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Relation between Cytoskeleton, Hypo-Osmotic Treatment and Volume Regulation in Ehrlich Ascites Tumor Cells

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Summary. Pretreatment with cytochalasin B, which is known to disrupt microfilaments, significantly inhibits regulatory volume decrease (RVD) in Ehrlich ascites tumor cells, suggesting that an intact microfilament network is a prerequisite for a normal RVD response. Colchicine, which is known to disrupt microtubules, has no significant effect on RVD. Ehrlich cells have a cortical three-dimensional, orthogonal F-actin filament network which makes the cells look completely black in light microscopy following immunogold/silver staining using anti-actin antibodies. After addition of cytochalasin B, the stained cells get lighter with black dots localized to the plasma membrane and appearance of multiple knobby protrusions at cell periphery. Also, a significant decrease in the staining of the cells is seen after 15 min of RVD in hypotonic medium. This microfilament reorganization appears during RVD in the presence of external Ca^{2+} or Ca^{2+}-ionophore A23187. It is, however, abolished in the absence of extracellular calcium, with or without prior depletion of intracellular Ca^{2+} stores. An effect of increased calcium influx might therefore be considered. The microfilament reorganization during RVD is abolished by the calmodulin antagonists pimozide and trifluoperazine, suggesting the involvement of calmodulin in the process. The microfilament reorganization is also prevented by addition of quinine. This quinine inhibition is overcome by addition of the K^+ ionophore valinomycin.

Key Words volume regulation · Ehrlich ascites tumor cells · microfilament · Ca^{2+} and Ca^{2+}-ionophore A23187 · quinine · calmodulin

Introduction

The mechanisms by which cells sense osmotically induced changes in turgor pressure, volume and/or intracellular composition, and transduce those signals into a regulatory response is only partly understood. Several factors have been assigned to a regulatory function, e.g., Ca^{2+}, calmodulin, cAMP, eicosanoids, polyphosphoinositide metabolism, protein kinases, and the microfilament network (*see* Hoffmann, Simonsen & Lambert, 1993).

In isolated membrane vesicles from lymphocytes (rabbit thymocytes), the expected increase in K^+ permeability in response to either cytoplasmic Ca^{2+} increase or hypotonic swelling was absent, suggesting that an intact cytoarchitecture is required for the RVD response (Grinstein, Du Pre & Rothstein, 1982). That some contractile or elastic structure could play a part in volume control under isosmotic conditions was already suggested more than twenty years ago by Kleinzeller (1965, 1972). Moreover, cytochalasin B, which is known to disrupt microfilaments, inhibited RVD in *Necturus* gallbladder epithelial cells (Foskett & Spring, 1985), in isolated axons of the green crab *Carcinus maenas* (Gilles et al., 1986) and in mouse peritoneal macrophages (Galkin & Khodorov, 1988). Previous studies also demonstrated that cytochalasin B strongly affects the volume regulation processes of PC12 pheochromocytoma cells and T2 fibrosarcoma cells. In addition, hypo-osmotic conditions induce a complete reorganization of the microfilament network of these cells (Cornet, Delpire & Gilles, 1987, 1988; Cornet, Isobe & Lemanski, 1992). Osmotically induced changes in F-actin organization were later confirmed in shark rectal gland (Ziyadeh, Mills & Kleinzeller, 1992). A possible role for microtubules and microfilaments during volume regulation has also been suggested in cultured rat pheochromocytoma cells and in Ehrlich ascites cells (Delpire et al., 1985).

Mills and Skiest (1985) and Mills and Lubin (1986) have analyzed the role of the cytoskeleton in cell volume control using MDCK cells. They suggested that the state of organization of F-actin, which can be influenced by changes in the levels of cAMP, could have an effect on membrane elements

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that play a role in volume control processes. Stretch-activated channels activated also after cell swelling have recently been demonstrated in different cell types, including Ehrlich cells (Christensen & Hoffmann, 1992) or PC12 cells (Cornet, Ubl & Kolb, 1993), and coupling of the cytoskeleton with stretch-activated channels has been proposed by Sachs (1987). Changes in the organization of F-actin induced by hypotonic stress have been demonstrated in a cultured mammalian cell (Cornet et al., 1987, 1992).

In the present investigation we demonstrate that cytochalasin B also inhibits volume regulation in Ehrlich ascites tumor cells after transfer to hypotonic medium, and that a significant reorganization of actin occurs during RVD in Ehrlich cells. The changes in microfilament organization are caused by an increase in Ca^{2+} resulting from an increased Ca^{2+} influx. Calmodulin seems to be involved in microfilament reorganization.

Abbreviations

BSA: bovine serum albumin; DMSO: dimethylsulfoxyde; EGTA: ethylene-glycol-bis-(β-amino-ethyl-ether)N,N,N′,N′-tetraacetic acid; GAR-G5: goat anti-rabbit IgG coupled to 5-nm gold particles; HEPES: N-2-hydroxy-ethyl-piperazine-N′-2-ethane sulfonic acid; IBMX: isobutyl-methyl-xanthine; NGS: normal goat serum; MOPS: 3-(N-morpholino-propane)sulfonic acid; PBS: phosphate-buffered saline; PIPES: 1,4-piperazine-bis-(ethane sulfonic acid); TBS: tris-buffered saline; TES: N-tris(hydroxymethyl)methyl-2-amino-ethanesulfonic acid; Tris: tris(hydroxymethyl)aminomethane.

Materials and Methods

Cell Culture and Incubation Media

Ehrlich ascites tumor cells (hyperdiploid strain) were maintained in white female Theiller mice by weekly intraperitoneal transplantation and harvested eight days after transplantation. They were then suspended in standard incubation solution containing heparin (25 IU/ml) and washed twice by centrifugation (700 × g; 45 sec) with standard saline solution prior to a 40-min preincubation. For immunocytochemical visualization, washed Ehrlich ascites tumor cells were fixed for 30 min in standard saline medium on glass coverslips, previously coated with poly-α-ornithine (0.1 mg/ml H_2O, 30 min), and then washed three times with standard saline solution.

The standard saline solution (300 mOsm) had the following composition (in mM): Na^+: 150; K^+: 5; Ca^{2+}: 1; Mg^{2+}: 1; Cl^-: 150; SO_4^{2-}: 1; inorganic phosphate: 1; MOPS: 3.3; TES: 3.3 and HEPES: 5; pH: 7.4. Hypotonic medium (150 mOsm) was prepared by 1:1 dilution of the standard medium with distilled water containing buffers in the same concentration as in the standard saline solution. NMDG medium was prepared by substituting Na^+ with N-methyl-D-glucammonium$^+$ while $NaNO_3$ medium was prepared by substituting the Na^+ and the K^+ salts of NO_3^- for NaCl and KCl.

Reagents

Ionophore A23187, quinine hydrochloride, trifluoperazine, IBMX, EGTA, quinine hydrochloride (Sigma Chemical), colchicine (Merck, FRG), trifluoperazine (Lundbeck, Denmark) and pimozide (Janssen Biochemica, Denmark) were added to the salines from stock solutions in absolute ethanol. EGTA was added from a stock solution adjusted to pH 7.2 with Tris base. Cytochalasin B was dissolved in DMSO prior to its addition to the salines. The final concentration of DMSO in salines never exceeded 0.1% at which concentration, DMSO had no effect on the cytoskeletal organization nor on the osmotic behavior of the cells. Colchicine and cytochalasin B were added to isosmotic salines for a 30-min preincubation before hypo-osmotic treatment.

Cell Volume Measurements

Volume measurements of Ehrlich ascites tumor cells were carried out by electronic cell sizing using a Coulter counter model ZB equipped with a Coulter Channellyzer (C-1000). The tube orifice diameter was 100 μm. An aliquot of the cell suspension was diluted 500-fold with filtered experimental medium (Millipore, poresize 0.45 μm) to give a final cell density of 85,000 to 95,000 cells per ml (equivalent to a cytocrit of about 0.03%). The mean cell volume (arbitrary units) was calculated as the median of the cell volume distribution curves. Absolute cell volumes were obtained using polystyrene latex beads (12.9 μm diameter, Coulter Electronics) as standards.

Immunocytochemistry

Cytoplasmic microtubule and microfilament networks were immunocytochemically stained for light microscopy following the Immunogold/Silver Staining method (IgSS) (De Mey et al., 1981). Cells were fixed and simultaneously permeabilized for 15 min in 0.5% glutaraldehyde plus 0.2% Triton X-100 in PIPES buffer (PIPES: 65 mM; HEPES: 25 mM; EGTA: 10 mM; $MgCl_2$: 2 mM; pH 6.9). Samples were washed three times in PIPES buffer, further permeabilized in 0.5% Triton X-100 in PIPES buffer for 30 min and treated two times 10 min each with $NaBH_4$ (1 mg/ml PIPES buffer). They were then washed again for two times 5 min each in PIPES buffer and immersed in TBS (Tris: 20 mM; NaCl: 154 mM; NaN_3: 20 mM; pH 8.2). Specimens were incubated in 5% NGS in TBS plus 0.1% BSA for 30 min prior to immunolabeling; 70 μl of rabbit polyclonal anti-tubulin or anti-actin antibodies (Bio-Yeda, Israel) at a dilution 1:20 in 0.2% NGS in TBS plus 0.1% BSA were applied for 2½ hr. Controls were incubated in same buffer without antibody. Cells were then washed in TBS + 0.1% BSA three times 10 min each, and incubated overnight in a 1:10 dilution of GAR-G5 (Amersham Int., UK) in TBS plus 0.1% BSA. Samples were washed again three times 10 min each as before, and finally rinsed two times 5 min each in PBS (NaCl: 140 mM; KCl: 2.7 mM; KH_2PO_4: 1.5 mM; Na_2HPO_4: 16.3 mM; pH 7.4). Postfixation was done in 0.5% glutaraldehyde in PBS for 15 min. Cells were rinsed again in PBS two times 3 min each and in distilled water, three times 3 min each. Silver enhancement was then carried out with an "IntenSE Silver Enhancement kit" (Am-

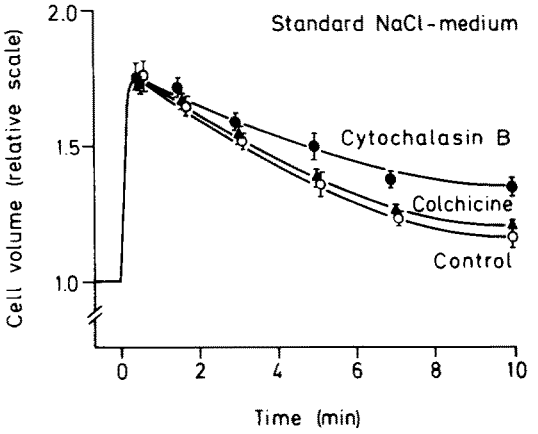

Fig. 1. Cell volume of Ehrlich ascites tumor cells after transfer to hypotonic NaCl medium (○) or to hypotonic NaCl medium in presence of 50 μM cytochalasin B (●) or 0.1 mM colchicine (▲). Cells suspended in isotonic standard NaCl medium were treated with the drugs for 30 min before transfer to hypotonic medium. The cell volume was followed with time in a Coulter counter cell sizing system and given relative to the cell volume measured in isotonic NaCl medium. Results are shown as the mean of six separate experiments ±SEM.

Table 1. Effect of cytochalasin B (50 μM) on the initial volume recovery following swelling in hypotonic $NaCl^-$, $NMDGCl^-$, and $NaNO_3$-medium*

Medium	Initial rate of volume recovery (relative to control)	*n*
NaCl	0.76 ± 0.11	5
NMDGCl	0.76 ± 0.04	2
$NaNO_3$	0.67 ± 0.10	5

* Experimental protocol as in Fig. 2. The initial rate of RVD (fl/min) was obtained from lines fitted to four to six values taken within 1 and 4 min after shift in osmolarity. The rates are given relative to controls without cytochalasin. Values are ± SEM.

ersham). Samples were finally dehydrated and mounted for light microscopy.

Results

Figure 1 shows that cytochalasin B (50 μM), which is known to disrupt the microfilaments (*see* Stossel, 1989), decreases the rate of RVD in Ehrlich ascites tumor cells. On the other hand, colchicine (0.1 mM), a drug well known to disrupt microtubules in numerous cells (Borisy & Taylor, 1967), only causes a slight insignificant inhibition of the volume regulatory process in Ehrlich cells (Fig. 1).

From Fig. 2 and Table 1, it is seen that removal of Na^+ or substitution of all intra and extracellular

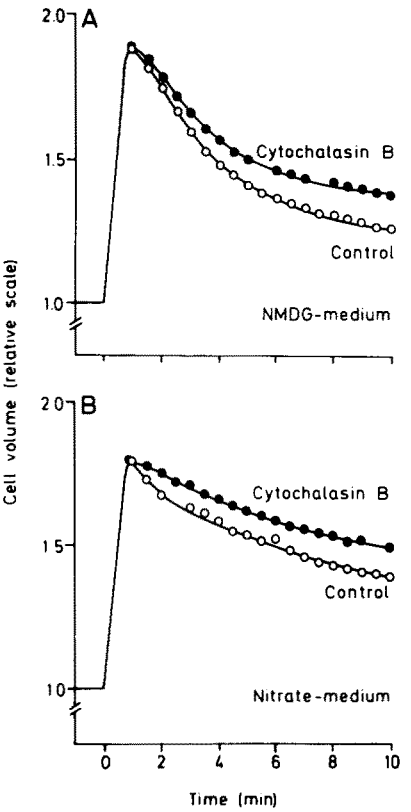

Fig. 2. Inhibition by cytochalasin B of regulatory volume decrease in Ehrlich cells after swelling in hypotonic NMDG medium (*A*) and in hypotonic $NaNO_3$ medium (*B*). Cells, preincubated in isotonic standard NaCl medium or in isotonic $NaNO_3$ medium for 30 min or more, were at time zero diluted with hypotonic NMDG medium (*A*) or hypotonic $NaNO_3$ medium (*B*), respectively. In the case of NO_3 cells, an additional wash of cells was performed after 15-min preincubation in order to remove excess Cl^-. In the case of cytochalasin B, the cells were treated for 3 min with 50 μM before the hypotonic shock. The cell volume was measured and shown as described in Fig. 1. The figure is representative of three independent experiments.

Cl^- with NO_3 does not affect the inhibition of RVD provoked by cytochalasin B. It is well known that KCl-cotransport as well as Na,K,2Cl-cotransport are both strictly Cl^- dependent and do not transport NO_3 (Hoffmann, Lambert & Simonsen, 1986). Thus, the inhibition of RVD by cytochalasin B is on the activation of the K^+ and the Cl^- channels. Figure 2 also demonstrates that 3-min preincubation with cytochalasin B is sufficient to result in inhibition of RVD. The inhibition after 3-min preincubation with 50 μM cytochalasin B is estimated at 24% in hypotonic Na^+-free NMDG medium, 33% in hypotonic Cl^--free $NaNO_3$ medium and 24% in hypotonic NaCl medium (*see* Table 1) compared to the 34% found in hypotonic NaCl medium after 30-min preincubation with 50 μM cytochalasin B (Fig. 1).

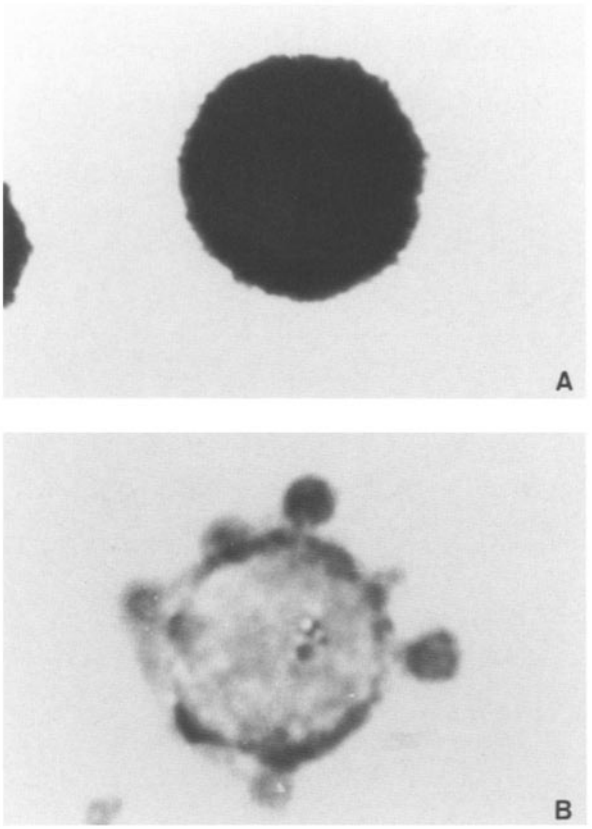

Fig. 3. Microfilament network, stained by the IgSS method using anti-actin antibodies of Ehrlich ascites tumor cells (*A*) maintained in isosmotic conditions (300 mOsm) and (*B*) incubated during 30 min in an isosmotic saline containing 50 μM cytochalasin B. (×2500).

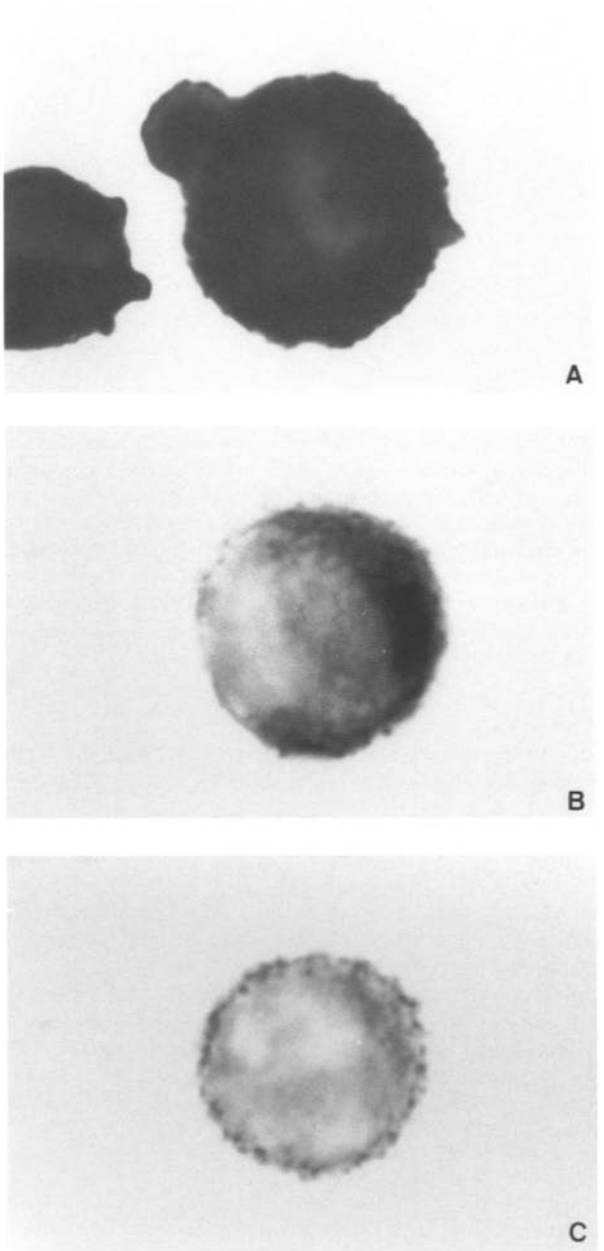

Fig. 4. Microfilament network, stained by the IgSS method using anti-actin antibodies of Ehrlich ascites tumor cells submitted to hypotonic NaCl medium (150 mOsm) for 1 min (*A*); 5 min (*B*) or 15 min (*C*). (×2500).

In Ehrlich ascites tumor cells, microfilaments, as seen by the IgSS method using anti-actin antibodies, appear as a dense network distributed in the whole cytoplasm (Fig. 3*A*). In the presence of cytochalasin B a dramatic change can be observed in the microfilament organization (Fig. 3*B*). The cytoplasmic staining is greatly reduced, but distinct patches of actin remain at the cell periphery, as well as in the numerous blebs induced by the drug. With 0.1 mM colchicine, a complete disruption of the microtubule network is observed within 30 min, as seen by the IgSS method using anti-tubulin antibodies (*data not shown*).

Reduction of the extracellular osmolarity from standard 300 mOsm to 150 mOsm first leads to a rapid and transient formation of one or a few blebs which gradually disappear within 5 min (Fig. 4 *A* and *B*). In the period 1 to 15 min after transfer to hypotonic medium, a dramatic and progressive change is observed in the microfilament network organization (Fig. 4 *A–C*). After 15 min, the cytoplasm of the Ehrlich ascites cells appears poorly stained with patches of actin present essentially close to the plasma membrane (Fig. 4*C*). These osmotically-induced changes in the organization of the actin network are slightly different from those produced by cytochalasin B (*compare* Fig. 3*B* with Fig. 4*C*).

In contrast, application of hypo-osmotic shocks

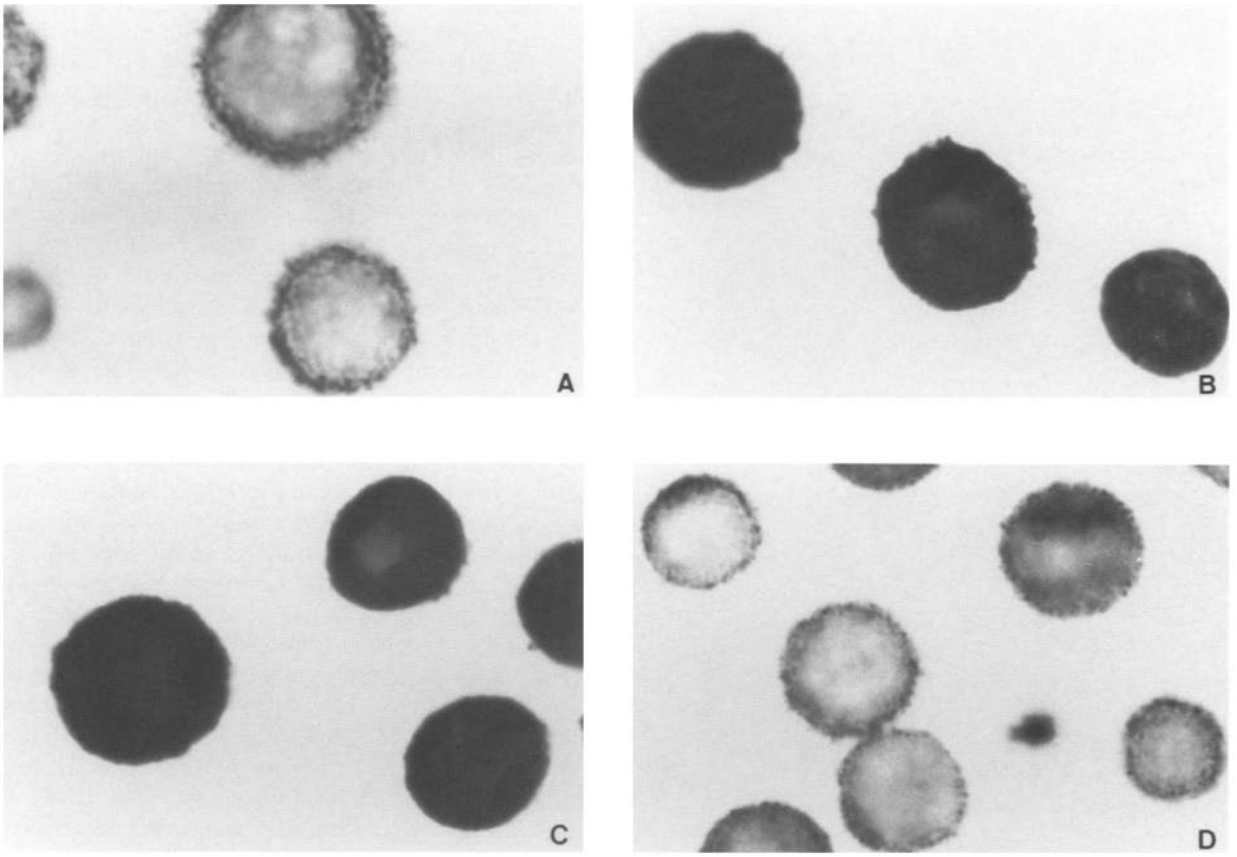

Fig. 5. Microfilament network, stained by the IgSS method, of Ehrlich ascites tumor cells (*A*) incubated during 15 min in control hypo-osmotic conditions; (*B*) incubated during 15 min in a hypo-osmotic calcium-free medium in the presence of 0.5 mM EGTA. (*C* and *D*) Before staining, and in order to deplete the internal stores in calcium, cells have been submitted to a prolonged preincubation in an isosmotic calcium-free medium in the presence of EGTA (0.5 mM) for 15 min and then treated with calcium ionophore A23187 (2 μM) for 15 min and incubated again for 1 min in the presence of EGTA. After such prolonged preincubation, Ehrlich cells have been incubated for 15 min in (*C*) a calcium-free hypo-osmotic medium in the presence of 0.5 mM EGTA; or (*D*) a hypo-osmotic medium in the presence of 0.5 mM calcium added 1 min after reducing the extracellular osmolarity. (×825).

does not induce any significant changes in the structure or density of the microtubule network of Ehrlich ascites tumor cells as seen by the IgSS method, using anti-tubulin antibodies (*data not shown*). Thus, changes in the organization of microfilaments, but not in the microtubules, seem to be associated with volume regulation.

Figure 5 shows that in a Ca^{2+}-free hypotonic medium containing 0.5 mM EGTA, the osmotically-induced microfilament reorganization is not seen (*compare* Fig. 5*A* and *B*). In addition, in order to deplete the internal Ca^{2+} stores, Ehrlich ascites cells have been incubated in an isosmotic Ca^{2+}-free medium in the presence of EGTA (0.5 mM) for 15 min. They were then treated with Ca^{2+} ionophore A23187 (2 μM) for 15 min and incubated again for 1 min in the presence of EGTA. After such a prolonged preincubation, application of a hypo-osmotic shock in a Ca^{2+}-free medium plus EGTA does not result in any changes in microfilament organization (Fig. 5*C*). Addition of 0.5 mM $CaCl_2$ 1 min after changing the extracellular osmolarity, however, results in a reduced staining (Fig. 5*D*), indicating that the actin network has now been reorganized. Addition of ionophore A23187 (2 μM) in the presence of high external concentration of Ca^{2+} (1 mM), added at time zero (Fig. 6*B*) or 1 min after reduction in the osmolarity, leads likewise to a rapid modification in the organization of the microfilament network. Table 2 gives the rate of RVD in these various groups and summarizes the staining of the cells as a function of the various treatments. It is seen that reduction in cellular Ca^{2+} reduces the rate of "channel mediated" RVD, i.e., the amount of KCl lost via the conductive channels. On the other hand, increasing cytosolic Ca^{2+} by exogenous addition of Ca^{2+} or by

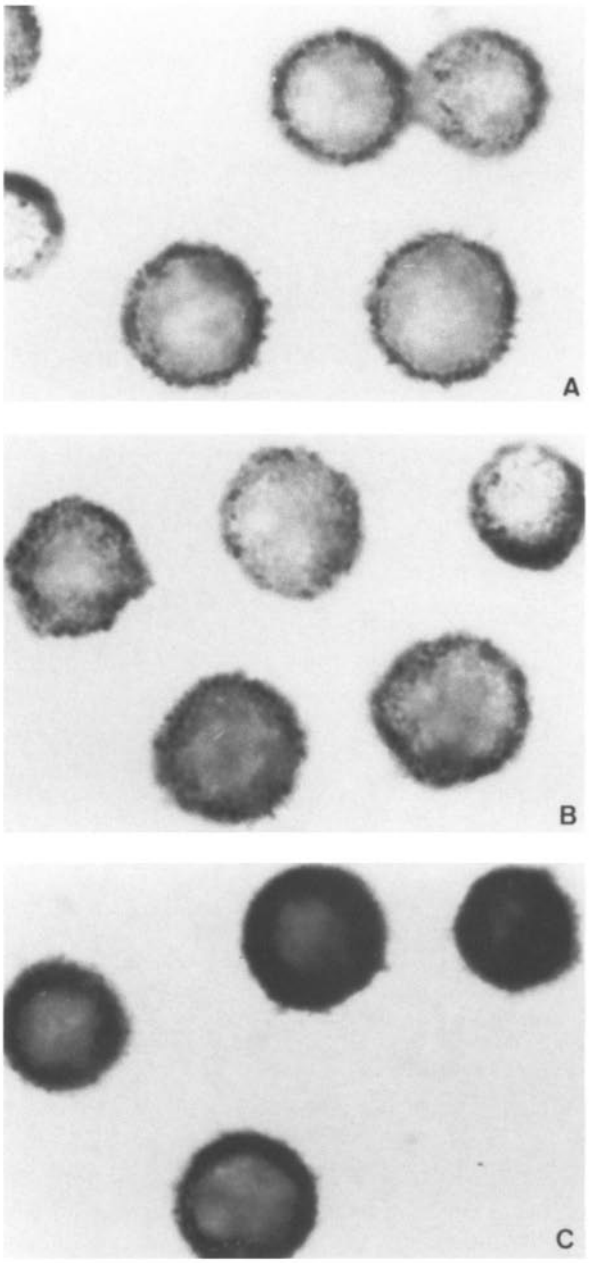

Fig. 6. Microfilament network of Ehrlich ascites tumor cells (*A*) after 1 min incubation in isosmotic conditions in the presence of 1 mM calcium and 2 μM calcium ionophore A23187; (*B*) after 1-min hypo-osmotic shock in the presence of 1 mM calcium and 2 μM calcium ionophore A23187; (*C*) after 15-min incubation in a calcium-free isosmotic medium in the presence of 0.5 mM EGTA and 2 μM calcium ionophore A23187. (×825).

the Ca^{2+} ionophore A23187 significantly increases the rate of RVD (Table 2). This confirms that Ca^{2+} also plays a significant role for the RVD. Addition of Ca^{2+}-ionophore A23187 under isotonic conditions results in a rapid modification in the organization of the microfilament network. If the cells were preincubated in Ca^{2+}-free, EGTA-containing isosmotic medium, addition of A23187 causes only a minor change in staining (*compare* Fig. 3*A* with Fig. 6*C*) indicating that no significant change has taken place in the organization of microfilaments, i.e., Ca^{2+} entry seems to be important in microfilament reorganization.

A number of drugs reported to inactivate the Ca^{2+}-binding protein calmodulin has been demonstrated to inhibit RVD in Ehrlich ascites tumor cells (Hoffmann, Simonsen & Lambert, 1984). To see whether calmodulin is involved in the Ca^{2+} effect on the microfilament reorganization, inhibitors of calmodulin regulated processes were used. Figure 7 demonstrates that both pimozide (diphenylbutyl piperidine, Fig. 7*A*) and trifluoperazine (Fig. 7*B*) inhibit the reorganization of microfilament system during RVD in Ca^{2+}-containing media, indicating the involvement of calmodulin in the process.

To see whether the shrinking process, which occurred during RVD and after addition of A23187, as such could be responsible for the observed changes in the filament system, we added 42.5 mM sucrose to Ehrlich cells suspended in isosmotic incubation media. This has previously been demonstrated to cause a cell shrinkage in Ehrlich ascites tumor cells (Hendil & Hoffmann, 1974). However, no microfilament reorganization could be observed in such conditions (*result not shown*).

Figure 8 shows that addition of quinine (20 μM), which is a potent inhibitor of the Ca^{2+}-dependent K^+ transport pathway implicated in the volume regulation process of Ehrlich ascites tumor cells (Valdeolmillos, Garcia-Sancho & Herreros, 1982; Hoffmann et al., 1986), blocks the microfilament reorganization under RVD. However, when the K^+ ionophore valinomycin (1.2 μM) is added to the osmotically swollen cells in the presence of quinine, the quinine inhibition of RVD is overcome (Hoffmann et al., 1984, 1986) and the microfilament system is now gradually being reorganized (Fig. 8*B*). It has previously been shown that addition of quinine to the Ehrlich cells in isotonic medium leads to a strong depolarization of the cell membrane potential which is reversed to a marked hyperpolarization after a subsequent addition of a K^+ ionophore (Lambert, Hoffmann & Jørgensen, 1989). The depolarization induced by addition of quinine results in a decreased driving force for Ca^{2+} uptake which might explain why we see no changes in microfilament organization. The hyperpolarization after addition of the K^+ ionophore valinomycin is equivalent to an increased driving force for Ca^{2+} influx and could explain the concomitant reorganization of the microfilament system.

Finally, we investigated the implication of a

Table 2. Effect of Ca^{2+} on the rate of "channel-mediated" RVD[a] and microfilament reorganization

	Initial rate of volume recovery (relative scale)	Anti-actin immunogold/silver Staining		
		Time after hypotonic shock		
		1 min	5 min	15 min
Control	1	Dark	Lighter	Light
Pimozide[b]	0.23 ± 0.03[c] $n = 4$	Dark	Dark	Dark
Ca^{2+}-free medium[b] (no Ca^{2+} added)	0.75 ± 0.09[d] $n = 6$	Dark	Dark	Dark
Ca^{2+}-free medium[e] (1 mM Ca^{2+} added at 1 min)	1.88 ± 0.08 $n = 5$	Dark	Lighter	Light
A23187 (2 μM)[f] added at 1 min	8.1 ± 0.4 $n = 6$	Dark	Light	Light

[a] Cells preincubated in isotonic medium for 40 min or more were at time zero diluted 500-fold with hypotonic medium with half osmolarity. The cell volume was followed with time in a Coulter counter system. The initial rate of volume recovery (fl/min) for control cells was estimated as described in Table 1. In the case of ionophore A23187- or Ca^{2+}-stimulated cells, the initial rate was estimated from cell volumes taken within the first minutes following addition of A23187 or Ca^{2+}. Values are ± SEM.

[b] The cells were preincubated in isotonic $NaNO_3$ medium containing Ca^{2+} (1 mM) or Ca^{2+}-free (0.5 mM EGTA) and at time zero transferred to hypotonic Ca^{2+}-containing (0.5 mM) or Ca^{2+}-free (0.5 mM EGTA) $NaNO_3$ medium, respectively. The experiments were performed in NO_3^--media in order to avoid interference with the KCl cotransport system.

[c] 10 μM was added at the time of osmotic cell swelling to NO_3 cells in the presence of 0.5 mM Ca^{2+}.

[d] Values from Kramhøft et al., 1986. The rate of RVD in Ca^{2+}-depleted cells is given relative to the rate of RVD in Ca^{2+}-containing control cells.

[e] Cells were preincubated in isotonic Ca^{2+}-free NaCl medium (0.5 mM EGTA) and at time zero transferred to hypotonic Ca^{2+}-free NaCl medium. Ca^{2+} (1 mM) was added at the time of maximal cell swelling, i.e., $t = 1$ min. The rate of RVD in Ca^{2+}-treated cells is given relative to the rate of RVD in Ca^{2+}-free control cells.

[f] Cells were preincubated in isotonic NaCl medium (1 mM Ca^{2+}) and at time zero transferred to hypotonic NaCl medium (0.5 mM Ca^{2+}); 2 μM Ca^{2+}-ionophore A23187 was added at $t = 1$ min. The rate of RVD in A23187-treated cells is given relative to the rate of RVD in Ca^{2+}-containing control cells.

cAMP system in the initiation of microfilament reorganization. Ehrlich ascites cells were incubated in presence of 0.1 mM IBMX, which increases the intracellular level in cAMP by inhibiting its degradation by the phosphodiesterase (Appleman et al., 1982). In isosmotic conditions, the drug did not induce any modification of the microfilament pattern (*data not shown*). In reduced extracellular osmolarity, the actin reorganization observed in presence of IBMX was similar to the one induced in control hypo-osmotic conditions (*data not shown*). Thus, cAMP seems not to be involved in the microfilament changes.

Discussion

Microtubules do not appear to be implicated in the volume regulatory response of Ehrlich ascites tumor cells. Reduction of the extracellular osmolarity does not induce any significant change in their structure or density (*data not shown*) and high doses of colchicine, which disrupt the microtubules, have no significant effect on RVD (Fig. 1).

On the contrary, the microfilament network appears to play a role in the regulatory response of Ehrlich cells following cell swelling in hypotonic medium. Cytochalasin B is known to inhibit actin assembly by inhibiting the addition of actin monomers to microfilaments (*see* Fig. 3; Mac Lean-Fletcher & Pollard, 1980; Stossel, 1989). Pretreatment of Ehrlich cells with that drug inhibits the RVD response (Fig. 1). Removal of Na^+ or substitution of all intra and extracellular Cl^- with NO_3 does not affect the inhibition of RVD provoked by cytochalasin B (Fig. 2, Table 1). Since the KCl as well as the Na,K,2Cl cotransport are both strictly Cl^- dependent and do not transport NO_3 (Hoffmann et al., 1986), it is concluded that the inhibition of RVD by cytochalasin B is on the activation of the K^+ and the Cl^- channels. Cytochalasin B was found to

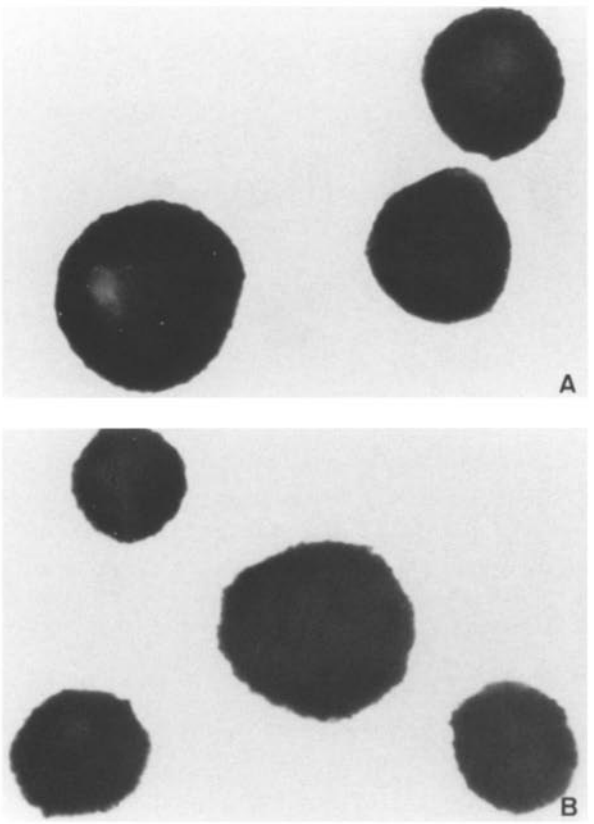

Fig. 7. Microfilament network of Ehrlich ascites tumor cells submitted to 15-min hypo-osmotic shock in the presence of (*A*) 4 μM pimozide or (*B*) 10 μM trifluoperazine, added at zero time. (×825).

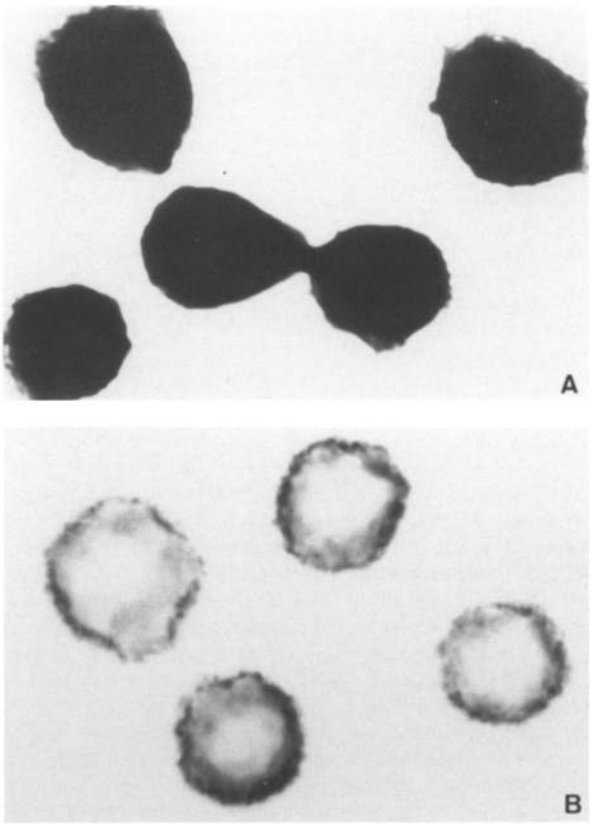

Fig. 8. Microfilament network of Ehrlich ascites tumor cells submitted to 15-min hypo-osmotic shock in the presence of (*A*): 20 μM quinine hydrochloride or (*B*) of 20 μM quinine hydrochloride plus 1.2 μM valinomycin, added at zero time. (×825).

inhibit RVD by 75% in gallbladder epithelial cells from *Necturus maculosus* (Foskett & Spring, 1985), to completely abolish the volume readjustment phase in isolated axons from *Carcinus maenas* (Gilles et al., 1986) and of mouse peritoneal macrophages (Galkin & Khodorov, 1988), and to inhibit RVD by some 80% in T2 fibrosarcoma cells (Cornet et al., 1988). It also completely modifies volume regulatory processes in PC12 pheochromocytoma cells (Cornet et al., 1988, 1993).

These results strongly suggest that an intact microfilament network is a prerequisite for the activation of ion transporting systems activated during cell volume regulation.

Two possible mechanisms by which microfilaments can regulate ion channels are: (*i*) by insertion of channels from a cytoplasmic store into the membrane, as proposed by Foskett and Spring (1985) and by Lewis and Moura (1982). A vesicle insertion mechanism has also been proposed in different epithelial cells by Van Rossum and Russo (1981). The ion transport pathways are supposed to exist in vesicles located in the cytoplasm. Recent ultrastructural studies, however, showed that there is no significant change in the amount of cytoplasmic vesicles in cultured PC12 or Ehrlich ascites cells submitted to hypo-osmotic conditions (Delpire et al., 1985; Cornet et al., 1992). (*ii*) by acting as the mechanical transducer by which membrane-stretch induces channel activation. Ion channels activated by membrane-stretch, and presumably attached to cytoskeletal strands, have been detected in a variety of vertebrate cells (for review *see* Sachs, 1988; Erxleben, Ubl & Kolb, 1989; Rugolo et al., 1989; Morris, 1990). This prevalence of stretch-activated channels suggests that these channels might constitute part of the volume-sensing signal transduction mechanisms. Furthermore, several recent studies have demonstrated that stretch-activated channels are activated during volume regulatory processes (Christensen, 1987; Ubl, Murer & Kolb, 1988, 1989; Christensen & Hoffmann, 1992; Cornet et al., 1993). It was therefore suggested that these stretch-activated channels,

probably linked with components of the cytoskeleton, could be involved in volume regulation, perhaps as mechanotransducers in some sort of cell volume sensor (Christensen, 1987; Ubl et al., 1988, 1989; Christensen & Hoffmann, 1992; Cornet et al., 1993).

Microfilament organization is dramatically affected by hypo-osmotic treatment (Fig. 4). Electron microscopic studies, IgSS method and use of rhodaminyl-phalloidin, which is known to stain F-actin specifically (Wulf et al., 1979), demonstrated in PC12 cells that the osmotic shock does not induce a complete depolymerization of the actin network, but rather a progressive and biphasic reorganization of the polymerized actin (Cornet et al., 1988, 1992). Considering that the same IgSS method was used for the present investigations, and since the actin staining and appearance of cells are different under osmotic shock or after cytochalasin B treatment, an osmotically-induced reorganization of F-actin can also be considered in Ehrlich cells, instead of a drastic network disruption. The osmotically-induced changes in the organization of Ehrlich ascites microfilament network likewise seem to occur gradually with time (Fig. 4 *A–C* and Table 2), and at a time course parallel to the volume recovery processes (*see* Fig. 1). Similar data have first been demonstrated for PC12 and T2 cells (Cornet et al., 1988), and later confirmed in shark rectal gland (Ziyadeh, 1992). It is possible that fine ultrastructural modifications, and more specific modifications induced at the level of membrane-microfilament interactions, could appear during the first minutes of the osmotic shock, thereby interfering with the regulatory processes. Such thin structural alterations are unfortunately undetectable, even with the more recent techniques for electron microscopy (Cornet et al., 1992). However, some previous studies reported that during the first minutes of osmotic shock, important modifications appear in the appearance, length and density of the microvilli which normally cover the surface of Ehrlich ascites tumor cells (Hoffmann, Lambert & Simonsen, 1988). Similar changes were observed in human lymphocytes and PC12 cells (Cheung et al., 1982; Cornet et al., 1992). Microvilli are known as anisotropic structures supported by a submembranous lattice of highly aligned microfilaments (*for review,* Schliwa, 1986). The observed changes in the microvilli pattern after 1 min (*see* Hoffmann et al., 1988), therefore, demonstrate that important modifications of the structural organization of the submembranous microfilament network are already induced within the first minutes following the osmotic shock.

Calcium concentration is known to be an important factor for the regulation of the formation and growth of the actin filaments (*see* for examples Yin et al., 1981; Pollard et al., 1982; Stolz & Bereiter-Hahn, 1988; Stossel, 1989). In most cells investigated, Ca^{2+} seems to be involved in the RVD response following cell swelling, but the nature of the role played by Ca^{2+} is far from clear. For recent reviews of the role of Ca^{2+} in cell volume regulation *see* Pierce and Politis (1990) and Hoffmann et al. (1992). In several cell types, extracellular Ca^{2+} and Ca^{2+} entry across the cell membrane are prerequisites for the swelling-induced RVD response. In other cell types the volume response is unaffected by removal of external Ca^{2+}, and in these cells, swelling-induced release of Ca^{2+} from internal stores has been proposed to be involved in the RVD response. In Ehrlich ascites tumor cells, it has been suggested that internal Ca^{2+} as well as Ca^{2+} entry are important in RVD (*see* Hoffmann et al., 1992). Table 2 summarizes the effect of Ca^{2+} and calmodulin on RVD and disassembly of the actin filaments in Ehrlich cells. It can be seen that depletion of intracellular Ca^{2+} prior to a hypotonic shock in Ca^{2+}-free medium reduces the loss of KCl via the K^+ and Cl^- channels to 75% of the loss in the presence of Ca^{2+}. Under similar conditions, the microfilament reorganization normally induced by the osmotic shock is completely abolished (Fig. 5*C*, Table 2). The present experiments, moreover, demonstrate that changes in microfilament organization are abolished during RVD in the absence of extracellular calcium (Fig. 5*B*) even without prior Ca^{2+} depletion. On the contrary, increasing the intracellular calcium concentration by addition of Ca^{2+} or by the Ca^{2+}-ionophore A23187 plus Ca^{2+} accelerates the RVD response two and eight times, respectively, and induces, in less than 1 min, an actin reorganization similar to the one seen after cell swelling (Fig. 6, Table 2). An increase of the membrane permeability to calcium in hypo-osmotic conditions (Wong & Chase, 1986), as well as a stretch activation of nonselective cation channels which are permeable to Ca^{2+} have recently been demonstrated (Christensen, 1987; Ubl et al., 1988, 1989; Christensen & Hoffmann, 1992). Thus, it is likely that extracellular Ca^{2+} is the trigger of the actin reorganization observed after osmotic cell swelling.

In the presence of the anti-calmodulin drugs pimozide and trifluoperazine, changes in microfilament organization after cell swelling are abolished (Fig. 7 and Table 2), indicating that calmodulin plays an important role in microfilament reorganization as well as in the activation of the ion channels. Calmodulin is known to be involved in the regulation of microfilament formation and organization in a variety of cell types (Glenney, Bretscher & Weber, 1980; Pritchard & Moody, 1986; Sobue & Sellers, 1991). The present results are in agreement with previous

observations in sea urchin coelomocytes submitted to hypo-osmotic conditions, where the observed microfilament reorganization is demonstrated to be regulated via the calcium-calmodulin complex (Henson & Schatten, 1983).

The microfilament reorganization normally seen under hypotonic conditions is prevented by addition of quinine (Fig. 8*A*). Quinine is a potent inhibitor of the volume activated K^+ channels and is known to inhibit RVD and the concomitant loss of KCl in a variety of cell types (Grinstein et al., 1982; Sarkadi et al., 1985; Hoffmann et al., 1984, 1986; Delpire, Cornet & Gilles, 1991). In the Ehrlich cells, addition of quinine also leads to a depolarization of the resting membrane potential (Lambert et al., 1989). During RVD the increase in the Cl^- permeability exceeds the concomitant increase in the K^+ permeability and the cell membrane potential depolarizes (Lambert et al., 1989). It is, therefore, likely that the depolarization after cell swelling is even stronger in the presence than in the absence of quinine and that this strong depolarization prevents the influx of Ca^{2+} and thereby the reorganization of microfilaments. Lifting the quinine-induced inhibition of the K^+ channels by addition of the cation-ionophore valinomycin enables osmotically swollen Ehrlich cells to volume regulate (Hoffmann et al., 1984). Concomitantly, microfilament organization is modified (Fig. 8*B*). Imposing a high cation permeability to Ehrlich cells in which the K^+ channels are blocked by quinine results in a strong hyperpolarization of the membrane potential (Lambert et al., 1989) and the effect of valinomycin on the microfilament system (Fig. 8*B*) is, therefore, likely to be the result of an increased influx of Ca^{2+}. It should be noted, however, that K^+ and Cl^- might also interfere with the formation of actin filaments (Wegner & Neuhaus, 1981; Pardee et al., 1982). Thus the lack of KCl loss in the presence of quinine could, alternatively, be part of the quinine effect.

Some authors suggested the existence of a concomitant control of the microfilament organization and of the cell volume in isosmotic conditions by cyclic adenosine 3′,5′-monophosphate (cAMP) in MDCK cells (Mills & Skiest, 1985; Mills, 1987). Many studies indeed demonstrated that an increase of the intracellular level in cAMP induces important modifications in the microfilament arrangement (Albertini & Herman, 1984; Westermark & Porter, 1982; Chaldokov et al., 1989). No significant variation of the microfilament organization in Ehrlich ascites cells was, however, observed in isosmotic conditions in the presence of IBMX, and the drug did not cause any changes in the normally osmotically induced microfilament reorganization (*data not shown*). It is, therefore, not likely that cAMP is involved in the microfilament reorganization in Ehrlich cells during RVD.

Conclusively, we suggest the following model. Cell swelling leads to hydrolysis of PIP_2 (Christensen et al., 1988). PIP_2 breakdown would be predicted to facilitate tight association between cytoplasmic profilin and actin, thereby favoring disassembly of actin filaments (*see* Stossel, 1989). PIP_2 hydrolysis generates an increase in cytosolic IP_3 which is suggested to release Ca^{2+} from internal stores (*see* Hoffmann et al., 1993). In addition, cell swelling activates Ca^{2+} permeable, nonspecific cation channels (Christensen & Hoffmann, 1992) which leads to Ca^{2+} influx. The rise in cytosolic Ca^{2+} impairs the growth of actin filaments (*see* Stossel, 1989). Thus, the observed reorganization of the microfilament system during RVD can be explained as a consequence of PIP_2 breakdown and an increase in intracellular free Ca^{2+}, which in turn results from IP_3 release and stretch-activation of Ca^{2+} permeable, nonselective channels.

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