Free and Polymerized Tubulin in Cultured Bone Cells and Chinese Hamster Ovary Cells: The Influence of Cold and Hormones

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ABSTRACT Free and polymerized tubulin were measured in bone cells and Chinese hamster ovary (CHO) cells cultured on plastic substrata. Polymerized tubulin was stabilized in a microtubule-stabilizing medium (MSM) containing 50% glycerol and separated from free tubulin by centrifugation. Tubulin content was assayed in both fractions by the colchicine-binding assay. The measured degree of polymerization in both bone cells and CHO cells varied with stabilization conditions. The degree of polymerization in attached cells was found to increase up to 73% during the first 20 min after addition of MSM at 24°C, and remained constant thereafter. Stabilization at 0°C resulted in a decrease down to 62% in the degree of polymerization during the first 20 min after addition of the MSM, which again remained constant thereafter. Confluent bone cells maintained at 0°C for 1 h before stabilization contained significantly less polymerized tubulin than control cells kept at 37°C using stabilization both at 0°C and at 24°C. Changes in bone cell morphology induced by incubation of cells with prostaglandin E_1 or E_2, parathyroid hormone, and dibutyryl cyclic AMP were not associated with a change in the degree of tubulin polymerization. This was confirmed morphologically by immunofluorescence using affinity-purified tubulin antibodies: microtubules in hormone-treated cells were not noticeably reorganized when compared to microtubule organization in control cells. They were, however, squeezed closer together in cellular pseudopods due to the altered cell shape. This altered cell shape appears to be correlated with disorganization of the microfilament system, since microfilaments, detected using affinity-purified actin antibodies, did alter drastically their appearance and distribution after hormone addition.

Recently, a number of hormones that are known to elevate intracellular cAMP have been shown to elicit a shape change in a variety of cell types (14, 20, 21, 22). This hormone- and nucleotide-induced change in morphology has been postulated to be dependent on microtubules and/or microfilaments (14). The latter possibility has been questioned (5, 14).

Cyclic nucleotides, in particular cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP), and Ca^{++} have been implicated in the regulation of assembly and disassembly of the microtubular system in cells (17; see reference 32 for review). However, some discrepancies are apparent. For example, Weissmann et al. (30) found in complement-stimulated human neutrophils that treatment with cAMP in combination with theophylline seemed to have an inhibitory effect on microtubule assembly. In contrast, the observations of Porter et al. (21) indicate that dibutyryl cAMP (DBcAMP) stimulates microtubule assembly in Chinese hamster ovary (CHO) cells. A similar discrepancy exists with regard to the role of cAMP in the change in shape occurring in activated platelets, which has been suggested to result from reorganization of microtubules (4, 31). Although Steiner (28) suggested recently that cAMP binds to and promotes polymerization of platelet tubulin in vitro, Kenney and Chao (13) have shown that cAMP did not alter the proportion of assembled microtubules and free tubulin when it was added to freshly isolated, intact platelets.

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Pipeleers et al. (18–20), using the finding that glycerol and DMSO stabilize microtubules (8), have developed a rapid assay for quantification of polymerized and depolymerized forms of tubulin in tissues in vivo and in isolated rat pancreatic islets, human lymphocytes, and human platelets. The technique is based on stabilization of both polymerized and depolymerized tubulins, and quantification of both forms by the colchicine-binding assay (2, 33).

In this paper, we have used a similar assay to determine whether hormone-induced changes in cell shape in cultured bone cell populations are associated with changes in the amount of detectable assembled tubulin. We have attempted to validate the assay in a number of ways. The results reported emphasize the importance of checking incubation conditions when using this assay in new test systems. Using conditions that, in our opinion, are most likely to allow a true estimate of the degree of tubulin polymerization, we found no change in equilibrium between polymerized and depolymerized tubulin after hormone stimulation of bone cells in vitro. In a subsequent series of experiments, cytoskeletal structures were studied morphologically using affinity-purified actin and tubulin antibodies. The results showed that, when bone cells assumed a stellate morphology after the addition of hormones, microtubules did not reorganize noticeably, but the microfilaments rearranged strikingly. Both the biochemical and the morphological data therefore indicated that hormone-induced changes in cell shape in cultured bone cell populations are not the result of a shift in equilibrium between polymerized and nonpolymerized tubulin.

MATERIALS AND METHODS

Biochemical Studies

BONE CELLS: Calvaria from fetal rats (Wistar Canada Breeding Laboratory, Ottawa, Canada) were dissected aseptically, minced under sterile conditions in 0.1 M sodium phosphate, pH 7.4, containing 0.15 M NaCl, and incubated with stirring at 37°C in an enzyme mixture containing collagenase, elastase, and DNAse (23). Eight calvaria were used per ml of enzyme mixture. Cells were isolated by five sequential 20-min digestions. After each digestion the suspension containing the cells was removed, filtered through a stainless steel screen (200 mesh), and the protease activity was stopped with an equal volume of fetal calf serum (FCS; Flow Laboratories, Inc., Rockville, MD). The cells were collected by centrifugation for 10 min at 200 *g* and resuspended in alpha minimal essential medium (α-MEM) containing 15% FCS, 100 μg/ml penicillin-G (Sigma Chemical Co., St. Louis, MO), 50 μg/ml Gentamycin (Sigma Chemical Co.) and 0.3 μg/ml Amphotericin B (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA). The five isolated cell populations were pooled, plated in 100-mm Falcon 3003 dishes (Becton, Dickinson and Co., Cockeysville, MD), and incubated at 37°C in a humidified atmosphere of air plus 5% CO_2. The medium was changed every 3 or 4 d. After 7–14 d some of the cultures were detached from the dishes by treatment with 0.25% trypsin (1:250; Difco Laboratories, Detroit, MI) in citrate saline supplemented with 1% glucose and plated at a density of 3×10^5 cells per dish to obtain first subcultures. After 7 d some of these were subcultured again to obtain second subcultures. Only primary cultures and first and second subcultures were used.

CHINESE HAMSTER OVARY (CHO) CELLS: CHO cells were obtained originally from Dr. P. Stanley (Department of Medical Genetics, University of Toronto). The cells were cultured in 100-mm Falcon plastic petri dishes in 10 ml of α-MEM supplemented with 15% FCS and antibiotics and incubated as described above. Twice a week the cells were detached from the dishes by treatment with trypsin, as described in the preceding section, and plated at a density of about 5×10^6 cells per dish. The medium was changed every day. For some experiments CHO cells were grown as a suspension (10^8 cells per 150 ml α-MEM supplemented with 15% FCS and antibiotics) in spinner flasks for 24 h at 37°C in an atmosphere of air plus 5% CO_2.

HORMONES: Bovine parathyroid hormone (PTH, TCA powder, 179 U/mg) was obtained from Wilson Laboratories (Chicago, IL). PTH was dissolved in 5 mM acetic acid to a final concentration of 200 U/50 μl and stored at −80°C. Cultured bone cells were treated with 4 U/ml for 1 h. Prostaglandin E_1 and E_2 (PGE_1, PGE_2) were kindly supplied by Dr. John E. Pike (Upjohn Co., Kalamazoo, MI). Either PGE_1 or PGE_2 was dissolved in 95% ethanol (1 mg/ml) and stored at −20°C. The hormone was added to the culture medium in a concentration of 2.5 μg/ml for 1 h. Bone cells were incubated with these hormones in the absence of FCS. Control cultures were incubated in the vehicles used to dissolve the hormones. N^6,O^2-dibutyryl adenosine 3′,5′-cyclic monophosphate (DBcAMP) was purchased from Sigma Chemical Co. and added to the culture medium in a concentration of 1 mM.

STABILIZATION OF POLYMERIZED TUBULIN: The medium was removed from the cultures and replaced by a microtubule-stabilizing mixture (MSM) composed of 50% glycerol, 5 mM $MgCl_2$, 0.1 mM EGTA, 0.3 mM guanosine triphosphate (grade II-S, Sigma Chemical Co.), 10 mM sodium phosphate, pH 6.8. The cultures were rinsed twice and stabilized in an excess of MSM at room temperature (22°–24°C) for 20 min unless stated otherwise. In some experiments MSM as described by Filner and Behnke (8) was used (50% glycerol, 10% dimethyl sulfoxide, 5 mM $MgCl_2$, 10 mM sodium phosphate, pH 6.8). The cells were harvested with a rubber scraper in 150–200 μl MSM, homogenized with a motor-driven Teflon pestle operated at 600 rpm (two strokes up and down), and centrifuged at 26,000 *g* in a Sorvall RC2-B centrifuge (17,000 rpm in a rotor SS-34 in 1.0 ml tubes; Dupont Instruments-Sorvall Biomedical Div., Dupont Co., Newtown, CT) at 20°C for 30 min. After centrifugation the supernatants were removed, their volumes measured using a 500-μl Hamilton syringe (Hamilton Co., Reno, NV), and the amount of tubulin was assayed. The pellets were resuspended in 200 μl of ice-cold 20 mM sodium phosphate, pH 6.8 (PNa) containing 0.2 M NaCl and 0.3 mM GTP for 20 min to depolymerize tubulin (absence of microtubules was checked by means of electron microscopy) and subsequently centrifuged at 26,000 *g* at 0°C for 30 min. The supernatants were removed and used for determination of tubulin content. Samples of 10^7 CHO cells grown for 24 h in spinner flasks as a suspension were collected by centrifugation for 10 min at 200 *g*. The medium was removed, and the cells were resuspended in 300 μl of MSM and processed as described above.

COLCHICINE-BINDING ASSAY: The relative amount of tubulin in the supernatants was determined by a modification of the colchicine-binding assay described by Borisy (2). Tritiated colchicine (ring C, methoxy-[^{3}H]) was purchased from Amersham Corp. (Arlington Heights, IL). Nonradioactive colchicine (Sigma Chemical Co.) was added to adjust the specific activity to 0.46 mCi/mmol. After addition of colchicine to a final concentration of 6.5×10^{-6} M, the first and second supernatants were incubated for 1.5 h in darkness at 37°C (saturation of binding sites was reached with a colchicine concentration of 5×10^{-6} M). After incubation, the mixture was cooled on ice and filtered through a stack of circular DEAE-impregnated Whatman DE81 paper filters (W & R Balston, Ltd., England). The stacks were washed seven times with 2 ml 20 mM PNa, pH 6.8, containing 0.1 M NaCl. The filters were removed, added to 7 ml of PCS solubilizer (Amersham Corp.), and the radioactivity bound to each filter was determined. The total amount of tubulin-[^{3}H]-colchicine complex formed during incubation at 37°C was determined as follows. The radioactivity adsorbed to the subsequent filters of a stack of five filters decreased logarithmically (Fig. 1). The amount of radioactivity that would have been bound to a filterstack composed of filters was estimated by means of linear regression analysis after logarithmic transformation of the cpm per filter. This method diminished variation between samples due to differences in efficiency of adsorption of the tubulin-colchicine complex to the filterstack. In all following experiments, the radioactivity bound to a stack of three filters was used to calculate these values. All results were corrected for background which was determined by filtering samples of [^{3}H]-colchicine solution through the stack. Colchicine binding activity in the final pellets was negligible. Only 0.80% (SD = 0.14; n = 12) of the total bound radioactivity in the cell preparations was found in these pellets.

During preliminary studies using crude high-speed rat brain supernatant, it was found that glycerol markedly interfered with binding of colchicine to tubulin

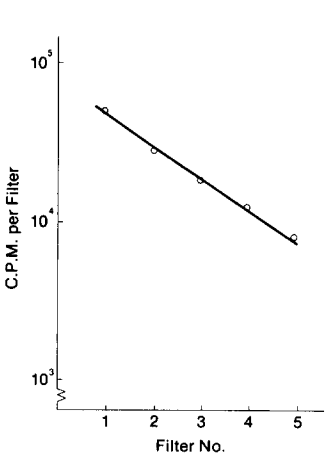

FIGURE 1 Adsorption of tubulin-bound [^{3}H]colchicine to five subsequent filters of a filterstack.

(see also, 7, 12, 16, 25). The initial binding capacity as determined by a time-decay binding assay (33) was less in glycerol-containing samples than in glycerol-free samples, despite the fact that the decay rate of the colchicine-binding activity at 37°C was slowed down under the influence of glycerol. Because in our assay procedure the concentration of glycerol in the first supernatants of the cell preparations was 50% and that in the second supernatants ~0–5%, systematic errors would have been introduced. Hence, all preparations were adjusted to the same concentration of glycerol (25%) before incubation with colchicine at 37°C by diluting the first supernatant 1:1 with PNa and the second supernatant with MSM. The rate of decay of the colchicine-binding activity in first and second supernatants, both containing 25% glycerol, was similar. In six experiments the average half-life was 4.4 h (SD = 1.2) for the first supernatant and 3.9 h (SD = 1.2) for the second supernatant. This difference was not significant (Student's t-test for paired observations, $t = 0.584$; $df = 5$; $p > 0.20$).

Immunofluorescence Studies

Freshly trypsinized (0.01% trypsin (Worthington Biochemical Corp., Freehold, NJ) in citrate saline) bone cells were plated onto glass cover slips and allowed to attach for at least 24 h in medium as above. 12–18 h before hormone treatment, the medium was replaced by fresh medium without FCS. Prostaglandin E_2 (PGE_2, final concentration 2.5 μg/ml) was added and cells were incubated at 37°C for 1 h. Cells either untreated or treated with hormone were fixed for 5 min at −10°C in methanol and then rinsed in PBS. Rabbit antibodies to actin or tubulin were added at ~0.05 mg/ml for 45 min at 37°C. After rinsing in PBS, preabsorbed fluorescein-labeled goat anti-rabbit IgG's were added at approximately 0.5 mg/ml for 45 min at 37°C. Finally, cells were rinsed in PBS and mounted in Moviol 4-88 (Canadian Hoechst Ltd., Montreal, PQ). The affinity-purified rabbit antibodies to porcine brain tubulin and chicken gizzard actin and the procedures for immunofluorescence experiments have been described in detail (34).

RESULTS

Variations in the Assay Method

(*a*) CELL ATTACHMENT: In our initial experiments with bone cell cultures, the cells were harvested and homogenized within 30 s after addition of MSM at room temperature. The percentage of polymerized tubulin in these cultures varied from 20 to 60%, with an average value of 39% (Table I). It was observed that under the influence of MSM the cells became firmly attached to the bottom of the culture dish in some but not all cultures. This was noted when the cells were scraped from the bottom of the dishes. In the firmly attached cultures 32% of the tubulin was found to be in a polymerized state, whereas for the cultures in which the cells could easily be removed from the dishes the percentage of polymerized tubulin was 52%.

(*b*) EFFECT OF INCUBATION TIME IN MSM: When bone cells were removed before the addition of MSM or after a 5-min incubation in MSM, we never encountered firmly attached cells. It was therefore decided to test the effect of time of incubation in MSM at room temperature on the amount of polymerized tubulin (Fig. 2). The amount of polymerized tubulin increased during the first 20 min and remained constant thereafter.

(*c*) EFFECT OF CELL ATTACHMENT: To test whether this phenomenon is observed only in cells cultured and assayed in monolayers, CHO cells grown as monolayers and as suspension cultures were tested for the degree of tubulin polymerization after stabilization in MSM for 30 s and 20 min (Table II). A significant increase in the amount of polymerized tubulin during stabilization was observed in cells cultured in monolayers but not in cells grown as a suspension. Identical results were obtained when cultures were stabilized according to Filner and Behnke (9) in MSM, which contains 10% DMSO but no EGTA and GTP (results not shown).

(*d*) EFFECT OF STABILIZATION TEMPERATURE: One possible explanation for the increase of polymerized tubulin during incubation of the cells in MSM at room temperature may be that under the influence of glycerol, free tubulin in the cells aggregates. This possibility was investigated by incubating confluent cultures of bone cells for different time periods in ice cold MSM and then transferring them to 37°C for 30 min. The results, given in Fig. 3, demonstrate that at 0°C the amount of polymerized tubulin decreased from ~69% after 5 min to 63% after 20 min of incubation, and remained constant thereafter. When cells, stabilized for 5 or 30 min in MSM at 0°C, were transferred to 37°C for 30 min, repolymerization of tubulin occurred (Fig. 3). Thus MSM does not prevent tubulin from depolymerizing or repolymerizing under these conditions.

(*e*) TUBULIN POLYMERIZATION AFTER HOMOGENIZATION: The stability of the polymerized and nonpolymerized states at 0°C and room temperature after homogenization in MSM was tested as follows. Confluent cultures of bone cells were treated with ice-cold MSM for 30 min and then homogenized. The homogenate was incubated further at 0°C or at 24°C. As is shown in Fig. 4, the degree of polymerization remained essentially constant at 24°C, but decreased progressively at 0°C. In all further experiments, the homogenized tubulin preparations were kept at room temperature.

(*f*) EFFECT OF TUBULIN CONCENTRATION ON THE OBSERVED DEGREE OF POLYMERIZATION: We also tested

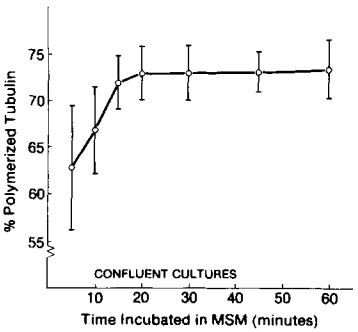

FIGURE 2 Percentage of polymerized tubulin in confluent cultures of bone cells incubated for various time intervals in MSM at room temperature. Bars indicate standard deviations (three cultures per time interval).

TABLE I

Degree of Tubulin Polymerization in Confluent Cultures of Bone Cells Stabilized for 30 S in MSM at Room Temperature

	% Polymerized tubulin ± SD	No. of cultures
All cultures	39 ± 13	21
"Firmly attached" cultures	32 ± 8*	14
"Loosely attached" cultures	52 ± 8*	7

Culture and assay conditions are described in the text.
* Significantly different from one another, $p < 0.001$.

TABLE II

Percentage of Polymerized Tubulin in CHO Cells Cultured in Suspension or in Monolayers and Stabilized in MSM for 30 S or 20 Min

	Duration of incubation in MSM (24°C)*	
	30 s	20 min
Suspension cultures	68.9 ± 5.2 (3)	68.7 ± 4.7 (3)
Confluent monolayers	53.4 ± 10.2 (3)‡	74.6 ± 2.6 (4)‡

* Assay and incubation conditions are described in the text. Results represent the mean ± standard deviation. The number of measurements is given in brackets.
‡ Significantly different from one another, $p < 0.01$.

whether the tubulin concentration affected the observed degree of polymerization. Confluent cultures of bone cells were treated with MSM for 30 min. The cells were pooled and homogenized. Aliquots ranging from 20–250 μl were diluted with MSM to a final volume of 250 μl and the degree of tubulin polymerization was determined in all samples. A positive correlation was found between the percentage of polymerized tubulin and the concentration of tubulin expressed as total bound cpm (first and second supernatants combined) per μl of cell homogenate (Fig. 5; $r = 0.78$; $df = 6$; $p < 0.05$). This indicates that at low concentration polymerized tubulin depolymerizes in MSM at room temperature.

Effect of Treatment with Cold on the Degree of Tubulin Polymerization

As "stabilization" of polymerized tubulin in the cell layers at 0°C and 24°C resulted in different values, we decided to test both procedures on cells treated with cold. When confluent cultures of bone cells were placed on ice for 1 h and then stabilized in MSM at 0°C for 30 min, 46% of the tubulin was in a polymerized form (Table III). In cultures not pretreated with cold but also stabilized in ice-cold MSM for 30 min, 62% of the tubulin was in a polymerized state. When cells pretreated with cold were "stabilized" in MSM at room temperature, 69% of the tubulin was found polymerized, slightly less than the value of 75% obtained for cells not treated with cold (Table III). Loss of polymerized tubulin in nonstandardized cultures at 0°C is in agreement with the well-known observation that polymerized microtubules may depolymerize at low temperature (eg. reference 16; 6 for review).

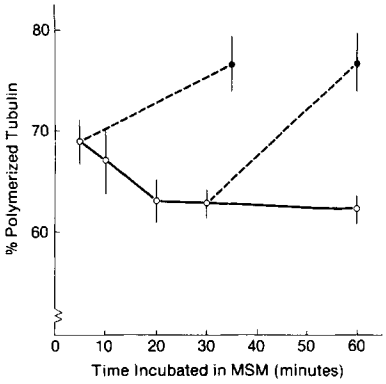

FIGURE 3 Percentage of polymerized tubulin in confluent cultures of bone cells incubated for varying times in ice-cold MSM. The cultures were rinsed twice with MSM at 0°C and subsequently incubated on ice. After 5 or 30 min some of the cultures were further incubated at 37°C for 30 min. Bars indicate standard deviations (five cultures per time interval distributed over three experiments). (○) 0°C, (●) 37°C.

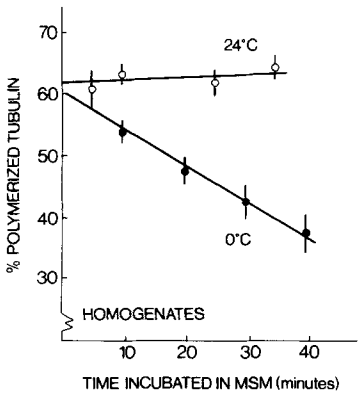

FIGURE 4 Influence of temperature on the degree of tubulin polymerization in homogenates of bone cells. Confluent cultures (5×10^6 cells) were stabilized in ice-cold MSM for 30 min. The cells were harvested, homogenized in 200 μl of MSM, and incubated at room temperature or 0°C for various time intervals. The homogenates were centrifugated at 20°C and processed as described in Materials and Methods. Bars indicate standard deviation (two or three cultures per treatment per time interval distributed over two experiments).

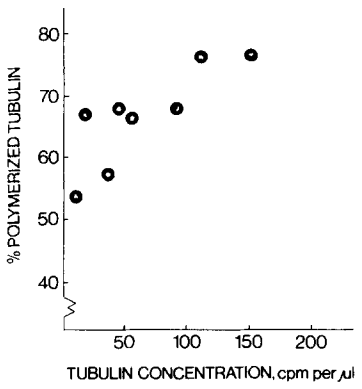

FIGURE 5 Relationship between concentration of tubulin and its degree of depolymerization. Confluent cultures of bone cells were treated with MSM for 30 min at room temperature. The cells were pooled and homogenized. Samples of 20–250 μl were diluted with MSM to a final volume of 250 μl and assayed for free and polymerized tubulin. The concentration of tubulin is expressed as total cpm bound (first and second supernatants combined) per μl of cell homogenate.

TABLE III

Influence of Cold Treatment on the Percentage of Polymerized Tubulin in Cultured Bone Cells§

Treatment	30 min MSM	% Polymerized tubulin, ± SD(N)
none	0°C	62.4 ± 3.5 (8)*
	24°C	75.2 ± 3.1 (8)*
1 h at 0°C	0°C	45.7 ± 2.8 (8)‡
	24°C	69.0 ± 1.2 (4)‡

* Significantly different from each other, $p < 0.01$.
‡ Significantly different from each other, $p < 0.001$.
§ Experimental details are described in text.
The number of determinations is given between brackets.

Effect of PGE_1, PTH, and DBcAMP on Tubulin Polymerization in Bone Cells

PTH and DBcAMP induced a transient change in morphology (from a spherical into a stellate shape) of bone cells incubated in a serum-free medium (14, 23). We determined the degree of tubulin polymerization in cultured bone cells treated with PGE_1, PTH, or DBcAMP. The percentage of stellate cells and the percentage of polymerized tubulin is given in Table IV. In these experiments, PGE_1 or DBcAMP caused >90% of the cells to change shape, whereas only 34% of the cells treated with PTH changed their shape. Despite these striking changes in morphology, in none of the treated cultures did marked changes in the degree of tubulin polymerization occur.

Morphological Studies on the Integrity of the Microtubule System in Hormone-treated, Stellate Cells

Because no change could be observed biochemically in the degree of tubulin polymerization in cells induced to become stellate by hormone treatment, we decided to study the integrity of the microtubule system in the stellate cells using immuno-

TABLE IV

Effect of Parathyroid Hormone, Dibutyryl cAMP, and Prostaglandin E_1 on Morphological Transformation and Degree of Tubulin Polymerization in Confluent Monolayers of Bone Cells

Treatment	% Stellate shaped cells*	% Polymerized tubulin‡
PGE_1, 2.5 μg/ml	94	77.3 ± 4.1 (12)
Control	6	73.7 ± 4.7 (12)
DBcAMP, 1 mM	94	78.1 ± 2.9 (13)
Control	2	75.9 ± 3.8 (13)
PTH, 4 U/ml	34	73.7 ± 4.2 (4)
Control	9	72.3 ± 0.9 (4)

* Cells were counted 60–90 min after addition of PGE_1 (2.5 μg ml^{-1}), DBcAMP (1 mM), PTH (4 U ml^{-1}) or their respective vehicles. The cultures were coded and randomized. Cells were counted in randomly chosen areas of 0.36 mm^2 in three dishes per treatment (~200 cells per dish) by means of an eyepiece grid at a magnification of × 200. The effect of treatment was tested using the chi-square test. Results represent the mean of three determinations.

‡ Cultures were preincubated for 12 h in the absence of FCS. The drugs were added 1 h before stabilization in MSM. Control cultures received vehicles only. Results are given as mean ± standard deviation. The number of measurements is given between brackets.

fluorescence. Fig. 6 *a* and *d* shows control osteoblastlike cells fixed and stained with rabbit tubulin antibodies (Fig. 6 *a*) or rabbit actin antibodies (Fig. 6 *d*) followed by fluorescein-labeled goat anti-rabbit IgG's. Treatment for 1 h with 2.5 μg/ml PGE_2 induced these cells to assume the stellate shape. No detectable alteration in microtubule integrity could be seen in the stellate cells (Fig. 6 *b* and *c*) but a striking rearrangement of actin-containing microfilaments was noted (Fig. 6 *e*).

DISCUSSION

A number of attempts have been made to determine the size of the pools of free and polymerized tubulin in tissues in vivo and cells grown in vitro. Though various assays have been used, including radioimmunoassays (11, 15), measurement of the apparent tubulin content from PAGE (see reference 9 for review), and estimation of the amount of tubulin by the colchicine-binding assay (2, 33), all are difficult to utilize to determine an absolute value for the tubulin: microtubule content (see reference 9 for discussion). In this report, we have been interested in comparing the ratio of unpolymerized versus polymerized tubulin, and thus, have concentrated on validating the stability of the two pools under our assay conditions, which rely on the use of glycerol-DMSO-based buffers. In tissues and cells previously analyzed in this way, stabilization has been done routinely at room temperature.

We have found that the observed degree of tubulin polymerization in bone cells and CHO cells cultured on a solid substratum varied widely and depended entirely upon the stabilization conditions used. During prolonged "stabilization" in MSM at room temperature the detectable amount of polymerized tubulin increased for the first 20 min in MSM. A comparable phenomenon may be that observed by Rebhun et al. (24) who, when studying the effect of glycols on marine eggs, observed a rapid increase in both volume and birefringence of the mitotic apparatus. One possible explanation for this phenomenon could be that part of the free tubulin pool polymerized into either microtubules or other aggregates during stabilization in MSM. An alternate explanation could be that the stabilization of the polymer is not complete until 20 min at room temperature. In support of the first explanation is the observation that glycerol does enhance the process of microtubule assembly in vitro in tubulin preparations at or above room temperature (26). Pipeleers et al. (20) have demonstrated that polymerization of tubulin in vitro is dependent on the tubulin concentration, and accordingly always used a buffer to tissue volume ratio >10 for tissue homogenization in MSM. It seems likely therefore that polymerization of intracellular tubulin during stabilization in MSM, but before homogenization, can indeed affect the observed degree of tubulin polymerization. Rubin and Weiss (25) could not observe a significant difference in the numbers of microtubules in sections of MSM-treated and control CHO cells. It may well be, however, that other forms of aggregation of tubulin are induced by glycerol. That nonspecific aggregates of tubulin may exist has been suggested by Weisenberg (29) and also by Staprans and Dirksen (27).

"Stabilization" of polymerized tubulin at 0°C resulted in an apparent loss of polymerized tubulin in agreement with the work of Pipeleers et al. (18–20). It has been reported that treatment with cold does not induce depolymerization of polymerized tubulin in CHO cells (25), although it was not clear in that study what temperature was used during incubation in MSM of cells treated with cold. We cannot exclude the possibility that at 0°C some aggregation of tubulin still may occur. Grisham and Wilson (10) reported that in the presence of GTP, EGTA, and $MgCl_2$ (without addition of glycerol) polymerization of tubulin into cold stable microtubules may occur in ice-cold 80,000 *g* supernatants of bovine brain.

Homogenized tubulin preparations were stable at room temperature, when at least 5 × 10^6 cells were homogenized in 200 μl MSM. Significant depolymerization occurred at room temperature when the tubulin concentration was lowered by diluting the extracts with MSM. However, when homogenized tubulin preparations were stored at 0°C, significant depolymerization occurred, even at the higher tubulin concentrations.

Bearing in mind a number of the reservations outlined above, it may be difficult or impossible to determine the absolute amounts of free or polymerized tubulin by this technique. This is illustrated by the results in Table III, in which it can be seen that, although pretreatment with cold resulted in a decrease in the amount of detectable polymerized tubulin when compared with controls, the values of the ratio were clearly dependent on the temperature during stabilization in MSM. Nevertheless, the approach does seem valid for comparing changes in the ratios of the two pools in response to certain stimuli provided control and experimental cultures are handled identically during the stabilization step and subsequently.

We have found, in agreement with Miller et al. (14), that a dramatic change in bone cell morphology occurs within 1 h after addition of PGE_1, PTH, or DBcAMP in serum-free medium (22). It was reported that this conversion of bone cells from a spherical into a stellate shape could be blocked by colchicine (14), suggesting that the process is microtubule-dependent. We have found that the changes in bone cell shape induced by hormone or DBcAMP are not accompanied by changes in the degree of tubulin polymerization under assay conditions employing "stabilization" at room temperature. Our immunofluorescence studies following over 30 min–6 h the change from well-spread to stellate and back to well-spread, have indicated that no striking rearrangements in microtubules are detectable during hormone-induced morphological changes (Fig. 6, this paper; and, Aubin, J. E., E. Alders, and J. N. M. Heersche. 1982. *Exp. Cell Res.* In Press.). On the other hand,

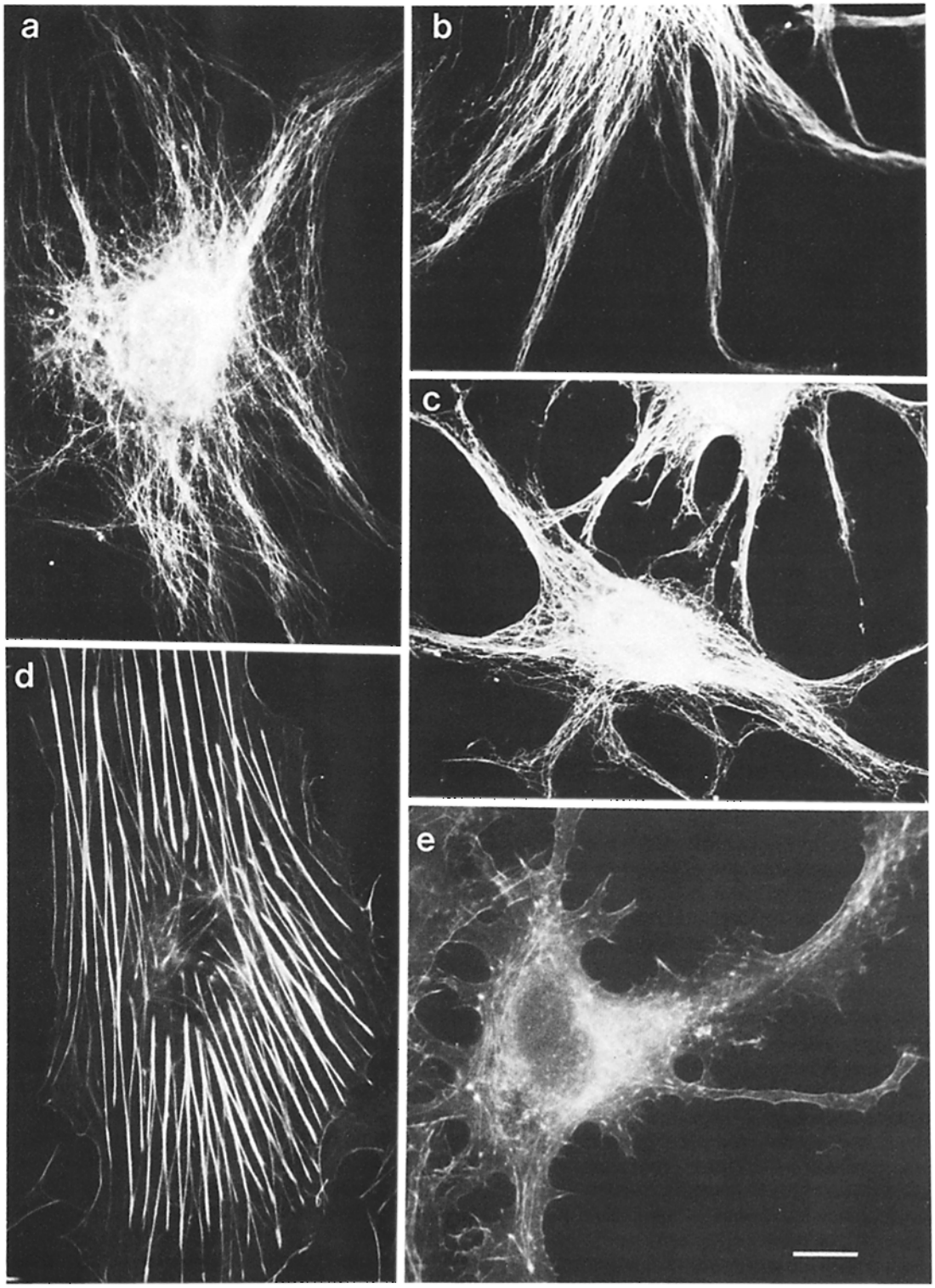

FIGURE 6 Osteoblastlike cells either untreated (*a*,*d*) or treated with 2.5 μg/ml PGE_2 for 1 h (*b*, *c*, and *e*) were labeled with rabbit tubulin (*a*–*c*) or actin (*d* and *e*) antibodies followed by fluorescein-labeled goat anti-rabbit IgG's. Normal radial arrays of microtubules were evident in untreated cells (*a*); intact microtubules were easily visible in stellate-shaped cells, although microtubules were squeezed closer together in pseudopods (*c*, edge of cell in *b*). The actin-containing stress fibers seen in untreated cells (*d*) were totally disrupted in PGE_2-treated cells in which only smaller and shorter bundles or aggregates were visible against a more diffusely staining background (*e*). Bar, 15 μm. × 685.

during retraction of the cytoplasm after hormone addition, the microfilament system as labeled with actin antibody was strikingly altered. Stress fibers were largely or totally disrupted during the shape changes, leading to small actin-containing bundles or aggregates that were disorganized and unaligned.

Different mechanisms have been proposed previously to explain morphological changes induced by DBcAMP. Based on electron microscopy analyses, it was suggested that DBcAMP caused assembly and alignment of the microtubule system in CHO cells during reverse transformation. A similar

observation, though not quantitatively evaluated, has been made by Borman, Dumont, and Hsie (3). On the other hand, an immunofluorescence analysis using antibodies to myosin and to tubulin suggested that DBcAMP did not cause striking rearrangement of microtubules in CHO cells, but did cause a major reorganization of myosin into myosin-containing stress fibers (1). In a number of other systems, disparate observations have been made with respect to no effect on, or increased polymerization of, microtubules (13, 30, see Introduction). It is not clear why these apparent discrepancies occur. Our data would suggest that microtubules are not reorganizing directly in response to hormones. The data obtained by structural analysis would suggest that reorganization of microfilaments is responsible for these shape changes. It should be noted, however, that the shape changes induced by hormone can be inhibited by breaking down microtubules using such agents as colcemid before hormone treatment (Alders, E., and J. E. Aubin, unpublished; reference 14). Thus, microtubule depolymerization may lead secondarily to disruption of microfilament organization, suggesting that, in some situations, the two systems are interconnected.

We thank Drs. M. Osborn and K. Weber for actin and tubulin antibodies used in this study. WB was supported by a fellowship from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.)

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Received for publication 13 January 1982, and in revised form 9 July 1982.

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