytosis, is not an important component of RVD in RCCT-28A cells. On the other hand, it is possible that cell swelling stimulated exocytosis and increased membrane area but that the change was too small to detect. Furthermore, it must also be considered that swelling stimulated parallel increases in exocytosis and endocytosis such that cell capacitance remained unchanged. Additional experiments are required to explore these alternative hypotheses.
In preliminary experiments we observed that diacylglycerol production by RCCT-28A cells is stimulated by the hypotonic bath solution and that calphostin C, an inhibitor of protein kinase C, and pertussis toxin block the swelling-induced activation of the 305 pS Cl- channel (4). Furthermore, calphostin C and pertussis toxin prevented disruption of actin filaments in cells exposed to the hypotonic solution.2 These observations are consistent with previous studies on RCCT-28A cells in which we demonstrated that the pertussis toxin-sensitive G protein, G_{o,3}, and protein kinase C activate the 305 pS Cl- channel (20). Accordingly, our preliminary data suggest that cell swelling activates Cl- channels by a sequential signaling pathway that includes cell swelling, G_{o,3}, protein kinase C, and disruption of F-actin.

It also must be considered that other signaling pathways may contribute to swelling-induced activation of the 305 pS Cl- channel. Cell swelling increases intracellular calcium in some cells, and it is known that a rise in intracellular calcium activates Cl- channels either directly or indirectly via calmodulin or calmodulin-dependent protein kinases (16). Furthermore, exposure to a hypotonic solution increases the production of arachidonic acid and its metabolites and increases cyclic AMP. Thus, other signaling mechanisms, not yet examined, may also be involved in RVD in RCCT-28A cells.

Finally, changes in the conformation of the Cl- channel protein due to alterations in channel-actin cytoskeleton interactions may also regulate channel activity. Ion channels and transporters are linked to actin via spectrin and ankyrin in many cell types (33, 63–72). This interaction is known to anchor these integral membrane proteins in specific domains within a cell; however, it is not known whether cytoskeletal proteins directly affect transporter function. As integral membrane proteins, ion channels have numerous membrane-spanning domains that are stabilized by electrostatic attractions and subtle conformations. Alteration of these conformations by changes in the actin-based cytoskeleton may change the physical dimensions of the ion channel protein, alter its conformation, and open or close the channel pore. Adenylly cyclase, a membrane-spanning protein with an ion channel motif and function, is activated by mechanical deformation of the cell, resulting in a 100% increase in intracellular concentrations of cyclic AMP (16). This activation is enhanced by cytoschalin and by microtubule-disrupting agents such as colchicine and vinblastine (73, 74). However, the mechanism whereby actin regulates transmembrane proteins is unknown and is a challenge for future research.

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