Control of Cell Volume in the J774 Macrophage by Microtubule Disassembly and Cyclic AMP

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ABSTRACT   We have explored the possibilities that cell volume is regulated by the status of microtubule assembly and cyclic AMP metabolism and may be coordinated with shape change. Treatment of J774.2 mouse macrophages with colchicine caused rapid microtubule disassembly and was associated with a striking increase (from 15-20 to >90%) in the proportion of cells with a large protuberance at one pole. This provided a simple experimental system in which shape changes occurred in virtually an entire cell population in suspension. Parallel changes in cell volume could then be quantified by isotope dilution techniques. We found that the shape change caused by colchicine was accompanied by a decrease in cell volume of ~20%. Nocodozole, but not lumicolchicine, caused identical changes in both cell shape and cell volume. The volume loss was not due to cell lysis nor to inhibition of pinocytosis.

The mechanism of volume loss was also examined. Colchicine induced a small but reproducible increase in activity of the ouabain-sensitive Na^+,K^+-dependent ATPase. However, inhibition of this enzyme/transport system by ouabain did not change cell volume nor did it block the colchicine-induced decrease in volume. On the other hand, SITS (4'acetamido, 4-isothiocyano 2,2' disulfonic acid stilbene), an inhibitor of anion transport, inhibited the effects of colchicine, thus suggesting a role for an anion transport system in cell volume regulation.

Because colchicine is known to activate adenylate cyclase in several systems and because cell shape changes are often induced by hormones that elevate cyclic AMP, we also examined the effects of cyclic AMP on cell volume. Agents that act to increase cyclic AMP (cholera toxin, which activates adenylate cyclase; IBMX, an inhibitor of phosphodiesterase; and dibutyryl cyclic AMP) all caused a volume decrease comparable to that of colchicine. To define the effective metabolic pathway, we studied two mutants of J774.2, one deficient in adenylate cyclase and the other exhibiting markedly reduced activity of cyclic AMP-dependent protein kinase. Cholera toxin did not produce a volume change in either mutant. Cyclic AMP produced a decrease in the cyclase-deficient line comparable to that in the wild type, but did not cause a volume change in the kinase-deficient line. This analysis established separate roles for cyclic AMP and colchicine. The volume decrease induced by cyclic AMP requires the action of a cyclic AMP-dependent protein kinase. Colchicine, on the other hand, induced a comparable volume change in both mutants and wild type, and thus does not require the kinase.

Although volume and shape are probably the most basic characteristics that distinguish animal cells, the relationship of cell volume regulation to shape has been relatively unexplored. However, it is evident that the number and arrangement of surface folds are determined by cell volume. Moreover, a primary change in cell volume would generally force a shape change. Thus, it seemed possible to us that the regulation of cell shape would be highly coordinated with control of cell volume. If so, then, because microtubules are generally believed to be significant determinants of cell shape (1, 25), the state of microtubule assembly may be a determinant of cell volume.

In the J774.2 macrophage cell line, as in a variety of other

leukocytes, microtubule disassembly causes a rapid and predictable change in cell shape; a large bulge or protuberance develops in >90% of these cells. The presence of this shape change in an entire cell population allows the study of their volume by physiological approaches. Its rapid development allows the effect of growth on cell volume to be ignored. Thus, the J774.2 cell line provides a relatively simple system to explore the interaction of shape and volume control.

It is shown here, using an isotope-dilution technique, that changes in volume occur roughly parallel to the extent of microtubule disassembly and the frequency of protuberance formation. Because peptide hormones, acting via cyclic AMP and associated kinase, have both acute and long-term effects on cell morphogenesis (9, 10), we have also studied the effect of cyclic AMP on cell volume and its relationship to the volume change associated with microtubule disassembly.

MATERIALS AND METHODS

Cells

The J774.2 variant (19) of the J774 mouse macrophage cell line originally isolated by Ralph and Nakoinz (26) was used. Two J774.2 mutants were also used: J7H1, deficient in cyclic AMP-dependent protein kinase, and CT2, deficient in adenylate cyclase. These lines were isolated and characterized by Dr. B. Bloom and Dr. O. Rosen and their colleagues (28) and kindly provided to us.

Media

Cells were grown in Dulbecco's modified Eagle's medium with 20% horse serum on nontissue culture plastic dishes. Cultured in this way the cells remain essentially in suspension during growth. Before each experiment, the cells were removed from the plates by gently pipetting medium onto the dishes and aspirating the detached cells, then centrifuged at room temperature for 5 min, resuspended in fresh complete medium at concentrations of 1–3 × 10^6/ml, and held for 30 min at 37°C. In this and subsequent manipulations, care was taken to use complete medium and to perturb the cells as little as possible so as to maintain constancy of the cellular environment and thus minimize volume change in control cell populations.

Immunofluorescence

Immunofluorescence labeling of microtubules was obtained using a goat antitubulin (3) and a modified procedure derived from Osborn and Weber (23). Monolayers on cover slips were briefly rinsed at room temperature with stabilization buffer containing 0.1 M PIPES (piperazine-N,N'-bis 2-ethane sulfonic acid), pH 6.9, 1 mM EGTA, and 4% polyethylene glycol '6000' and incubated at 37°C for 1 min in the same buffer containing 0.5% Triton X-100. The cover slips were carefully drained, placed in methanol at −15°C for 5 min, rinsed in phosphate-buffered saline (PBS), and covered for 30 min at 37°C with 100 μl of the goat antiserum diluted 1:100. Cover slips were then rinsed with PBS and incubated for 30 min at 37°C with fluorescein-labeled rabbit anti-goat immunoglobulin (N. L. Cappel Laboratories, Inc., Cochranville, Pa.) diluted 1:40. The result was photographed through a Zeiss ×63 phase Neofluor objective onto Kodak Tri-X film.

Volume Measurement

This was determined by isotope dilution of markers for total water ([^{3}H]H$_2$O) and extracellular space ([^{14}C]inulin). Cell suspensions (300 μl of 1–3 × 10^6/ml) in culture medium were layered into 1.5-ml conical plastic tubes (Walter Sarstedt, Inc., Princeton, N. J.) over silicone oil of density less than that of the cells and greater than that of the medium (Versilube F50-General Electric Co., Medical Systems Div., Milwaukee, Wis.) and centrifuged rapidly in an Eppendorf 3200 microcentrifuge (Brinkmann Ins., Westbury, N. Y.). The tube bottom containing the cell pellet was then sliced off for determination of radioactivity or other parameters. Each experimental measurement was done in triplicate. Pellets contained 3–9 × 10^5 cells. The basic method has been used extensively before (22, 36) and depends on the stripping away of most of the extracellular fluid as the cells are pelleted through the oil. We showed that the pelleted cell protein was directly proportional to the volume of cell suspension centrifuged. After correction for extracellular fluid, ^{3}H$_2$O recovered was also directly proportional to the volume of cells centrifuged. Corrections for extracellular fluid averaged

~10–15% of the total pellet [^{3}H]H$_2$O. However, this was sufficiently variable that extracellular fluid was determined in every sample.

^{86}Rb Uptake and Cellular Electrolytes

Isotope or ion contents were determined on cell pellets centrifuged through silicone oil as described above and corrected for contaminating extracellular fluid. ^{86}Rb was determined by direct gamma counting, ^{36}Cl by beta scintillation counting, and K$^+$ and Na$^+$ by atomic absorption spectrophotometry.

For ^{86}Rb uptake rates, samples were taken every 2 min for 10 min. ^{86}Rb$^+$ uptake was linear over this time. Regression equations were calculated from the raw data after subtraction of background counts, and the slopes were compared by one-way analysis of variance.

^{36}Cl was added in trace amounts to the medium, and aliquots of the cell suspension were sampled every 3 min for 36 min. An approximate plateau in cell ^{36}Cl was attained at about 12 min and increased only 10% over the additional interval. Control and colchicine-treated cells behaved similarly.

Fluid Pinocytosis

The method of Walter et al. (35), in which the uptake of fluorescein-labeled dextran is measured, was used. Cells were suspended in medium containing 1 mg/ml fluorescein isothiocyanate (FITC)-dextran (Sigma Chemical Co., St. Louis, Mo.). The cells were pelleted, washed, solubilized in 0.1% SDS, and fluorescence was measured in a Hitachi-Perkin-Elmer MP4 spectrofluorometer (Hitachi America, Ltd., New York, N. Y.). As described (35), FITC-dextran uptake is linear with time and dextran concentration with an adsorptive component of uptake, obtained by immediate cell separation after mixing, or <2% of the 1-h uptake. The actual volume pinocytized was calculated from the fluorescence of a known quantity of the supernatant medium.

RESULTS

Rapid Microtubule Disassembly Induced by Colchicine in J774.2 Macrophages

We first showed that colchicine in the doses used caused rapid microtubule disassembly over the time-course of our experiments. Fig. 1 shows a series of immunofluorescence photographs of representative J774.2 incubated on monolayers with 10 μM colchicine at 37°C. It is seen that some microtubule disassembly is apparent after 30 min and is essentially complete by 45 min. The typical protuberant shape of microtubule-depleted J774.2 is also clear from these photographs. It should be pointed out that the volume studies were performed with cells in suspension, not monolayer. However, the development of a cell protuberance (see below), which has been correlated quantitatively with microtubule disassembly in several cell types (20, 21), is virtually identical for J774.2 in suspension or monolayer.

Antimicrotubule Agents Cause a Decrease in Cell Volume

Incubation of J774.2 macrophages with colchicine produced a consistent decrease in cell volume. In wild-type J774.2 incubated with 10 μM colchicine, the decrease was 20% at 45 min (Table I). The time-course of the volume decrease was accelerated at high as compared to low colchicine concentrations. Fig. 2 depicts the average time-course of seven experiments at 1 and 10 μM colchicine. This pattern may be expected from the probable mechanism of colchicine action which depends on drug penetration and combination with free microtubule subunits leading to gradual microtubule disassembly (37). Nocodazole and podophyllotoxin (10 μM), chemically distinct antimicrotubule agents, have identical effects on cell volume, whereas lumicolchicine (10 μM), a photochemical derivative of colchicine without antimicrotubule properties, has no effect on cell volume.
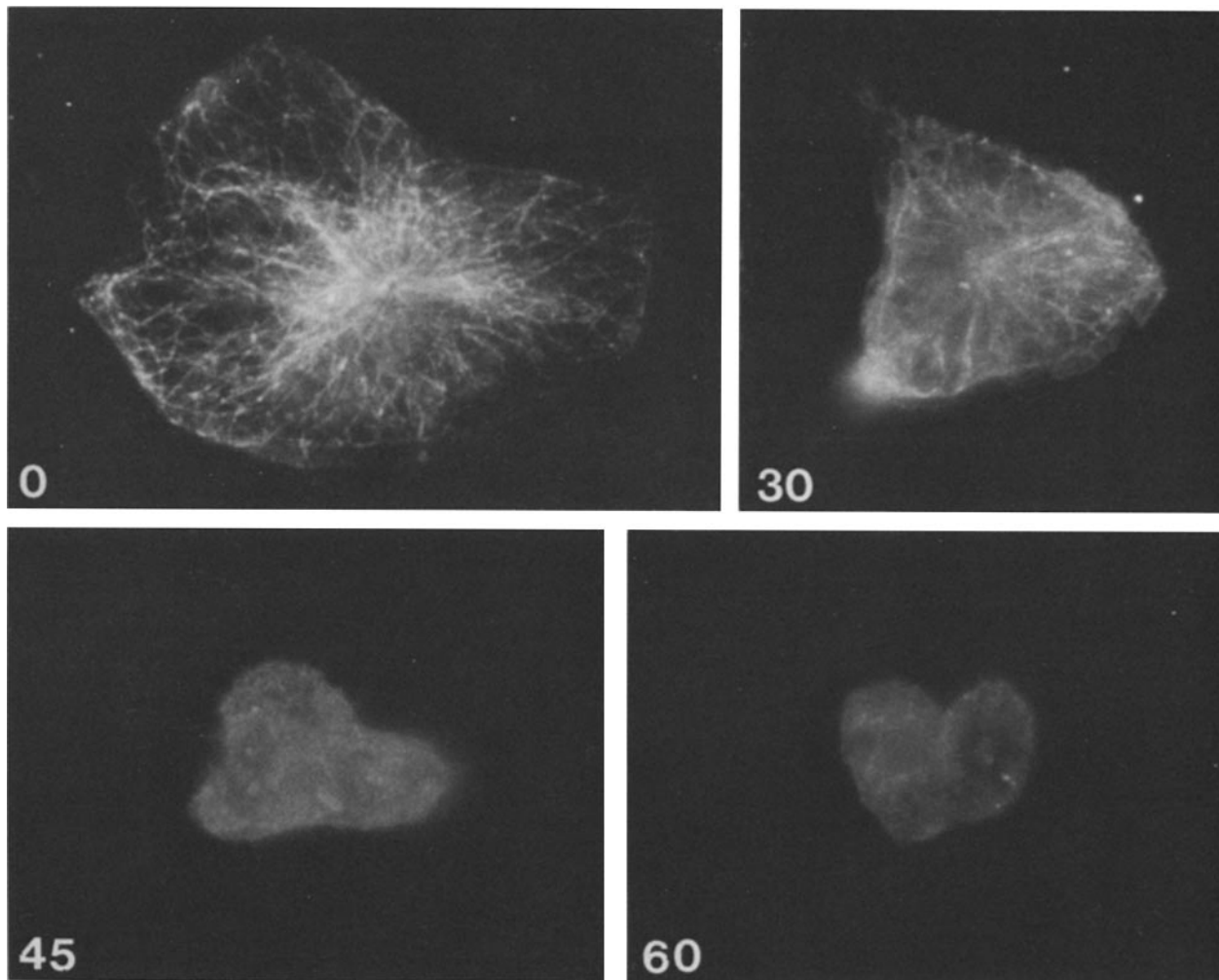
FIGURE 1 The distribution of microtubules on representative J774.2 macrophages after colchicine treatment. Monolayers of J774.2 were incubated with 10^{-6} M colchicine for the times indicated (0-60 min) and processed for immunofluorescence as described in Methods.

Relation of Volume Decrease to Inhibition of Pinocytosis

It has previously been shown by Steinman et al. (33) for cultured macrophages and L cells that during pinocytosis 50–200% of the surface membrane and significant volume may be interiorized per hour. It was therefore conceivable that the impairment of pinocytosis in J774.2 cells by colchicine (35) may substantially influence cell volume. The magnitude of pinocytosis is, however, too small to account for the volume changes observed. Thus, the amount of fluid pinocytized per hour calculated from FITC-dextran uptake was 0.097 (±0.013) $\mu l/10^6$ cells per h (13 experiments) in control macrophages or <5% of the total cell volume, and falls to 0.062 (±0.014) $\mu l/10^6$ cells per h or 3% in the presence of colchicine. The difference, or 2% of cell volume, although significant ($P < 0.001$) constitutes only a small part of the colchicine-induced volume change of 20%.

Relation of Shape Change to Volume Change

As noted, treatment of leukocytes, including J774 macrophages, with antimicrotubule agents leads to a distinct shape change: formation of a protuberance underlain with microfilaments (1). In the presence of ligands such as concanavalin A

TABLE I

Effect of Colchicine on Macrophage Cell Volume ($\mu l/10^6$ cells)

	n	Control	Colchicine	Change
			10 μM	%
J774.2 (wild type)	13	2.1 (±0.12)	1.69 (±0.1)*	−20 (±1.8)
Protein kinase deficient	13	1.32 (±0.08)	1.1 (±0.06)*	−17 (±2)
Adenylate cyclase deficient	11	1.5 (±0.11)	1.31 (±0.09)‡	−13 (±1.2)

In these experiments cell suspensions were incubated with or without 10 μM colchicine for 1 h, and volume was measured as described in Methods. n = number of experiments. Mean (SEM). Statistical significance of difference from paired controls by t test.
* $P < 0.001$.
‡ $P < 0.01$.

(Con A), a ligand cap is formed over the protuberance (1, 20). In neutrophils (20) or lymphocytes (21), where microtubules can be systematically counted by electron microscopy, this capping can be precisely correlated with inhibition of microtubule assembly. When J774 are incubated with colchicine, a striking parallel is seen between the rates of protuberance formation and decrease in volume (Fig. 3), although protuber-
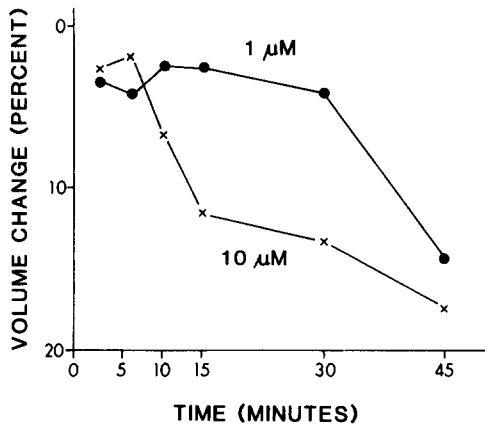
FIGURE 2 Effect of colchicine on cell volume. In these experiments, triplicate samples of cell suspension ($4-8 \times 10^5$ cells/250 μ/liter suspension) were layered over oil and spun as described in Methods. [^{3}H]H_2O and [^{14}C]inulin were added to cell suspensions at the beginning of the incubation time, and the cell volume was determined at the times shown. Isotope equilibration is essentially instantaneous. The graph depicts the mean of seven experiments for each concentration (1 and 10 μM of colchicine). 5 μM colchicine (not shown here) gave an effect intermediate between those of the two concentrations shown. Control suspensions showed increases in volume of 0–4% over the course of the experiment as would be expected from the continued growth of cells in complex medium.[1] Standard errors for all points are 2–3%. Volume changes were calculated relative to control cells at comparable times of incubation.
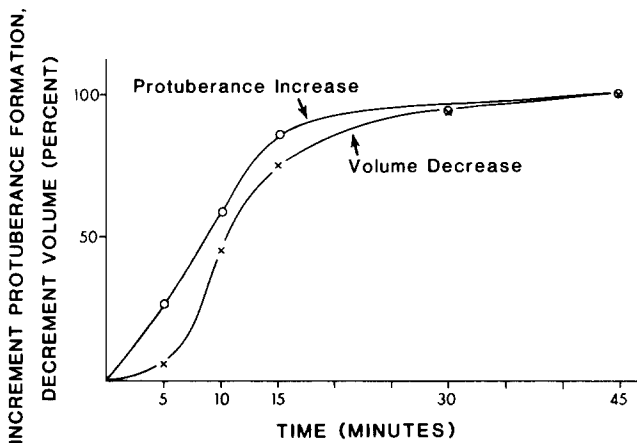

FIGURE 3 Time-course of protuberance formation (○) and of volume change (×) after 5 μM colchicine. The values are normalized to facilitate comparison of the time-course of shape and volume changes. Protuberance formation was determined at the indicated times by phase microscopy of cell suspensions. Examination of cells incubated without colchicine showed that under the standard conditions of cultivation there was a spontaneous tendency to protuberance formation of 15–20%. However, this remained constant over the hour of the experiment, and the colchicine-induced change was calculated after subtraction of this background. Each point of both protuberance formation and volume change is the mean of seven separate experiments.

ance formation begins slightly before a volume change is evident. The parallel development of volume change and protuberance formation was also evident over a range of colchicine (1–10 μM) concentrations but followed a variable time-course,

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[1] Cell cycle c. 15–18 h, Oliver and Berlin, unpublished observations.

developing more rapidly at the higher concentrations and then remaining stable over a relatively long period. This dose dependency of the rate of protuberance formation has been described before in detail for neutrophils (18).

No Cell Lysis nor Loss of Cell Protein during Volume Change

We considered that drug treatment or shape change might be associated with the loss of cell constituents. To test whether this was a possible mechanism of volume change in J774.2 cells, we measured the protein pelleted with, and without, colchicine treatment. No protein loss was detectable over a 60-min incubation period with or without colchicine treatment, despite a 19% decrease in cell volume. This rules out the loss of nonsedimenting shed material. To examine the alternative possibility that shed material could reseal and pellet with intact cells, we examined the number and size distribution of particles in the Coulter counter (Coulter Electronics Inc., Hialeah, Fla.). Particles must have high electrical impedance (be enclosed by intact membrane) to be counted. No increase in the number of small elements was found after colchicine, and in no case did the fraction of subcellular particles exceed 5% of the total particle number (although very small particles of a size below the threshold of the discriminator would not be counted). Importantly, although the size distribution of cells with or without colchicine could not be directly compared because of the induced shape change, no difference was found in the total count of cells with or without colchicine (the ratio of the cell number in the control group/to the cell number in the colchicine group = $1.00 \pm 0.02:1$ [mean SEM; nine experiments]). In all cases, $>95\%$ of cells excluded trypan blue. Thus, the observed volume decrease did not result from a drug-induced cell lysis.

Activation of Ouabain-sensitive Na^+-,K^+-dependent ATPase by Colchicine

A critical role for the Na^+,K^+-ATPase in volume regulation was postulated many years ago in the formulation of the so-called "pump and leak" hypothesis (15). There is, moreover, precedent for the belief that colchicine or microtubule disassembly can activate membrane enzymes, shown first in the work of Malawista and colleagues in which colchicine appears to activate neutrophil adenylate cyclase (18, 29). The effect of colchicine on the Na^+,K^+-ATPase was therefore examined.

Rodent cells generally require higher concentrations of cardiac glycosides than other mammalian cells to specifically inhibit the Na^+,K^+-dependent ATPase (2). Thus, we first established that ouabain effectively blocked this transport mechanism in J774.2 mouse macrophages. We determined $^{86}Rb^+$ uptake with and without preincubation for 15 min with ouabain, and K^+ content after incubation for 1 h. As shown in Table II (lines 3–4), $^{86}Rb^+$ influx was decreased $\sim 80\%$ by 1–2 mM ouabain. Correspondingly, intracellular K^+ concentration fell $\sim \frac{2}{3}$ over the hour that encompassed the duration of our volume experiments (Table II, last column). $^{+86}Rb^+$ influx is thus a valid indirect assay of the Na^+,K^+-ATPase.

After a 15-min preincubation in colchicine, $^{86}Rb^+$ uptake was stimulated $\sim 20\%$ (Table II, line 5). It was also demonstrated that the enhanced $^{86}Rb^+$ uptake after colchicine was indeed due to Na^+,K^+-ATPase activation and not to the activation or opening of some other channel. Thus, the colchicine stimulation was completely blocked by ouabain (Table II, line

TABLE II

Effect of Ouabain on $^{86}Rb^+$ Influx and K Content of J774.2
Macrophages

	n	$^{86}Rb^+$ Influx change*	Intracellular K^+
		%	mM
1. Control§	18	0	153 ± 9‡
2. Ouabain, 0.1 mM	5	−58*	99 ± 11
3. Ouabain, 1.0 mM	4	−78*	57 ± 5
4. Ouabain, 2.0 mM	14	−82*	
5. Colchicine, 10 μM, 15-min incubation	5	+22*	193 ± 17
6. Colchicine, 10 μM, 60-min incubation	3	0	—
7. Colchicine, 10 μM, 15 min + ouabain 2 mM	5	−83*	—

* Difference from control significant, $P < 0.0001$.
‡ Each experimental value is the mean of quadruplicate samples.
§ No drugs, incubation times as in drug-treated samples. n = number of experiments.

7), returning to levels obtained with ouabain in the absence of colchicine. The colchicine stimulation was not apparent after a 60-min incubation (Table II, line 6). Thus, this small but statistically significant stimulation has roughly the same time-course as the colchicine-induced volume change. However, we show below that the colchicine stimulation of the ouabain-sensitive ATPase is not related to volume regulation in J774.2 macrophages.

Colchicine-induced Volume Decrease Does Not Require a Ouabain-sensitive Na^+-,K^+-ATPase

Despite the strong inhibition of K^+ transport, treatment of J774.2 with ouabain produces little or no change in volume (+ 0.2 ± 3% SEM, nine experiments at 45 min). No loss of cells nor decreased viability as judged from trypan blue exclusion was evident over the period of incubation. Thus, volume regulation in macrophages is little influenced by profound inhibition of Na^+,K^+-ATPase. This finding adds to the growing body of evidence largely obtained in osmotically stressed tissues (reviewed by Rorive and Gilles [27]), that the ouabain-sensitive Na^+,K^+-ATPase is inadequate to explain volume regulation (12, 24).

More importantly, ouabain fails to block the colchicine-induced decrease in cell volume, and actually enhanced it slightly (to 22%, seven experiments).

Colchicine-induced Volume Change is Inhibited by 4'acetamido, 4 isothiocyano 2,2' disulfonic acid stilbene (SITS)

Several ionic pumps have been implicated in water transport, and a variety of drugs have been used in attempts to block their function. We examined two inhibitors of anion transport in erythrocytes and cultured cells (5, 34) for their effects on cell volume in J774.2 macrophages. DIDS (4,4' diisothiocyano, 2,2' disulfonic acid stilbene) produced large and immediate increases in cell volume and eventually cell lysis. This suggests an essential transport system blocked by DIDS. However, as is well known, cell swelling is often an indication of cell damage or metabolic inhibition. In any event, this swelling obviously

complicated a study of the role of DIDS-sensitive transport system in the colchicine-induced change. Surprisingly, SITS (4' acetamido, 4 isothiocyano 2,2' disulfonic acid stilbene), a closely related derivative of DIDS but with less tendency to form covalent bonds, had no effect on volume. However, SITS completely blocked the colchicine effect. Fig. 4 shows that for at least 45 min SITS leads to only trivial negative volume changes. In this series, colchicine alone caused a 14% decrease in volume. SITS completely blocked this decrease. The dose of SITS required was fairly critical: 5 mM did not inhibit, 10 mM inhibited completely. The free concentration of the drug is undoubtedly less because it will combine with serum proteins present in the medium. Some preliminary experiments suggest that <50% of the drug remains dialyzable after incubation in the complex medium used. Thus, it appears that microtubule disassembly may activate a specific SITS-sensitive transport system. The ionic basis of the effect is under investigation. In any event, the role of this transport system in volume regulation in noncolchicine-treated cells is unclear. The absence of a volume change in the presence of SITS alone may signify simply that other transport systems have increased their activities to compensate for the blocked SITS-sensitive system.

Extracellular Calcium is Not Essential for Volume Regulation

Over a 1-h period, addition of the calcium chelator EGTA at 5–10 mM did not appreciably alter cell volume nor did it block the colchicine-induced volume reduction. EDTA also had no effect.
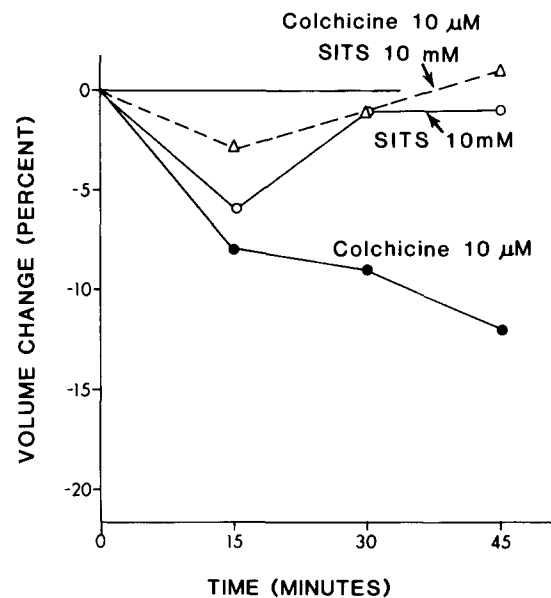

FIGURE 4 Effect of SITS on the colchicine-induced volume change. The reagents, 10 mM SITS (O), 10 μM colchicine (●), and 10 mM SITS + 10 μM colchicine (△), were added at time zero. The cell suspensions were then placed in a 37°C water bath and agitated frequently for ~10 min to ensure that the SITS had properly dissolved. 25 mM HEPES buffer (pH 7.4) was added to the medium before the start of the experiment to minimize pH fluctuations. The SITS was used in complete, protein-containing medium. As noted in the text, a large part of the SITS becomes tightly coupled to the protein under these conditions. The precise SITS-free concentration is thus unknown. The importance of stabilizing cell volume through the use of the normal complex medium was given overriding consideration.

Nature of the Intracellular Ionic Change

We have measured intracellular potassium (K⁺) and sodium (Na⁺) by atomic absorption spectroscopy and intracellular chloride (Cl⁻) by equilibration with ³⁶Cl. The absolute content of K⁺ is unchanged or slightly increased by colchicine. Its concentration (assuming equal distribution in all cellular compartments) is increased after volume reduction (Table II, line 5 and Table III). The absolute Cl⁻ content, on the other hand, is reduced by ~20%. This approximately equals the degree of volume change and, therefore, intracellular Cl⁻ concentration is essentially unchanged (Table III). Due to the small concentration of intracellular Na⁺ in J774.2 and the comparatively large contribution of extracellular Na⁺ to the Na⁺ of cell pellets, data of comparable accuracy for Na⁺ are not yet available. However, it is probable that some decrease in Na⁺ occurs with colchicine treatment as would be predicted from the Cl⁻ loss, because the other major cation, K⁺, is retained. Therefore, it would appear that the volume change is associated with a loss of both Cl⁻ and Na⁺ presumably by a SITS-sensitive transport system.

Role of Cyclic AMP (cAMP) in Volume Regulation

The motivation for exploring the role of cAMP in volume regulation was two-fold. First, it is well known that peptide hormones are known to cause elevation of cAMP, and that cAMP itself causes shape changes (e.g., 14) including the phenomenon of so-called "reverse transformation" (9, 10). Second, in neutrophils, drug-induced microtubule disassembly in the presence of the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX) causes a rise in endogenous levels of

cAMP (29) and an activation of adenylate cyclase in macrophages (8). Thus, we wished to know whether the colchicine-induced volume change could be mimicked by cAMP.

We found (Table IV, 1st column) that, indeed, in wild-type J774.2 exogenous dibutyryl cAMP, IBMX (which inhibits phosphodiesterase), and cholera toxin (which stimulates adenylate cyclase) decreased cell volume. The decrease was comparable to that obtained with colchicine.

The availability of J774.2 mutants that are known to be deficient in either cAMP-dependent protein kinase (designated J7HI) or adenylate cyclase (designated CT2) (28) enabled us to establish the specificity of the cAMP effect. We showed first (Table I and Table IV, top row) that colchicine causes a decrease in volume in both mutant lines that is similar to that of the wild type. By contrast, J7H1 failed to show a volume decrease in response to normal stimuli of cAMP synthesis, to dibutyryl cAMP or to any cAMP-related agent tested (Table IV, 2nd column). CT2 (Table IV, 3rd column) failed to show a response to cholera toxin, which acts via adenylate cyclase, or to IBMX. However, CT2 gave an appreciable decrease in volume in response to dibutyryl cAMP itself, which bypasses the cyclase step.

Thus, the colchicine-induced volume change clearly does not depend on the generation of cAMP nor on a cAMP-dependent protein kinase. At the same time, it becomes apparent that volume alterations may be stimulated by two separate mechanisms: (a) microtubule disassembly and (b) elevation of cAMP and activation of a cAMP-dependent protein kinase. The latter pathway constitutes the common pathway for stimulus by peptide hormones (which commonly induce shape changes). Thus, we would predict that rapid volume changes are likely accompaniments of hormone stimulation.

TABLE III

Effect of Colchicine on Intracellular [K⁺] and [Cl⁻]

	n	[K⁺]	With volume correction	[Cl⁻]	With volume correction
		mM *	mM	mM *	mM
Control	5	153 (±9)		44.4 (±11.0)	
Colchicine, 10 μM	5	161 (±14)	193‡ (±17)	34.8 (±8)	41.8 (±10)

Mean (±SE). n = number of experiments.
* Calculated on the basis of control cell volume.
‡ Significantly different from control (P < 0.05).

TABLE IV

Effect of Dibutyryl cAMP, IBMX, and Cholera Toxin Macrophage Cell Volume

	Mean percentage volume change*					
Agent	Wild type		Protein kinase deficient (J7H₁)		Adenylate cyclase deficient (CT₂)	
		n		n		n
Colchicine (10 μM)	−20 (±1.75)	13	−17 (±2)	13	−13 (±1.25)	11
Dibutyryl cAMP (0.46 mM)	−18 (±6)	3	+1 (±3)	5	−12 (±4)	2
IBMX (1.2 mM)	−17 (±3.5)	3	−1 (±5)	4	+4 (±1.5)	2
Cholera toxin	−18 (±0.3)*	3	0 (±2)‡	4	+1 (±4)‡	3

In this table the volume change was determined at the end of 1 h incubation with dibutyryl cAMP, isobutylmethylxanthine (IBMX), and cholera toxin. The percent effect of 10 μM colchicine is reproduced from Table I for comparison. 8 bromo-cAMP (0.5 mM), not shown here, produced a volume reduction of 15% (n = 4) in the J774.2 cell line but was not tested in the mutant cells. Choleragenoid, the inactive B subunit and competitive antagonist of cholera toxin (a gift of Professor R. Finkelstein, University of Texas) did not produce a volume change in J774.2. The volume decrease induced by dibutyryl cAMP was dose dependent; 0.11 mM, −6%; 0.23 mM, −12%; 0.46 mM, −18%. IBMX produced a 9% decrease at 0.15 mM (two experiments). Mean (±SEM); n = number of separate experiments.
* 100 ng/ml.
‡ 2.5 μg/ml.

The existence of these two mechanisms, which may act in concert, made it necessary to reexamine the failure of ouabain to block the colchicine-induced volume change. Because ouabain has been reported to activate adenylate cyclase (7, 8, 11, 16, 29, 31), hypothetically, ouabain might actually have blocked the colchicine effect but activated the cAMP pathway producing a volume change similar to that induced by colchicine in the wild type. The availability of mutants of the cAMP pathway that still decreased their volume in response to colchicine, allowed us to rule out this possibility. Thus, we found in both CT2 and J7H1 that (a) ouabain inhibits ^{86}Rb$^+$ influx to the same degree as in wild type, (b) as with the wild type, ouabain exerts little volume change when administered alone, and (c) ouabain has a similar enhancing effect on the colchicine-induced volume changes.

DISCUSSION

These studies have indicated two pathways that may be of physiological importance for volume regulation. First, agents that induce microtubule disassembly cause a rapid volume decrease that roughly parallels characteristic shape changes. Second, agents that elevate cAMP cause a volume decrease by a separate mechanism.

Although the general form of cells has long been believed to be related to the state of microtubule assembly, the observed changes have been long term and volume has not usually been specifically considered. We emphasize that the volume reduction induced by colchicine in our experiments is dose dependent and shared by other antimicrotubule agents but not by the photoderivative lumicolchicine that does not cause microtubule disassembly. We generally used a high concentration (10 μM) of colchicine to accelerate the shape change. We also have verified, by indirect immunofluorescence, that colchicine in the doses used does cause microtubule disassembly as expected from the development of cell protuberances (35). As discussed previously, protuberances occur normally. Their frequency is exaggerated by antimicrotubule agents. The structural similarity between the protuberance of cells in suspension and the uropod of motile cells is clear.[2] Thus, it may be predicted that similar volume changes occur in motile macrophages.

We recognize that the correlation of microtubule disassembly and decrease in cell volume does not prove that the pharmacological agents employed had no other action. Work in this and other laboratories has shown that tubulin can become membrane bound (4, 6). This tubulin could be the actual site of drug action. We also have emphasized previously that the effects of microtubule disassembly on surface topography and function may be indirect (3).

Previously, cell shape has been thought to be regulated directly by the cytoskeleton. Our data raise the possibility that volume regulation affects cell shape. One obvious mechanism is by determining the surface to volume ratio. However, it is also possible that the regulation of cell volume exerts some reciprocal effects on the cytoskeleton that, in turn, affects shape. For example, a 20% volume decrease of the whole cell may translate into a near doubling in concentration of constituents in the cytosol compartment. In two preliminary morphometric analyses of J774.2, 10 μM colchicine caused a 25% increase in the ratio of nuclear/cytoplasmic volume. This selective decrease in cytosol may underlie the slightly smaller decrease in volume caused by colchicine in the J774.2 mutants

compared with the wild type. The latter has a larger total volume per cell (Table I), most of which excess appears cytosolic, although more measurements of compartment volumes are required for definitive analysis. Such changes in the concentration of cytosolic constituents could, in turn, affect the assembly of fibrous proteins such as tubulin or actin, for example, in addition to other ion-sensitive cell functions (17, 30, 32).

The precise mechanism whereby colchicine induces volume changes has been only partly defined. Volume loss is associated with the loss of Cl$^-$ and probably Na$^+$ by a SITS-sensitive mechanism. K$^+$ content remains constant and its concentration, as calculated in total cell water, thus increases. The role of the ouabain-sensitive mechanism appears to be primarily in control of intracellular K$^+$: inhibition of Na$^+$,K$^+$-ATPase in the macrophage cell line was associated with a dramatic reduction in cell K$^+$ concentration, and presumably a rise in the cell Na$^+$ concentration, but no change in cell volume. The retention of K$^+$ in colchicine-treated cells may reflect the colchicine-induced activation of the ouabain-sensitive Na$^+$,K$^+$-ATPase, but this transport system is not required for the decrease in volume. It is conceivable that the increased cell Na$^+$ resulting from ouabain treatment provides an additional driving force to the SITS-sensitive mechanism with consequent increase of Cl$^-$ and Na$^+$ efflux and further volume reduction. The colchicine-related volume regulatory system is thus suggested to be poorly cation specific, using either Na$^+$ (which it prefers) or K$^+$ depending on availability. This supposed lack of cation specificity would be similar to that described for the behavior of kidney cortex slices swollen in salts of various cations (27).

Although the activation of the ouabain-sensitive Na$^+$,K$^+$-ATPase by colchicine did not prove to be essential for the volume change, the activation was nonetheless statistically highly significant and thus represents a novel effect of an antimicrotubule agent on membrane function. The activation was transient and is probably related to the high K$^+$ content following the volume change. Such changes in composition suggest that colchicine may bring about a membrane hyperpolarization, but this appears not to be the case.[3]

The second major pathway for volume regulation described here is revealed by treatment with cAMP or agents, such as cholera toxin, that enhance its endogenous production. The magnitude of volume change produced by this pathway is comparable to that obtained by microtubule disassembly. However, using mutant lines defective in cAMP metabolism, we show that the two pathways are readily separable. Thus, neither cAMP nor cholera toxin produces volume change in a protein kinase-deficient line, whereas cAMP, but not cholera toxin, produces a volume change in the adenylate cyclase-deficient line. Colchicine, on the other hand, induces comparable volume changes in both mutants and the parental line. These findings implicate a cAMP-dependent protein kinase in volume regulation by a second pathway that is presumably independent of the state of microtubule assembly (cAMP does not cause protuberance formation nor gross dissolution of microtubules by immunofluorescence). It is probable that the cAMP pathway is activated by a range of peptide hormones and other processes that depend on the cAMP-dependent protein kinase.

It is well to emphasize here the need to examine the relationship of microtubules and cAMP to volume regulation in other cell types. Just as agents that cause microtubule disassem-

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[2] J. M. Oliver and R. D. Berlin. Manuscript in press.

[3] McCaig and Berlin. Manuscript in preparation.

bly and peptide hormones induce large variations in shape, it may be expected that there will be a similar spectrum of volume changes. For example, Kregenow (13) has demonstrated a reproducible 4–5% increase in the volume of duck erythrocytes in response to cAMP, i.e., opposite to its effect on the J774.2 macrophage. In addition, it may be expected that there are variations between cell types in the specific membrane transport systems that effect volume regulation. Our observations establish the probability that processes such as microtubule assembly and cAMP metabolism, that are thought to affect cell shape, coordinately influence membrane events that affect cell volume.

In summary, direct physiological measurement of cellular volume in the J774.2 macrophage has revealed two pathways for volume regulation. Although certain implications of this regulation for morphogenesis and metabolism are already apparent, the study of control of cell volume is likely to develop a new level at which biochemical and physiological mechanisms can be integrated to explain cell shape and behavior.

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