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Modulation of a Volume-regulated Chloride Current by F-Actin

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Abstract. We have examined whether F-actin integrity is involved in activation of a volume-regulated Cl^- current (VRChlC) in B-lymphocytes. VRChlC activation was initiated in response to establishing a whole cell recording in the presence of a hyposmotic gradient. Parallel confocal microscopy experiments using Rhodamine-Phalloidin (R-P) as a specific marker of F-actin showed that the submembrane actin ring is reversibly disrupted in response to an hyposmotic gradient. Disruptions of cortical F-actin integrity by 50 μM cytochalasin B (CB) does not trigger activation of VRChlC under isosmotic conditions or potentiate the rate of activation when the osmolarity of the extracellular solution was decreased by 75%. However, incubation with CB increased the rate of VRChlC activation in response to a 90% hyposmotic gradient. Phalloidin, a stabilizer of F-actin, decreases the rate of VRChlC activation in response to a 90% gradient, but has no effect in response to a 75% gradient. These observations suggest that disassembly of cortical F-actin is not critical for VRChlC activation in B-lymphocytes. The integrity of cortical F-actin, however, can exert a modulatory effect on the rate of VRChlC activation in the presence of a hyposmotic gradient.

Key words: Volume regulation — F-actin — Cytochalasin B —Phalloidin — Myeloma — B-lymphocyte

Introduction

Mammalian cells are required to maintain their volume within a very narrow range when exposed to variations in external osmolarity. Although mammalian cells generally are not exposed to wide swings in external osmolarity, peripheral blood cells, including lymphocytes, are an exception. Blood cells are routinely exposed to large and rapid changes in extracellular osmolarity as they pass through renal medullary circulation. A variety of disease or trauma states may produce a transient or sustained change in osmolarity in many tissues (Law, 1991; McManus & Churchwell, 1994). Cells in other tissues which are not regularly exposed to changes in osmolarity, however, can also exhibit volume regulatory responses (e.g., Worrell et al., 1989; Häussler et al., 1994; Oike et al., 1994).

Acute exposure to a hypotonic solutions leads to cell swelling. Recovery to a normal cell volume is termed 'regulatory volume decrease' (RVD) (Law, 1991; Hoffman 1992) which occurs over a period of minutes. An initial response to swelling in many cells is to reduce the intracellular concentration of K^+ and Cl^- so that water will flow out of the cell, allowing the volume to return to normal. The outward flow of Cl^- through volume-regulated chloride channels (VRChlC) is thought to lead to the subsequent opening of voltage-sensitive K^+ channels (Grinstein et al., 1984; DeCoursey et al., 1985; Deutsch & Lee, 1988; Hoffman, 1992). VRChlC in lymphocytes are dependent on intracellular ATP and have an estimated single channel conductance on ≤ 2 pS (Lewis, Ross & Cahalan, 1993).

Several studies have shown that the architecture of the cytoskeleton changes in response to cell swelling in a variety of cell types (Mills, Schwiebert & Stanton 1994). Hyposmotic conditions, for example, result in changes in both microtubule and microfilament structure in pheochromocytoma cells, fibroblasts, and Ehrlich tumor cells (Foskett & Spring, 1985; Gilles, et al., 1986; Cornet, Ubl & Kolb, 1993). The relationship between changes in cytoskeletal integrity due to an osmotic challenge and regulation of mechanosensitive ion channels such as VRChlC is not fully understood. Cytoskeletal proteins such as F-actin have been reported to modulate volume-regulated currents in some systems, but not others (Oike et al., 1994).

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This study examines the role of a specific component of the cytoskeleton, F-actin, in activation of VRChlC. We address the hypothesis that VRChlC activity in myeloma cells is dependent on the disassembly of F-actin. In this study, we show, using a combination of electrophysiology and confocal microscopy, that disassembly of F-actin does not activate VRChlC in the absence of a hyposmotic challenge. Disassembly of F-actin, however, increases the rate of VRChlC activation in response to a hyposmotic challenge. Conversely, stabilization of F-actin with phalloidin decreases the rate of VRChlC activation. Thus, F-actin modulates VRChlC activity in the presence of an hyposmotic gradient, but is not essential for activation of VRChlC.

Materials and Methods

Cell Lines and Tissue Culture

RPMI 8226 myeloma cells are a continuous cell line that grows in suspension. Its shape can be approximated by a sphere, allowing less ambiguity in measurements of diameter or volume. These cells express a VRChlC similar to that previously described in cells ranging from *Xenopus* oocytes to mammalian cells in primary or continuous culture (e.g., Cahalan & Lewis, 1988; Kunzelman, Pavenstadt & Greger, 1989; McCann, Li & Welsh, 1989; Worrell et al., 1989; Solc & Wine, 1991; Doroshenko & Neher, 1992; Kubo & Okada, 1992; McDonald et al., 1992; Sorota, 1992; Tzeng, 1992; Yantorno et al., 1992; Chan et al., 1993; Diaz et al., 1993; Lewis et al., 1993; Stoddard, Steinbach & Simchowitz, 1993; Zhang et al., 1993; Ackerman, Wickman & Clapham, 1994; Kelly, Dixon & Sims, 1994; Nilius et al., 1994). VRChlC is outwardly rectifying under symmetrical chloride conditions (e.g., Worrell et al., 1989; Solc & Wine, 1991; Lewis et al., 1993), it is sensitive to the nonselective chloride channel blockers, DIDS, SITS, NPPB, and DPC (e.g., Worrell et al., 1989; Solc & Wine, 1991; Kubo & Okada, 1992; Lewis et al., 1993), and it has the anion selectivity of $I^- > Br^- > Cl^-$ > gluconate (Kubo & Okada, 1992; Lewis et al., 1993). The current amplitude of VRChlC typically develops over several minutes after exposure to a hypotonic solution.

Myeloma cells were grown in suspension with RPMI 1640 (Cell Grow of Fisher Scientific/Mediatech, Washington, DC) supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY) and were fed and split every 2–3 days. In preparation for recording, cells are washed 2× with RPMI 1640 without serum and then allowed to settle on an uncoated glass cover slip and washed with extracellular recording solution before experiments.

Solutions

Standard external recording solution contains (in mM): 155 NaCl, 2 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, pH 7.2. Intracellular solutions contain (in mM): 120 Cs^+-Glutamate, 10 HEPES, 4 ATP, pH 7.2 (CsOH) with free $[Ca^{++}]$ ~10 nM (0.1 $CaCl_2$, 1.1 EGTA). Changes in the composition of recording solutions are indicated in figure legends or text. Substitution of Cs^+ by TEA or inclusion of 0.2 mM GTP (in addition to ATP) in intracellular solution has no effect on activation of VRChlC. Intracellular solutions contain Cs^+-glutamate to eliminate any potential contamination of Cl^- currents by outward K^+ currents. The composition of these solutions results in an outward appearing current or inward Cl^- flux.

The osmolarity of all solutions was determined immediately before experiments with a vapor pressure osmometer (Wescor, Logan, UT) and was adjusted by addition of water (decreasing osmolarity of external solutions) or sucrose (increasing osmolarity of internal solutions). Relative osmolarity of external solutions is expressed as a percentage of the internal osmolarity ([external osmolarity/internal osmolarity] · 100). The activation of the current is dependent only on the osmotic gradient from internal solution to external solution. It is independent of whether the osmolarity of the extracellular solution is decreased with respect to the internal solution of the osmolarity of the internal solution is raised with respect to the external solution. Increasing the intracellular osmotic and ionic strength with CsCl, instead of sucrose, results in an equivalent activation of VRChlC. Current activation is also independent of the osmolarity of the media in which cells were grown.

Electrophysiological Recording

Macroscopic ionic currents were measured using the whole cell configuration of the standard patch clamp technique (Hamill et al., 1981). Pipettes were pulled from N51A glass (Garner Glass, Claremont, CA), coated with Sylgard (Dow Corning Corp. Midland, MI), and fire polished to give a final resistance of 2–6 MΩ, in the above recording solutions. Currents were recorded using an EPC9 amplifier (HEKA Electronik, Lambrecht, Germany), ITC-16 interface (Instrutech, Great Neck, NY) and accompanying acquisition and analysis software (Pulse & PulseFit, HEKA Electronik, Lambrecht, Germany) running on a Macintosh Quadra 700 or 800. Further analysis employed IGOR (WaveMetrics, Lake Oswego, OR) and Microsoft EXCEL (Microsoft, Redmond, WA). Junction potentials were nulled immediately before seal formation. Pipette and whole cell capacitance were automatically compensated and recorded. Whole cell capacitance and series resistance (R_s) were monitored throughout the recording. Normal cellular current convention is used when referring to the direction of current. In other words, *outward* current refers to *inward* Cl^- ion flow. Cells were treated with CB for 20 min or Ph for 30 min before recording was initiated in order to insure that F-actin structure was not undergoing any further change. The effect of either agent on VRChlC activation was consistent for an additional 80 min, after which time we terminated recording. All experiments were performed at room temperature (22°C ± 2).

In experiments using cells with large current amplitudes (>500 pA), series resistance compensation was employed. In these cases, 95% compensation with a 100 μs lag was used. Occasionally, a lower percentage compensation was required to prevent current oscillation. Cells exhibiting small current amplitudes (<500 pA) did not require series resistance compensation. In these cases, the voltage error involved is usually <5%. Electrophysiological results are based on records made from >100 cells.

Confocal Imaging

Confocal imaging of Rhodamine-Phalloidin (R-P: Molecular Probes, Eugene, OR) was used to specifically analyze labeled F-actin. Myeloma cells were treated with 100 μM hydroxyurea for 24 hr. Viable cells were isolated by separation on a ficoll gradient, and then washed in RPMI 1640 media in the absence of sera, resuspended in isosmotic recording solution and allowed to settle on cover slips coated with polylysine. Coverslips were divided into control and experimental groups and experimentally challenged (e.g., exposure to 50% hypos-

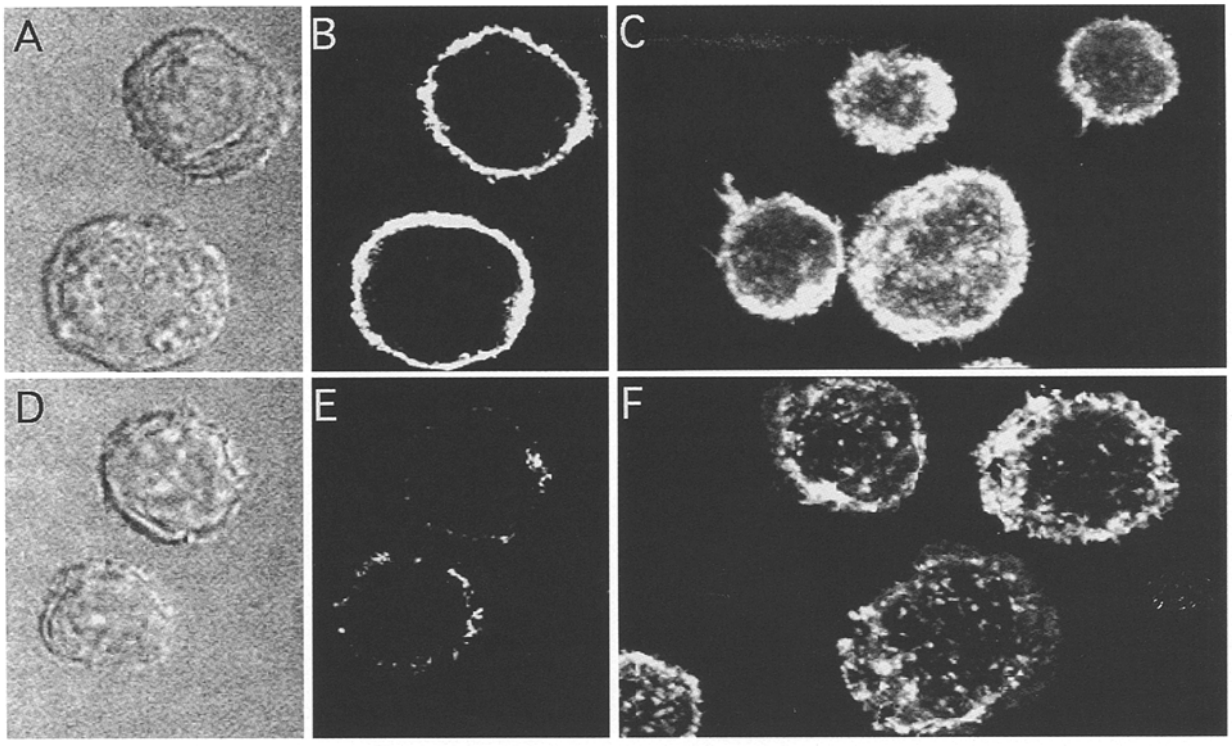
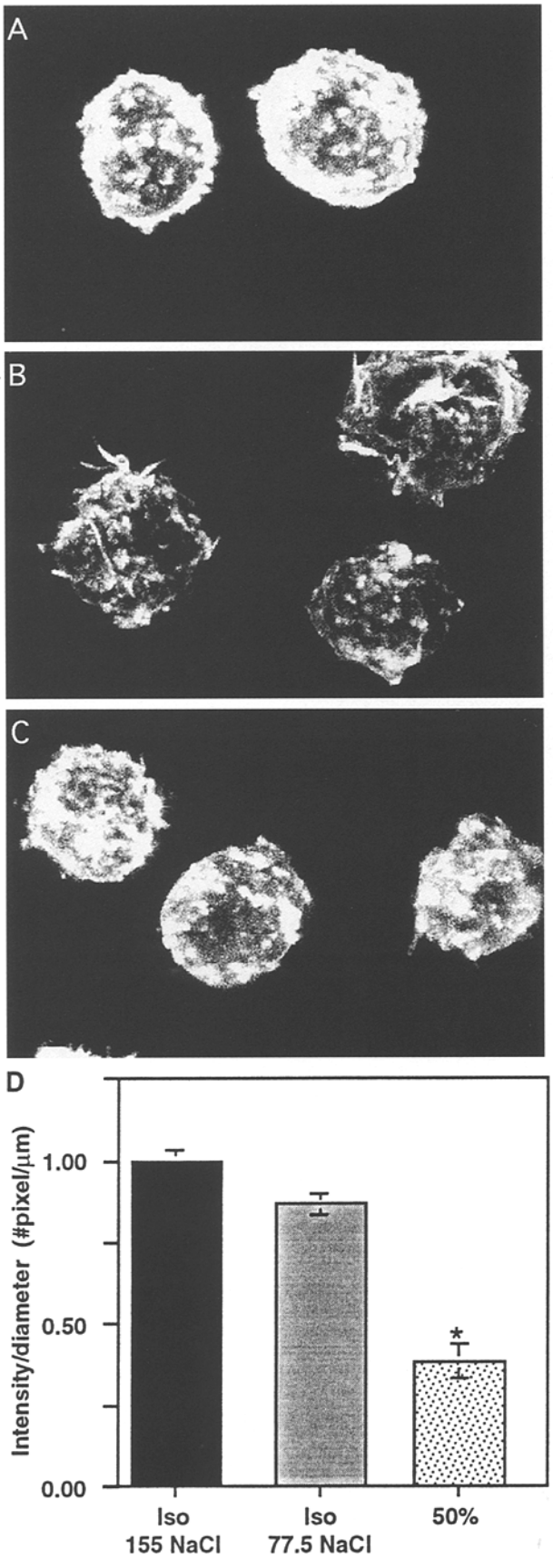


Fig. 1. Disassembly of F-actin by hyposmotic shock. Cells exposed to an isosmotic gradient (311 ± 5 mOsm: external solution) are shown in panels *A, B,* and *C.* Cells exposed to a 75% hyposmotic gradient (243/294 ± 5 mOsm: external/internal solution) in panels *D, E, F.* Panels *A* and *D* show the image obtained from cells using transmitted light. Panels *B* and *E* show confocal images of F-actin labeled with fluorescent R-P from a median plane of the same cells shown in Panels *A* and *D.* Panels *C* and *F* show the three-dimensional reconstruction (*see* Methods) of R-P labeled F-actin in cells from the same experiments as cells shown in panels *A* and *C.* F-actin appears as a cortical shell under isosmotic conditions that is disrupted in the presence of hyposmotic solutions. Quantification of R-P fluorescence from the experiment represented in Panels *A–F* is shown in Panel *G.* Fluorescence intensity of cells exposed to 75% hyposmotic solution was reduced by 52% when compared to cells maintained in isosmotic solutions. Pixel intensity was normalized to F-actin ring diameter. Average F-actin diameter (in μm) is (*A–C*) 18.9 ± 0.2, (*C–E*) 18.7 ± 0.2. n = 100 for each experimental condition. Bars indicate SEM and asterisks indicate a significance level of $P < 0.001$ (paired Student's *t*-test).

motic solution for 5 min and then returned to isosmotic solutions for recovery over 20 min) before being subsequently fixed in 3.7% paraformaldehyde in (mM): 150 NaCl, 5 Hepes, 3 $MgCl_2$ (pH 7.2), washed in phosphate-buffered saline (pH 7.0: 2 times, 3 min each), permeablized with 0.1% Triton and incubated with 300 nM R-P. Cells were then washed in PBS (5 times, 3 min each) and mounted using SlowFade (Molecular Probes, Lake Oswego, OR) to prevent fading. Staining and fixing was performed at room temperature (22°C ± 2). Confocal microscopy was performed using a BioRad MRC 600 confocal system with a 63× oil immersion lens fitted on a Zeiss inverted microscope. A YMS filter block was used in conjunction with a 3% laser and a direct filter for single scanned images or a Kalman (2 scan) for Z-series images. R-P images were acquired on a Gateway 2000 4DX 66 MHz computer using CoMOS (v. 3.02, BioRad software) and transferred to a Macintosh Quadra 800 for analysis with NIH Image and Adobe Photoshop. Images are made 0.36 μm apart along the Z-axis of cells and reconstructed to show R-P staining in the top half of the cell.

R-P staining was quantified by taking images through a plane at the center of a cell diameter and determining the mean of the number of pixels per cell, normalized to diameter of F-actin ring ({# pixels/cell}/{diameter of F-actin ring}, n = 100 cells per condition). Control cells treated only with isosmotic media prepared for R-P staining were included in each experiment. To control for any inherent variations in fluorescent intensity, gain and black level of the photomultiplier were carefully adjusted at the beginning of each experiment, so that pixel intensity did not saturate in control, isosmotic cells. Quantification of pixel intensity (R-P staining) using Microsoft EXCEL (Microsoft, Red-



mond, WA) is expressed as a percentage relative to the isosmotic control. Electrophysiological recordings and staining of F-actin with R-P were done on the same day, using the same population of cells. Imaging results are based on >20 experiments, using >10 different conditions and >8000 cells.

Results

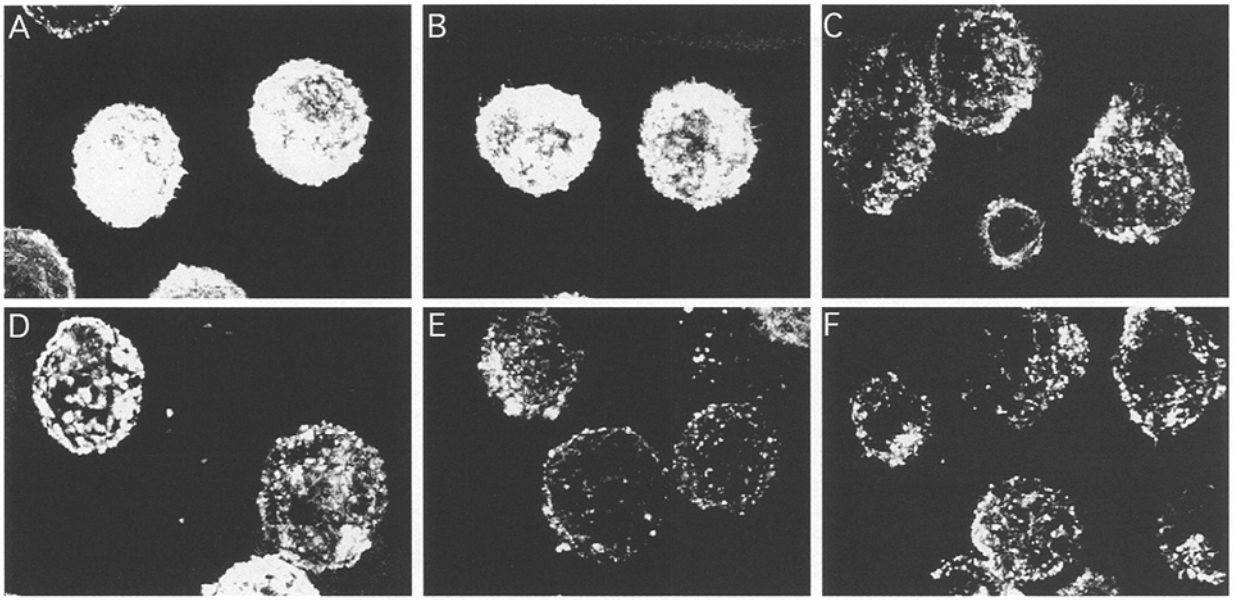
Cortical F-actin Integrity

Under isosmotic conditions, F-actin appears as a shell underneath the plasma membrane when visualized with rhodamine-labeled phalloidin (R-P) (Fig. 1). This cortical F-actin shell appears to be break under hyposmotic conditions (e.g., when osmolarity of external solution is 75% of control). The change in F-actin integrity can be quantified by measuring the average pixel intensity of the median plane taken through labeled cells (Fig. 1). Pixel intensity, which is related to the number of R-P binding sites, is decreased under hyposmotic conditions to 39 ± 7%, relative to isosmotic conditions. The loss of R-P labeling in cells exposed to hyposmotic solutions, quantified in Fig. 1*G*, suggests that the number of phalloidin binding sites is decreased. We interpret this loss to be to the loss of F-actin integrity because phalloidin binds with high affinity to the filamentous (i.e., F-) form of actin. Cortical F-actin integrity recovered within 5 min in cells returned to isosmotic solutions after a 5 min exposure to a 50% hyposmotic solution (*data not shown*). The average diameter of labeled F-actin rings does not appear to change in response to a hyposmotic challenge. For example, the average F-actin diameter (n = 100) for cells shown in Fig. 1 is 18.9 ± 0.2 μm in cells under isosmotic conditions and 18.7 ± 0.2 μm under hyposmotic conditions.

F-actin and Ionic Strength

The loss of cortical F-actin in response to a hyposmotic challenge is due to cell swelling and not the decrease in

Fig. 2. Disassembly of F-actin is unaffected when osmotic strength is maintained but ionic strength is reduced. Cortical F-actin cells exposed for 5 min to (*A*) an isosmotic gradient (317 ± 5 mOsm: external solution) was significantly greater than that of cells exposed to (*B*) at 50% hyposmotic gradient (150/300 ± 5 mOsm: external/internal solution). (*C*) Cells exposed for 5 min to external solutions containing half the normal concentration of NaCl Ringers but with sucrose added to the external solution to maintain the ionic strength (330 mOsm: external solution) were similar to control. (*D*) Fluorescence intensity was not significantly decreased in cells exposed to solutions containing reduced ionic strength but normal osmolarity. Average F-actin diameter (in μm) is control: 15.3 ± 0.2, 50% hyposmotic: 16.5 ± 0.2, 50% + sucrose: 17.4 ± 0.3, n = 100 for each experimental condition. Bars indicate SEM and asterisks indicate a significance level of $P < 0.001$ (paired Student's *t*-test).



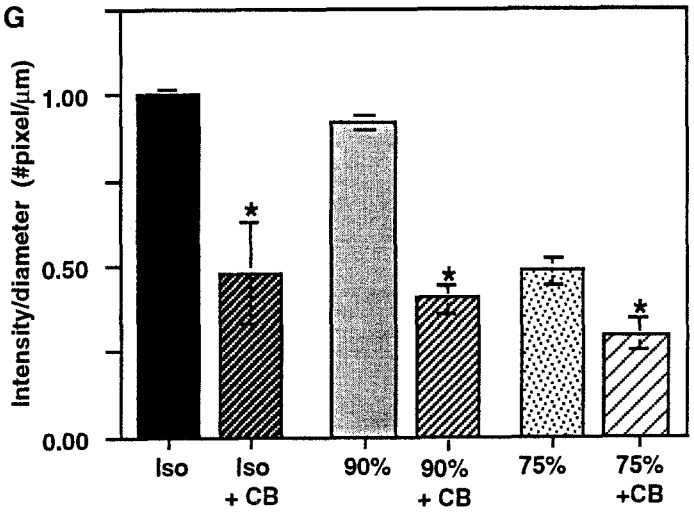


Fig. 3. F-actin is disassembled by cytochalasin B. Cells exposed to an isosmotic gradient (311 ± 6 mOsm: external solution) are shown in Panels *A* and *D*. Cells shown in Panels *B* and *E* were exposed to a 90% hyposmotic gradient (290 ± 7 mOsm: external solution). Cells shown in panels *C* and *F* were exposed to a 75% hyposmotic gradient (240 ± 2 mOsm: external solution). Cells in Panels *A, B* and *C* were exposed for 20 min to control solutions, whereas cells in Panels *D, E,* and *F* were exposed for 20 min to solutions containing 50 μM cytochalasin *B*. Cells in Panels *C* and *F* are from a different experiment than cells shown in Panels *A, B, D,* and *E*. Cortical F-actin integrity appears intact in the presence of a small (90%) hyposmotic gradient, but was obviously disrupted in the presence of a 75% hyposmotic gradient. Cortical F-actin integrity, however, was disrupted in the presence of cytochalasin B regardless of the osmotic gradient. Panel *G* shows the quantification of rhodamine-phalloidin fluorescence under experimental conditions shown in Panels *A–F*. Average F-actin diameter (in μm) is (*A*) 17.0 ± 0.3, (*B*) 16.1 ± 0.2, (*C*) 18.7 ± 0.2, (*D*) 15.6 ± 0.3 (*E*) 16.0 ± 0.3, (*F*) 17.7 ± 0.2. n = 100 for each experimental condition. Bars indicate SEM and asterisks indicate a significance level of $P < 0.001$ (paired Student's *t*-test).

external ionic strength. We determined that this is the case by assaying F-actin integrity with measurements of pixel intensity in cells incubated in an external solution diluted to 50% of normal ionic strength (155 mM to 77.5 mM) but with sucrose added to maintain osmolarity at 300 mOsm (Fig. 2). Cells do not swell appreciably under these conditions because solutions are approximately isosmotic with intracellular solutions. Normalized pixel intensity was significantly higher when the osmolarity was maintained with sucrose (86 ± 3% of control in isosmotic sucrose compared with 38 ± 2% of control in 50% hyposmotic solution). Note that pixel intensity is decreased to a similar level in 75% and 50% hyposmotic solutions (Figs. 1*G* and 2*D*).

Modulation by F-actin Disassembly

F-actin integrity can be disrupted by the application of cytochalasin B (CB). When cells are incubated in 50 μM CB under isosmotic solutions, the cortical F-actin integrity, as measured by R-P labeling, decreases to a level similar to that of cells exposed to hyposmotic solutions (Fig. 3). This decrease of R-P staining in the presence of

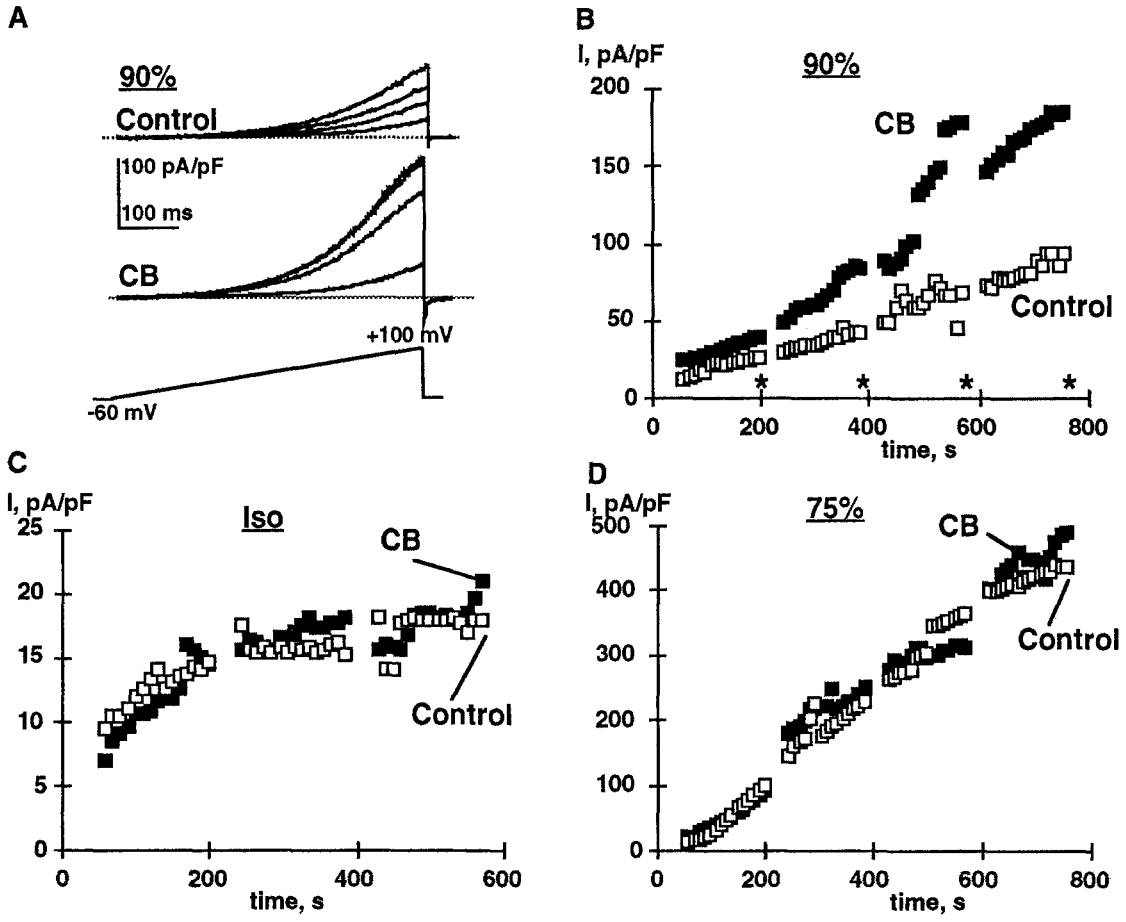


Fig. 4. VRChlC activation is enhanced by cytochalasin B. (*A*) Current amplitude in response to a linear voltage ramp from −60 to +100 mV increases with time in response to a 90% gradient. Current amplitude increases faster in the presence of CB. Asterisks (*) in Panel *B* indicate the time each current trace shown in Panel *A* was recorded. Time course of VRChlC current amplitude at 100 mV with (filled symbols) and without (open symbols) 50 μM CB was determined in cells exposed to (*B*) 90% hyposmotic gradient (290/322 ± 10 mOsm: external/internal solution, n ≥ 8), (*C*) isosmotic solutions (290/280 ± 10 mOsm: external/internal solution, n ≥ 6), and (*D*) 75% hyposmotic gradient (290/390 ± 10 mOsm: external/internal solution, n ≥ 9). Note that the range of the Y-axis is different in each panel. Points of time course represent average of peak currents elicited at +100 mV, with n ≥ 3.

CB remained constant over a 5 to 20 min period (*not shown*). Exposing cells to a 90% hyposmotic solution alone decreases the F-actin integrity only slightly to 85 ± 2% of isosmotic control. Addition of CB in the presence of a 90% hyposmotic solution, however, results in a significant decrease in F-actin integrity, 34% ± 2% of that in the 90% control. There is an additive relationship between CB and the osmotic strength because cells exposed to a 75% hyposmotic solution decrease the F-actin integrity to a lesser extent, 69% ± 2% of that in 75% hyposmotic solution alone (Fig. 3). Moreover, as indicated above, when CB is added to cell in isosmotic solutions R-P fluorescence decreases to 45% ± 3% of control.

To determine whether F-actin disassembly in response to a hyposmotic environment initiates VRChlC, currents in myeloma cells were recorded after cells were treated with 50 μM CB for ≥20 min. Recording of Cl^- currents from myeloma cells was initiated by establishing a whole cell patch configuration. Exposure of the cells to a hypo- or isosmotic gradient is also initiated by establishing of the recording configuration. VRChlC is activated over several minutes in response to a hyposmotic gradient but not an isosmotic gradient (e.g., Fig. 4). Cell swelling in response to a hyposmotic gradient can be observed by eye, with the cell taking on a "fried-egg" look (as the cell membrane increases in diameter and separates from the nucleus) over time. The volume of Jurkat T-lymphocytes has been shown to increase in response to a hyposmotic gradient (Ross, Garber & Cahalan, 1994). Our experimental conditions allow the recording of VRChlC with minimal contamination from cation currents (*see* Materials and Methods). Therefore, activation of VRChlC is measured as an increase in outward current amplitude (net inward Cl^- flux) under hyposmotic conditions.

Table 1. Averaged rates for activation of VRChlC in the presence and absence of F-actin modulators (50 μM CB, 15 μM Ph)

Condition	Rate pA/(pF − min)	r^2	n
0.90	1.00	0.91	8
0.90 + CB	2.33	0.88	10
0.75	1.00	0.98	10
0.75 + CB	1.02	0.95	9
0.90	1.00	0.94	8
0.90 + Ph	0.17	0.97	10
0.75	1.00	0.98	8
0.75 + Ph	0.81	0.87	12

The rate of VRChlC activation increases in the presence of CB but decreases in the presence of Ph when a 90% osmotic gradient is used. No change is observed if a 75% gradient is used. Slopes were determined using a linear regression fit of data points shown in Fig. 4 and 7 from 0 to 10 min and normalized to control values (in the absence of agent) with the same osmotic gradient. Recordings were made on paired control and treated cells on the same day. Goodness of fit to the data is shown as regression coefficient, r^2. Number of cells is given as n. Control conditions contained vehicle used for solubilization of CB (0.05% DMSO) or Ph (1.0% MeOH).

Table 2. Averaged reversal potentials and voltage-dependent inactivation parameters for VRChlC in the presence and absence of F-actin modulators (50 μM CB, 15 μM Ph)

Condition	V_R, mV	$V_{1/2}$, mV	Slope
0.90	−57 ± 2 (42)	85 ± 2 (26)	10 ± 1 (26)
0.90 + CB	−55 ± 2 (56)	92 ± 2 (24)	11 ± 1 (24)
0.75	−54 ± 1 (49)	96 ± 5 (17)	10 ± 1 (17)
0.75 + CB	−59 ± 1 (19)	98 ± 2 (24)	10 ± 1 (24)
0.90	−53 ± 2 (41)	84 ± 2 (5)	11 ± 1 (5)
0.90 + Ph	−54 ± 2 (60)	88 ± 2 (3)	9 ± 1 (3)
0.75	−58 ± 5 (42)	86 ± 2 (5)	12 ± 1 (5)
0.75 + Ph	−57 ± 3 (45)	82 ± 2 (4)	9 ± 1 (4)

Reversal potentials (V_R) were determined using a linear extrapolation of two paired current/voltage points on either side of current reversal. Calculated reversal potentials compared favorably with reversal potentials determined from current in response to a continuous linear change in voltage from −60 to 100 mV over 500 msec. Voltage-dependent inactivation was calculated as a function of a modified h-infinity function: $R = (R_1 - R_2)/(1 + \exp[(V - V_{1/2})/k]) + R_2$ where $V_{1/2}$ is the midpoint of inactivation, k is the slope factor, R_1 is the inactivation ratio at 0 mV and R_2 is the minimum plateau value of the inactivation ratio ($I_{\text{steady-state}}/I_{\text{peak}}$) at voltages ≥100 mV. Numbers in parentheses indicate total number of data points taken for averages. Errors are given as SEM. For all values, $P > 0.05$, paired Student's t test.

Current recordings in the presence of CB but in the absence of an osmotic gradient showed no activation of VRChlC (Fig. 4*C*). This result suggested that F-actin disassembly alone is not sufficient to activate macroscopic current. The rate at which VRChlC develops, however, in response to a small (90%) osmotic gradient increases 2.3-fold in the presence of CB (Fig. 4*A* and *B*). Changes in the rates of VRChlC activation as a function of osmotic gradient are given in Table 1. The effect of CB disappears in the presence of a 75% hyposmotic gradient (Fig. 4*D;* Table 1). Reversal potential of current recorded in the presence and absence of CB were similar (Table 2), suggesting that there is no change in the ionic selectivity of the VRChlC current. Thus, the rate of current development can be modulated by F-actin disassembly only in response to a mild (e.g., 90% hyposmotic) osmotic stress. These results indicate that VRChlC activation does not require intact F-actin but that disassembly of F-actin may exert a synergistic effect on the rate of VRChlC activation in the presence of an hyposmotic gradient.

VRChlC show a characteristic voltage-dependent inactivation. Figure 5 shows that this inactivation occurs, under control conditions or in the presence of CB, with a midpoint of around +90 mV (Table 2). This voltage-dependent inactivation in myeloma cells may not be physiologically relevant because it occurs at very depolarized voltages. Inactivation of this current, however, provides a characteristic of the conductance that can be used as an indication of changes in the inherent activity of VRChlC in the presence and absence of CB. The voltage-dependence of inactivation of VRChlC is not significantly altered in the presence of CB (Table 2, Fig. 5), although the rate of change in VRChlC current amplitude increased faster in the presence of CB.

Modulation of F-actin Stabilization

Phalloidin (Ph) is a fungal toxin which stabilizes F-actin filaments. We can use Ph to determine if stabilizing F-actin decreases VRChlC activity, in contrast to the effect of CB. Ph is taken up into certain cells by pinocytosis (Wieland & Faulstich, 1978; Barak et al., 1980; Taylor & Wang, 1980; Cooper, 1987). Experiments incubating cells in a high Ph concentration (15 μM) were used to determine if our myeloma cell line takes up Ph. Live (not fixed) myeloma cells were incubated with unlabeled Ph for 20 min. This incubation was followed by an iso- or 50% hyposmotic challenge (5 min) before fixation and staining with fluorescent labeled R-P as described above. In these experiments, we used R-P as an indicator of the binding of unlabeled Ph to F-actin in intact cells. Binding of unlabeled Ph should decrease R-P fluorescence due to a reduction in the number of available binding sites. R-P fluorescence in cells preincubated with Ph and treated with an isosmotic solution was significantly decreased, compared to control cells that had not been incubated with Ph (Fig. 6: pixel intensity of treated cells was 38% ± 9 of control). The intensity of R-P fluorescence is similar in cells pretreated with Ph, regardless of incubation in iso- or hyposmotic solu-

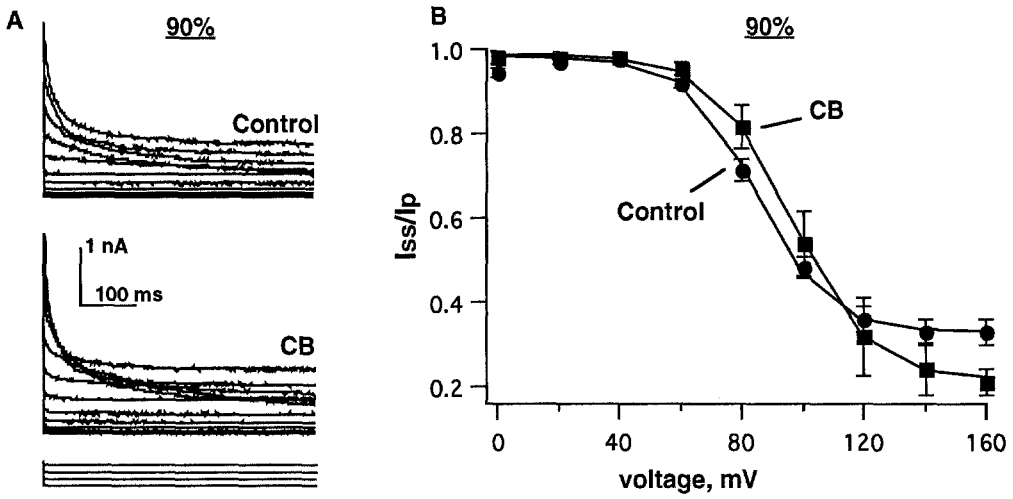


Fig. 5. Voltage-dependence of VRChlC is not altered by CB. (*A*) Current traces, recorded in the presence of a 90% osmotic gradient, are shown in response to a family of voltage pulses from −60 to + 160 mV (duration, 500 msec), from a holding potential of −60 mV. The time course of currents in control and 50 μM CB are similar. (*B*) Voltage-dependence of inactivation of VRChlC in a 90% hyposmotic gradient is similar in control (circles) and in the presence of CB (squares). Current inactivation was determined as a ratio of the current amplitude at steady state (I_{ss} at 475 ms after the initiation of a voltage step) over the instantaneous current amplitude (I_p at 5 msec after the initiation of a voltage step). The midpoints and slope factors were calculated from fit shown in solid line according to a modified h-infinity function as described in the legend of Table 2. Data points are averages of ⩾3 determinations, errors are given as SEM.

tions, however the pattern of R-P fluorescence is distinct in each condition (Fig. 6). This result indicates that externally applied Ph is taken up by myeloma cells, binds to, and stabilizes F-actin.

To determine the effect of F-actin stabilization on VRChlC activation, myeloma cells were incubated with 15 μM Ph for 20 to 60 min before initiation of whole cell current recording. The effect of Ph on VRChlC activation was in the opposite direction as that of CB (Fig. 7; Table 1). The rate at which VRChlC developed in response to a small (90%) hyposmotic osmotic gradient decreased 6.1-fold in the presence of Ph (Fig. 7*A* and *B;* Table 1). Like the effect of CB, the effect of Ph on the rate of current development disappears in the presence of a 75% hyposmotic gradient in 15 μM Ph (Fig. 7*C*). Ph, like CB, had no effect on the reversal potential or the voltage-dependence of VRChlC inactivation (Table 2).

Discussion

The major finding of this study is that F-actin, a major cytoskeletal element, modulates, but does not activate VRChlC in myeloma cells. Cytoskeletal modulation of VRChlC activity occurs only after the conductance has been activated and only in response to a mild osmotic stress. Modulation of VRChlC by F-actin appears to depend critically on the extent of the stimulus, which in this case is the osmotic gradient. The mild hyposmotic gradient we have used here is within normal physiological osmotic changes observed in the renal medullary circulation. The large hyposmotic gradient represents an extreme change.

There are many examples of modulatory shifts in biology. One example is that of facilitation of Ca^{++} channels (Artalejo, Adams & Fox, 1994). These Ca^{++} channels are silent when depolarized unless the appropriate stimulus, either strong predepolarization or increased intracellular cAMP, is given first. In analogy, VRChlC is "silent" in the absence of osmotic stimuli, even if F-actin is disassembled by CB, but activation is enhanced when F-actin integrity is disrupted by CB and mild stimulus is given. The role of F-actin integrity is reinforced by the opposite effects of CB and Ph on the activation of VRChlC. The modulatory role of F-actin on VRChlC activation is limited as indicated by the lack of effect in response to a strong stimulus.

Cytoskeletal Regulation of Ion Channels

Our results show that F-actin disassembly alone is not sufficient to activate macroscopic VRChlC activity in myeloma cells, in the absence of an osmotic gradient. In contrast, the activation of a large conductance (305 pS) volume-regulated anion channel in renal cortical collecting duct cells (Schwiebert, Mills & Stanton, 1994) and an "intermediate" conductance chloride channel in human skeletal muscle (Häussler et al., 1994) have been shown to be altered by agents which alter F-actin integrity in the absence of an osmotic gradient. Although single channel events are presumed to underlie macroscopic

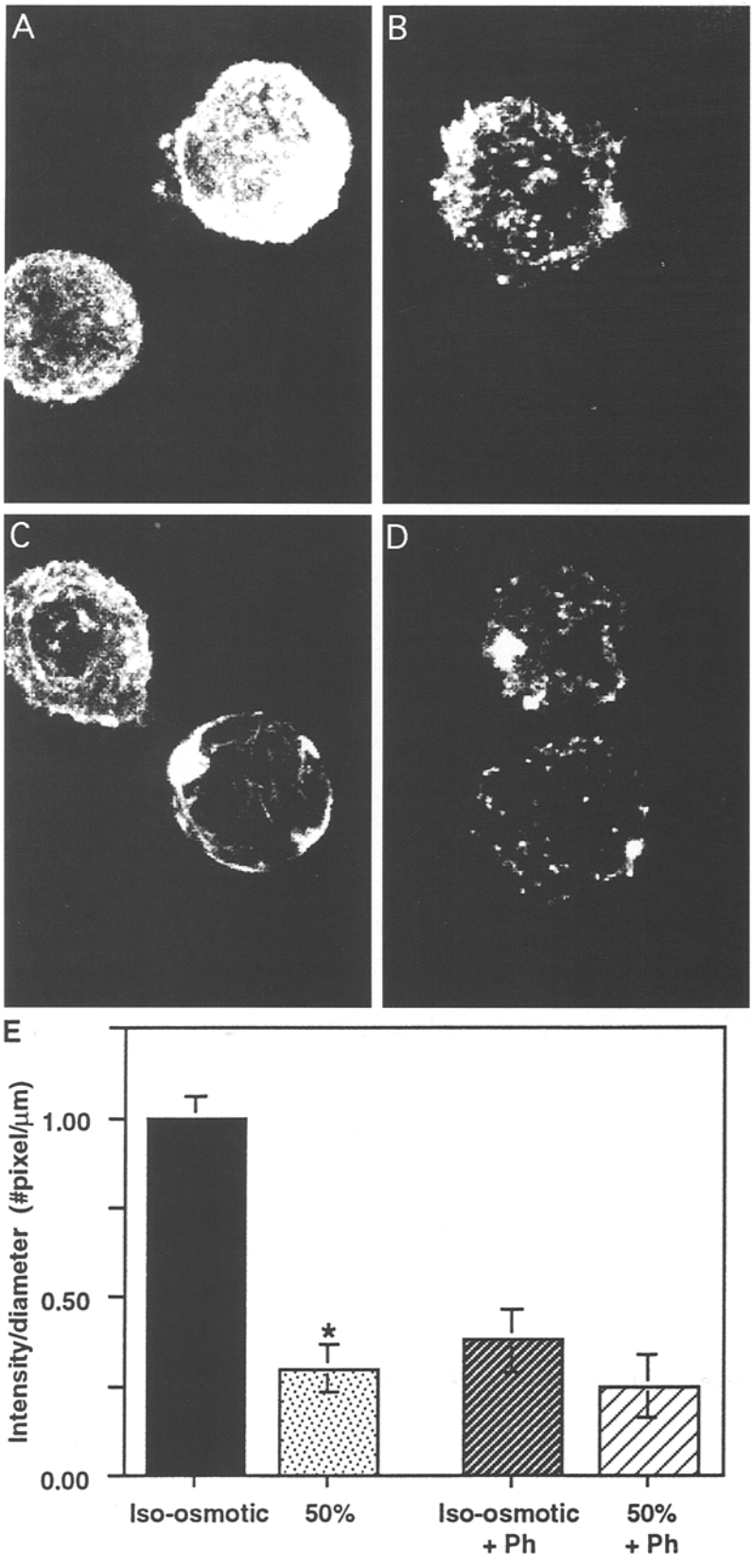


Fig. 6. External phalloidin is internalized. Cells were incubated for 20 min in (*A*) isosmotic solution (305 mOsm: external), (*B*) 50% hyposmotic solution (145 mOsm: external solution), (*C*) isosmotic solution + 15 μM Phalloidin, and (*D*) 50% hyposmotic solution + 15 μM Phalloidin. Cells were then fixed and stained with rhodamine-phalloidin. Under these conditions, unlabeled phalloidin blocks the binding of rhodamine-phalloidin as the unlabeled species entered the cell and is bound to F-actin, preventing the later binding of R-P. Panel (*E*) shows that a lower amount of fluorescence occurred, as expected, when cells are first incubated in external phalloidin. Average F-actin diameter (in μm) is isosmotic: 16.3 ± 0.2, 50% hyposmotic; 15.5 ± 0.2; isosmotic + 15 μM Phalloidin: 15.7 ± 0.2; 50% + 15 μM Phalloidin: 15.8 ± 0.2. n = 100 for each experimental condition. Bars indicate SEM and asterisks indicate a significance level of $P < 0.001$ (paired Student's *t*-test) compared to respective control.

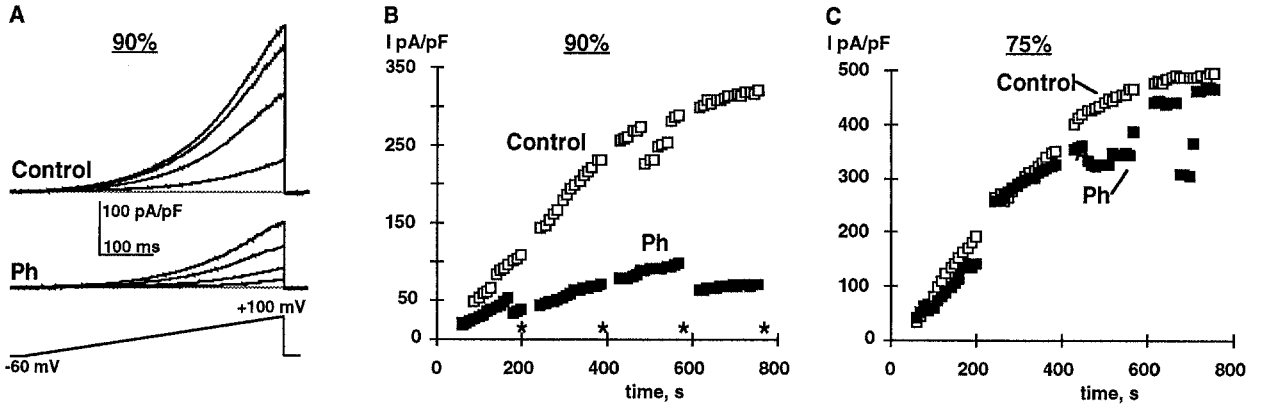


Fig. 7. VRChlC activation is inhibited by phalloidin. (*A*) Current amplitude in response to a linear voltage ramp −60 to +100 mV increases with time in response to a 90% gradient. Current amplitude increases faster in the presence of 15 μM external Ph. Asterisks (*) in Panel *B* indicate the time each current trace was recorded in Panel *A*. Time course of VRChlC current amplitude at 100 mV with (filled symbols) and without (open symbols) 15 μM Ph was determined in cells exposed to (*B*) 90% hyposmotic gradient (290/322 ± 10 mOsm: external/internal solution, n ≥ 8), (*C*) 75% hyposmotic gradient (290/390 ± 10 mOsm: external/internal solution, n ≥ 9). Note that the range of the Y-axis is different in each panel. Points of time course represent average of peak currents elicited at +100 mV, with n ≥ 3.

current, it is possible that a shift in the numbers of single channels that are open under isosmotic conditions in the presence of CB does not produce a significant change in the macroscopic VRChlC in myeloma cells. The single channel conductance underlying the macroscopic VRChlC recorded in lymphocytes is estimated to be ≤2 pS using stationary noise analysis (Lewis et al., 1993). This indicates that volume-regulated chloride channels in these cell lines may be distinct, although still members of a larger family of mechanosensitive ion channel proteins. It is therefore possible that the modulatory effects of cytoskeletal elements on the different Cl^- channels are similar yet distinct.

Oike et al. (1994) have shown that alteration of the cytoskeleton in endothelial cells does not regulate a volume-regulated chloride conductance. This chloride conductance in endothelial cells has similar characteristics to the VRChlC in myeloma epithelial cells, except that the voltage-dependent inactivation is much slower, taking place over hundreds of seconds (Nilius et al., 1994). These experiments, however, used a strong hyposmotic gradient (>60%). Results presented here suggest that this gradient may be too large to observe a modulatory effect of F-actin on VRChlC and that modulation of VRChlC in endothelial cells may be observed if a milder gradient is used.

The activity of other ion channels (e.g., amiloride-sensitive Na^+ channel) and transport proteins (e.g., Na^+/K^+ transporter) have been linked to cytoskeletal proteins such as ankyrin and spectrin. Activity of certain Ca^{++} and NMDA receptor channels also appears to be stabilized by F-actin (Johnson & Byerly, 1993; Rosenmond & Westbrook, 1993). Another cytoskeletal protein, filamin, an actin binding protein, is also required for the activation of a volume-regulated K^+ channel in a melanoma cell line (Cantiello et al., 1993). These proteins, in addition to many others, are found between the cellular membrane and cortical F-actin scaffolding and may provide a more direct link between integral membrane proteins, such as ion channels, and the cortical F-actin cytoskeleton. Thus, the modulatory effect of F-actin integrity on VRChlC activation may not be a direct result of changes in F-actin alone, but rather to changes, involving several proteins, in the overall cytoskeletal architecture of the cell.

Modulation of VRChlC by Cytoskeleton

VRChlC are part of a large functional family of ion channels that respond to membrane stress by activating or increasing macroscopic current amplitude. For example, in yeast spheroblasts, the activation of mechanosensitive cation channels is a function of membrane tension rather than pressure on the membrane (*reviewed* by Sackin, 1994). Membrane tension is a force which is distributed within the plane of the membrane, whereas pressure is orthogonal to the membrane. Thus, VRChlC may be activated directly by membrane tension, for example, or indirectly via the cytoskeleton.

One way to control or modulate membrane tension may be through the interaction of cytoskeletal proteins linked to integral membrane proteins. Intact cytoskeleton-protein linkages, however, would prevent a change in membrane tension, thereby decreasing VRChlC activation. Disassembly of the cytoskeleton and its linkages to integral membrane proteins, such as VRChlC, would allow the increase of membrane tension with a corresponding increase in VRChlC activation. Indeed, Kleinzeller and Ziyadeh (1990) have proposed a model in which linkages between the membrane and cytoskeleton are disrupted as a result of massive swelling.

Using such a physical mechanism, the results of this study can be interpreted as follows: VRChlC in myeloma cells requires membrane tension for activation. Sufficient membrane tension is developed in the presence of a hyposmotic gradient which causes the myeloma cell to swell. F-actin disassembly alone is insufficient to trigger activation of VRChlC because the required membrane tension is not developed. Once the cell begins to swell, membrane tension will increase thus activating additional ion channels underlying VRChlC. Cl^- ions move out of the cell because the resting potential is less than Cl^- equilibrium potential. The disassembly of F-actin allows membrane tension to increase faster thereby increasing the rate of VRChlC activation. Alternatively, if F-actin is stabilized, membrane tension does not develop as quickly, and the rate of VRChlC activation is relatively slower. Once VRChlC activation is proceeding at a maximum rate (e.g., under a 75% hyposmotic gradient) F-actin integrity no longer influences VRChlC activity, because such a large hyposmotic gradient results in the physical dissociation of F-actin and ion channel proteins in the cellular membrane, enabling VRChlC activation to proceed at a maximal rate. Cell swelling results in an increase in the membrane diameter measured in Jurkat cells (Ross et al., 1994) and is suggested in our results from the "fried-egg" look of very swollen cells. F-actin diameter, however, does not appear to increase under hyposmotic stress (this report). These observations suggest that cortical F-actin network, including associated cytoskeletal proteins, and the membrane will dissociate as cell swelling increases.

Disruption or stabilization of the cortical F-actin structure may also mediate VRChlC activity via an intracellular messenger. Intracellular $[Ca^{++}]$ is known to increase on initiation of cell swelling in certain cell types (Hoffmann & Simonsen, 1989). Intracellular $[Ca^{++}]$ in lymphocytes, however, does not appear to be involved in the volume regulatory response (Rink et al., 1983). Although VRChlC activity is independent of $[Ca^{++}]$, ATP is required to maintain the activity during prolonged whole cell recording (Lewis et al., 1993; Ross et al., 1994). Addition of GTP to the intracellular recording solution is not required for activity (*see* Materials and Methods), suggesting that a G-protein mediated mechanism is not involved. Together, these observations suggest that second messengers are not involved, although the effects of fatty acids and metabolites (e.g., arachidonic acid), which have been implicated in volume regulatory responses in other systems (Hoffmann & Simonsen, 1989), have not yet been tested in myeloma cells.

Implications for Volume Regulations

Disassembly of F-actin is apparent in cells isolated from shark rectal gland and in leukemic cells in response to acute hyposmotic stress (Ziyadeh, Mills & Kleinzeller, 1992). The disassembly of F-actin in shark rectal gland recovers gradually as the cell completes RVD. RVD can be inhibited by disrupting F-actin using CB, which results in the disassembly of F-actin (Cornet et al., 1993; Foskett & Spring, 1985; Gilles et al., 1986). The rate of recovery from swelling is much slower than normal in the presence of cytochalasin B or D.

The results presented here show that F-actin integrity in myeloma cells, like that from cells of other origin (Cornet et al., 1993; Foskett & Spring, 1985; Gilles et al., 1986; Ziyadeh et al., 1992), changes in response to a hyposmotic challenge. Cortical F-actin in myeloma cells decreases when the ionic and osmotic strength of the external solution is reduced. This change in F-actin integrity can be prevented by maintaining the osmotic strength of the external solution. F-actin integrity can also be restored by returning cells to an external medium of normal osmotic strength. The observation that F-actin disassembly alone does not activate VRChlC under isosmotic conditions suggests that the cytoskeleton and chloride conductance contribute to regulated volume control in myeloma cells. Other experiments suggest that these volume changes occur with a similar time course as cell swelling (Levitan and Garber, *unpublished*). F-actin disassembly is not essential to activation of VRChlC, but can potentiate VRChlC activity. In this manner, F-actin integrity may influence chloride-dependent volume regulation.

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References

Ackerman, M.J., Wickman, K.D., Claphman, D.E. 1994. Hypotonicity activates a native chloride current in *Xenopus* oocytes. *J. Gen. Physiol.* **103:**153–179

Artalejo, C.R., Adams, M.E., Fox, A.P. 1994. Three types of Ca^{2+} channel trigger secretion with different efficacies in chromaffin cells. *Nature.* **367:**72–76

Barak, L.S., Yom, R.R., Nothnagel, E.A., Webb, W.W. 1980. Fluorescence staining of the actin cytoskeleton with 7-nitrobenz-2-oxa-1,3-diazole-phallacidin. *Proc. Natl. Acad. Sci. USA* **77:**980–984

Cahalan, M.D., Lewis, R.S. 1988. Role of potassium and chloride channels in volume regulation by T lymphocytes. *Soc. Gen. Physiol. Series* **43:**281–301

Cantiello, H.F., Prat, A.G., Bonventre, J.V., Cunningham, C.C., Hartwig, J.H., Ausiello, D.A. 1993. Actin-binding protein contributes to cell volume regulatory ion channel activation in melanoma cells. *J. Biol. Chem.* **268:**4596–4609

Chan, H.C., Fu, W.O., Chung, Y.W., Huang, S.J., Zhou, T.S., Wong, P. 1993. Characterization of a swelling-induced chloride conductance in cultured rat epididymal cells. *Am. J. Physiol.* **265:**C997–C1005

Cooper, J.A. 1987. Effects of cytocholasin and phalloidin on actin. *J. Cell Biol.* **105:**1473–1478

Cornet, M., Ubl, J., Kolb, H.-A. 1993. Cytoskeleton and ion move-

ments during volume regulation in cultured PC12 cells. *J. Membrane Biol.* **133:**161–170

DeCoursey, T.E., Chandy, K.G., Gutpa, S., Cahalan, M.D. 1985. Voltage-dependent ion channels in T-lymphocytes. *J. Neuroimmunol.* **10:**71–95

Deutsch, C., Lee, S.C. 1988. Cell volume regulation in lymphocytes. *Renal Physiol. Biochem.* **3–5:**260–276

Diaz, M., Valverde, M.A., Higgins, C.F., Rucareanu, C., Sepulveda, F.V. 1993. Volume-activated chloride channels in HeLa cells are blocked by verapamil and dideoxyforskolin. *Pfluegers Arch.* **422:**347–353

Doroshenko, P., Neher, E. 1992. Volume-sensitive chloride conductance in bovine chromaffin cell membrane. *J. Physiol.* **449:**197–218

Foskett, J.K., Spring, K.R. 1985. Involvement of calcium and cytoskeleton in gallbladder epithelial cell volume regulation. *Am. J. Physiol.* **248:**C27–C36

Gilles, R., Delpire, E., Duchene, C., Cornet, M., Pequeux, A. 1986. The effect of cytocholasin B on the volume regulation response of isolated axons of the green crab *Carcinus maenas* submitted to hypoosmotic media. *Comp. Biochem. Physiol.* **85A:**523–525

Grinstein, S., Rothstein, A., Sarkadi, B., Gelfand, E.W. 1984. Responses of lymphocytes to anisotonic media: volume regulating behavior. *Am. J. Physiol.* **246:**C204–C215

Hamill, O.P., Marty, A., Neher, E., Sakmann, B., Sigworth, F.J. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pfluegers Arch.* **391:**85–100

Häussler, U., Rivet-Bastide, M., Fahlke, C., Müller, D., Zacher, E., Rüdel, R. 1994. Role of the cytoskeleton in the regulation of Cl^- channels in human embryonic skeletal muscle cells. *Pfluegers Arch.* **428:**323–330

Hoffman, E.K. 1992. Cell swelling and volume regulation. *Canadian J. Physiol. Pharm.* **70:**S310–313

Hoffman, E.K., Simonsen, L.O. 1989. Membrane Mechanisms in Volume and pH regulation in vertebrate cells. *Physiol. Rev.* **69:**315–382

Johnson, B.D., Byerly, L. 1993. A cytoskeletal mechanism for Ca^{2+} channel metabolic dependence and inactivation by intracellular Ca^{2+}. *Neuron* **10:**797–804

Kelly, M.E.M., Dixon, S.J., Sims, S.M. 1994. Outwardly rectifying chloride current in rabbit osteoclasts is activated by hyposmotic stimulation. *J. Physiol.* **475:**377–389

Kleinzeller, A., Ziyadeh, F.N. 1990. Cell volume regulation in epithelia with emphasis on the role of osmolytes and the cytoskeleton. *In:* Cell Volume Regulation. K.W. Beyenbach, editor. pp. 59–86. Karger, Basel

Kubo, M., Okada, Y. 1992. Volume-regulatory Cl^- channel currents in cultured human epithelial cells. *J. Physiol.* **456:**351–371

Kunzelman, K., Pavenstadt, H., Greger, R. 1989. Properties and regulations of chloride channels in cystic fibrosis and normal airway cells. *Pfluegers Arch.* **415:**172–182

Law, R.O. 1991. Amino acids as volume-regulatory osmolytes in mammalian cells. *Comp. Biochem. Physiol.* **99A:**263–277

Lewis, R.S., Ross, P.E., Cahalan, M.D. 1993. Chloride channels activated by osmotic stress in T lymphocytes. *J. Gen. Physiol.* **101:**801–826

McCann, J.D., Li, M., Welsh, M.J. 1989. Identification and Regulation of Whole-Cell Chloride Currents in Airway Epithelium. *J. Gen. Physiol.* **94:**1015–1036

McDonald, T.V., Nghiem, P.T., Gardner, P., Martens, C.L. 1992. Human lymphocytes transcribe the cystic fibrosis transmembrane conductance regulator gene and exhibit CF-defective cAMP-regulated chloride current. *J. Biol. Chem.* **267:**3242–3248

McManus, M.L., Churchwell, K.B. 1994. Clinical significance of cellular osmoregulation. *In:* Cellular and Molecular Physiology of Cell Volume Regulation. K. Strange editor. pp. 63–80. CRC Press, Boca Raton

Mills, J.W., Schwiebert, E.M., Stanton, B.A. 1994. The cytoskeleton and cell volume regulation. *In:* Cellular and Molecular Physiology of Cell Volume Regulation. K. Strange editor. pp. 241–258. CRC Press, Boca Raton

Nilius, B., Oike, M., Zahradnik, I., Droogmans, G. 1994. Activation of a Cl^- current by hypotonic volume increase in human endothelial cells. *J. Gen. Physiol.* **103:**787–805

Oike, M., Schwarz, G., Sehrer, J., Jost, M., Gerke, V., Weber, K., Droogmans, G., Nilius, B. 1994. Cytoskeletal modulation of the response to mechanical stimulation in human vascular endothelial cells. *Pfluegers Arch.* **428:**569–576

Rink, R.J., Sanchez, A., Grinstein, S., Rothstein, A. 1983. Volume restoration in osmotically swollen lymphocytes does not involve changes in free Ca^{2+} concentration. *Biochem. Biophys. Acta.* **762:**593–596

Rosenmond, C., Westbrook, G.L. 1993. Calcium-induced actin depolymerization reduced NMDA channel activity. *Neuron* **10:**805–814

Ross, P.R., Garber, S.S., Cahalan, M.C. 1994. Membrane chloride conductance and capacitance in Jurkat T lymphocytes during osmotic swelling. *Biophys. J.* **66:**169–178

Sackin, H. 1994. Stretch-activated ion channels. *In:* Cellular and Molecular Physiology of Cell Volume Regulation. K. Strange editor pp. 215–240. CRC Press, Boca Raton

Schwiebert, E.M., Mills, J.W., Stanton, B.A. 1994. Actin-based cytoskeleton regulates a chloride channel and cell volume in a renal cortical collecting duct cell line. *J. Biol. Chem.* **269:**7081–7089

Solc, C.K., Wine, J.J. 1991. Swelling-induced and depolarization-induced Cl^- channels in normal and cystic fibrosis epithelial cells. *Am. J. Physiol.* **261:**C658–C674

Sorota, S. 1992. Swelling-induced chloride-sensitive current in canine atrial cells revealed by whole-cell path-clamp method. *Circ. Res.* **70:**679–687

Stoddard, J.S., Steinbach, J.H., Simchowitz, L. 1993. Whole cell Cl^- currents in human neutrophils induced by cell swelling. *Am. J. Physiol.* **265:**C156–C165

Taylor, D.L., Wang, Y.-L. 1980. Fluorescently labels molecules as probes of the structure and function of living cells. *Nature* **284:**405–410

Tzeng, G.-N. 1992. Cell swelling increases membrane conductance of canine cardiac cells: evidence for a volume-sensitive Cl channel. *Am. J. Physiol.* **262:**C1056–C1068

Wieland, T., Faulstich, H. 1978. Amatoxins, phallotxins, phallolysin and antamanide: the biologically active components of poisonous Amanita mushrooms. *Crit. Rev. Biochem.* **5:**185–260

Worrell, R.T., Butt, A.G., Cliff, W.H., Frizzell, R.A. 1989. A volume-sensitive chloride conductance in human colonic cell line T84. *Am. J. Physiol.* **256:**C1111–C1119

Yantorno, R.E., Carre, D.A., Coca-Prados, M. Krupin, T., Civan, M.M. 1992. Whole cell patch clamping of ciliary epithelial cells during anisosmotic swelling. *Am. J. Physiol.* **262:**C501–C509

Zhang, J., Rasmusson, R.L., Hall, S.K., Lieberman, M. 1993. A chloride current associated with swelling of cultured chick heart cells. *J. Physiol.* **472:**801–820

Ziyadeh, F.N., Mills, J.W., Kleinzeller, A. 1992. Hypotonicity and cell volume regulation in shark rectal gland: role of organic osmolytes and F-actin. *Am. J. Physiol.* **262:**F468–F479