MICROTUBULE ASSEMBLY IN CULTIVATED GREENE MELANOMA CELLS IS STIMULATED BY DIBUTYRYL ADENOSINE 3':5'-CYCLIC MONOPHOSPHATE OR CHOLERA TOXIN

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ABSTRACT

Both dibutyryl cyclic AMP (DBcAMP) and cholera toxin promote the formation and elongation of processes of cultivated Greene hamster melanoma cells. The formation and maintenance of these processes, which contain many microtubules, are sensitive to colcemid and vinblastine. Tubulin was measured by [^{3}H]colchicine binding and by acrylamide gel electrophoresis. We found that DBcAMP or cholera toxin increases the ratio of polymerized to unpolymerized tubulin but not the total amount of tubulin per cell. The sum of the lengths of microtubules per unit area was significantly greater in cells treated with DBcAMP than in control cells. Our findings support the hypothesis that cyclic AMP promotes the elongation of cell processes by stimulating the assembly of microtubules from existing tubulin.

In addition to their effect on cell division (9, 39, 48), cyclic adenosine 3'5'-monophosphate and its dibutyryl derivative (DBcAMP) alter the morphology of many different cell types. Cyclic AMP induces the formation of long, narrow processes in mouse neuroblastoma (31, 43), Chinese hamster ovary cells (CHO) (25, 45), and fetal rat brain cells (46). Mouse sarcoma cells become spindly with elongated cell bodies and long, narrow processes (28). Microtubules apparently serve in both the formation and maintenance of these processes (10). Microtubules are abundant in thin sections of neuroblastoma "neurites" (44); vinblastine sulfate, a drug that forms crystalline structures with tubulin (3), inhibits the induction of long processes in neuroblastoma cells by DBcAMP (43).

The correlation between microtubules and cell processes suggests three possible mechanisms whereby cyclic AMP causes a morphological change. (*a*) Cyclic AMP permits or promotes the reorientation of assembled tubules. (*b*) Cyclic AMP stimulates the *de novo* synthesis of tubulin, shifting the equilibrium toward the assembly of microtubules. (*c*) Cyclic AMP stimulates the assembly of tubules without affecting the synthesis of tubulin.

We report here the effects of cholera toxin, which increases intracellular levels of cyclic AMP in melanoma cells (2, 36), and of exogenous DBcAMP on the morphology of cultivated Greene melanoma cells. We present evidence that (*a*) microtubules are involved in the formation

and maintenance of cyclic AMP-induced cell processes, (*b*) that microtubules observed in thin sections are significantly longer in cyclic AMP-treated cells than in untreated cells, (*c*) that the ratio of polymerized to free tubulin is significantly greater in cells treated with cyclic AMP than in untreated cells, and (*d*) that cyclic AMP does not increase the total amount of tubulin per cell. We conclude that either DBcAMP or cholera toxin stimulates process formation by promoting the assembly of microtubules from pre-existing tubulin. These observations have been presented previously in preliminary form (12).

MATERIALS AND METHODS

Cell Culture

A hamster melanoma described in 1958 by Greene (18) and maintained in Syrian Golden hamsters (obtained from Dennen) was adapted to cell culture. Cells were grown in minimal essential medium (Grand Island Biological Co., Grand Island, N. Y.), with the following additions: 5% fetal calf serum, 2.2 mg/ml sodium bicarbonate, and antibiotics consisting of either penicillin (199 U/ml) and streptomycin (100 μg/ml) or 50 μg/ml gentamycin (Schering Corp., Nutley, N. J.). Cells were grown as a monolayer in Falcon plastic flasks (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) at 37°C in a humidified atmosphere containing 5% CO_2. Culture medium was changed every other day.

Colchicine Binding Assay

Cells were harvested in CMF-EDTA Tyrode's solution and sedimented at 4°C. All subsequent operations were carried out at 4°C. Melanoma cells or tumor were washed twice in CMF-EDTA Tyrode's solution and homogenized or sonically disrupted in 2.0 ml of SMT (0.24 M sucrose, 0.001 M $MgCl_2$, 0.01 M Tris, pH 7.0). The homogenate was examined by phase microscopy to verify cell breakage. $CaCl_2$, final concentration 8 mM, was added to depolymerize tubules, and the homogenate was then centrifuged for 20 min at 2,500 *g*. The pellet was stored at 4°C and the supernate was centrifuged at 100,000 *g* for 60 min. The pellet from this step was resuspended in SMT (+8 mM calcium) (2ml/10^8 cells) and combined with the first pellet. 8 mM calcium did not influence the distribution of colchicine-binding activity in the supernate and pellet of the 100,000 *g* centrifugation. The resuspended pellet was again sonically disrupted. The 100,000 *g* supernate and the resuspended pellet were used in separate binding assays, and their protein concentrations were determined by the method of Lowry et al. (33). The colchicine-binding assay was performed as described by Borisy (4) in the absence and presence of 5×10^{-4} M vinblastine sulfate (Eli Lilly & Co., Indianapolis, Ind.). Binding per milligram protein was determined from decay curves of binding activity extrapolated to 0 incubation time (55). Colchicine binding in the supernate and pellets was standardized using the binding at 0°C as background. [^{3}H]colchicine (sp act 5-16 Ci/mmol) was obtained from New England Nuclear Corp., Boston, Mass.

DETERMINATION OF RATIO OF UNPOLYMERIZED TO POLYMERIZED TUBULIN: 2.1×10^7 cells were harvested in CMF-EDTA Tyrode's solution at 37°C. Pellets were resuspended in 0.3 ml MTS buffer (50% glycerol, 5% dimethyl sulfoxide, 0.5 mM EGTA, 0.5 mM guanosine triphosphate, and 0.5 mM $MgCl_2$ in 10 mM sodium phosphate buffer, pH 6.95) and sonically disrupted at room temperature until cells were no longer recognizable by phase microscopy. The homogenate was fractionated as described in Fig. 5 (41). The homogenized cells were centrifuged at 8,500 *g*, for 10 min at 25°C and the supernate (SN1) was kept at 0°C. The pellets were resuspended in 0.3 ml of TS buffer (0.25 M sucrose, 0.5 mM GTP, 0.5 mM $MgCl_2$, 0.05% bovine serum albumin in 10 mM phosphate buffer, pH 6.95) at 4°C and centrifuged at 8,500 *g*, 10 min, at 4°C. The supernate (SN2) contained tubulin disassembled from tubules by low temperature. The final pellet was resuspended (P2) in 0.3 ml of TS buffer. Colchicine binding activity of the SN1, SN2, and P2 fractions was measured by the method of Sherline et al. (49). 100 λ of each fraction were incubated at 30°C for 1.5 h with 10 λ of [^{3}H]colchicine (1.6×10^{-5} mg [^{3}H]colchicine, 1×10^5 cpm). 1 ml of activated charcoal (3 mg/ml distilled H_2O) was added to each sample. After 10 min, the samples were centrifuged at 1,100 *g* for 10 min. A 200 λ portion was removed from each sample, and radioactivity was determined in 10 ml of Triton X-100 fluor (4). Binding was directly proportional to the amount of protein in the range of 50-400 λ of homogenate for each of the fractions. SN1 represents free tubulin, SN2 represents assembled tubulin subsequently disassembled by low temperature, and P2 represents residual tubulin in the pellet not solubilized by the addition of cold TS buffer.

[^{3}H]Leucine Incorporation

[^{3}H]Leucine (sp act 33 Ci/mmol) was obtained from New England Nuclear Corp. Cells were cultured in medium containing 1 μCi/ml [^{3}H]leucine for 24 h, harvested in CMF-EDTA Tyrode's solution at 37°C, and sedimented. The pellet was resuspended in 2 ml of saline (4°C), and an aliquot (15 λ) was removed for measurement of cell number. Cells were lysed by freezing and thawing in distilled H_2O, and protein was precipitated with 5% trichloracetic acid (TCA). Incorporation of isotope into protein was determined by the method of Mans and Novelli (34) using glass fiber filters (Reeve Angel glass fiber filter grade 943 AH 214 Em). The filters were placed in Liquifluor, and radioactivity was measured in a Nuclear Chicago Spectrometer (Nuclear Chicago Corp., Des Plaines, Ill.).

Measurement of Cell Processes

Cell processes were measured at 100× magnification with a calibrated ocular micrometer using a Nikon MS-D inverted phase-contrast microscope.

Electron Microscopy and Estimation of Relative Number of Microtubules

Cells on a plastic substratum were fixed in 3% glutaraldehyde at 37°C, stained with 1% osmium tetroxide, dehydrated in ethanol, and embedded in Epon. They were sectioned in a plane parallel to their substratum with an LKB Ultrotome III equipped with a diamond knife. The ultrathin sections were stained with uranyl acetate and lead citrate and examined in a Hitachi HU-11 B electron microscope.

To compare the amount of microtubules in cells exposed to DBcAMP and untreated cells, the total length of microtubules in prints was measured and divided by the total cell area observed. Tubules in the cell bodies of untreated cells were compared with those in the processes of cells treated with DBcAMP for 16 h. Total area was calculated by tracing the cell areas from prints of final magnification of 40,000 onto paper and weighing the paper. The measurements are presented as length of tubules in centimeters per square centimeter.

Chemicals

$N^6,O^{2'}$-Dibutyryl adenosine-3',5'-cyclic monophosphoric acid, adenosine 3',5'-cyclic monophosphoric acid, cycloheximide, Colcemid and colchicine were obtained from Sigma Chemical Co., St. Louis, Mo., and vinblastine sulfate (Velban) was a gift from Eli Lilly & Company. Butyric acid obtained from Fisher was neutralized with 1N NaOH. Theophylline was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. Crude racemized MSH was provided by Dr. Saul Lande of this department.

CHOLERA TOXIN: Purified cholera toxin lot 0572 (Syn. choleragen, cholera exoenterotoxin, cholera enterotoxin, cholera permeability factor, PF) was prepared under contract for the National Institute of Allergy and Infectious Diseases by R. A. Finkelstein, Ph.D., The University of Texas Southwestern Medical School, Dallas, Texas (13). The toxin was dissolved in 0.152 M NaCl, 0.019 M NaH_2PO_4, 0.081 M NaH_2PO_4 at pH 7.15 to give a concentration of 200 μg/ml. The solution was passed through a Millipore filter (0.22 μm) and stored in 1-ml lots in sterile glass vials at −70°C. The activity of the toxin was not reduced by storage under these conditions for at least 10 mo, nor by three cycles of thawing and freezing.

Preparation of Lumicolchicine

Lumicolchicine and [^{3}H]methyl lumicolchicine were prepared as described by Wilson and Friedkin (56).

Acrylamide Gel Electrophoresis

Protein samples were reduced in 1% sodium dodecyl sulfate (SDS) and 2.5% mercaptoethanol. Electrophoresis was carried out on 7.5% acrylamide gels containing 0.1% SDS (2.5 ma/gel). Gels were stained with fast-green for quantitative estimation of protein (16) and scanned with a Beckman gel scanner (Acta C-III Model, Beckman Instruments, Inc., Palo Alto, Calif.) at 645 nm. Molecular weight determinations of tubulin were carried out using standards according to the method of Weber and Osborn (53). Guinea pigs' brain tubulin, isolated according to the method of Shelanski et al. (47), was used as a standard.

MEASUREMENT OF INTRACELLULAR CYCLIC AMP: Cells grown on plastic Falcon tissue culture dishes were rinsed twice with phosphate-buffered saline (pH 7.4) and the protein was precipitated with 5% TCA (4°C). The cells were scraped off the plastic in 5% TCA and the suspension was sedimented at 2,000 *g*, 4°C, for 20 min. Protein content of the pellets was measured by the method of Lowry et al. (33). The supernates were extracted five times with 10 ml each of ethyl ether and then passed through 3-cm columns of Dowex resin 1-8× (200–300 mesh) in 0.1 M formic acid. Cyclic AMP was eluted from the columns with 5 ml of 1 N formic acid and the eluates were lyophilized. Lyophilisates were resuspended in 2 ml of 0.1 N tris pH 7.4. Assays were performed on these samples with a cyclic AMP-binding protein (provided by Dr. John Pawelek of this department), according to the method of Brown et al. (6).

RESULTS

Effects of DBcAMP on Morphology of Melanoma Cells

Greene melanoma cells are polygonal and occasionally possess elongated cellular processes (Fig. 1 *a*). After 24 h of treatment with DBcAMP, the cells have more and longer narrow processes than control cells (Fig. 1 *b*).

In order to quantitate this effect, DBcAMP (1 mM) was added to Greene melanoma cells 24 h after plating (5 × 10^5 cells/flask), and cells were examined at intervals up to 72 h. Cells with processes projecting greater than 20 μm from the cell body were counted. 2–8% of a control population of cells had processes longer than 20 μm. Response to any treatment is expressed as the percentage of cells with processes 20 μm or longer above or below control level, which has been adjusted to zero (Table I).

Table I shows that, as early as 3 h after treatment with DBcAMP or DBcAMP plus theophylline (Table II), there was a significant increase in

the percentage of cells with long processes (20.12% ± 4.7%). The maximum percentage was observed between 16 and 18 h (34.4% ± 7.2%), and the level declined thereafter despite further addition of DBcAMP.

Analogues and components of DBcAMP were tested for their ability to induce process formation in Greene melanoma cells (Table II). cAMP (1 mM) plus theophylline acted like DBcAMP; AMP (1 mM) had no effect. Sodium butyrate (2 mM) stimulated process formation but significantly less so than DBcAMP or cAMP. Theophylline (6×10^{-4} M), an inhibitor of phosphodiesterase activity, potentiated the effect of cAMP but was ineffective alone.

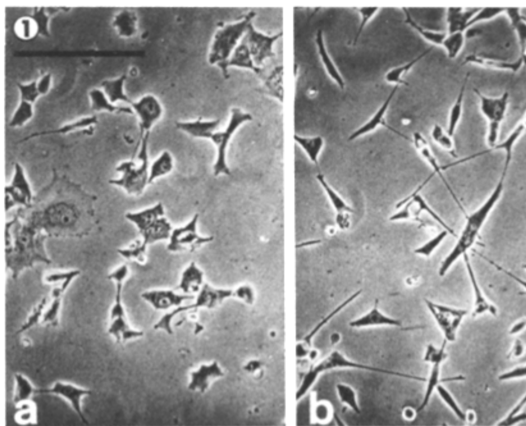

FIGURE 1 Phase-contrast photomicrographs of cultivated Greene melanoma cells. Cells were plated at 5.0×10^5 cells/flask (25 cm^2 area). (*a*) Control cells after 24 h. (*b*) Cells exposed to DBcAMP (1 mM) and theophylline (6×10^{-4} M), 24 h. Bar represents 200 μm.

Effect of Cholera Toxin and MSH on Morphology of Greene Melanoma Cells

Both cholera toxin (2, 36) and melanocyte stimulating hormone (MSH) (36, 57) are known to bind to the surface of Cloudman melanoma cells and to increase intracellular levels of cAMP. These compounds were tested for their ability to stimulate process formation in Greene melanoma cells. Table III shows that cholera toxin (1.25 μg/ml) stimulates process formation to roughly the same degree as DBcAMP (1 mM) while MSH (2×10^{-8} M) does so only after 40 h. Even at this time, the stimulation by MSH is only one-third of that of DBcAMP or cholera toxin. Cholera toxin causes a 10.5-fold increase in intracellular cyclic AMP while MSH stimulates only a 2.5-fold increase (Table IV) after 1 h of exposure.

Effect of Mitotic Inhibitors on Induction of Process Formation

The role of microtubules in the formation and maintenance of processes in DBcAMP-treated cell populations was explored with the use of the alkaloids vinblastine sulfate, Colcemid, and colchicine. Greene melanoma cells were treated with Colcemid (10^{-7} M) and DBcAMP (1 mM) or vinblastine sulfate (10^{-8} M) and DBcAMP (1 mM). Table I shows that both vinblastine sulfate and Colcemid completely blocked the induction of cell processes. Lumicolchicine (10^{-7} M) does not prevent the induction of processes by DBcAMP. The stability of DBcAMP-induced processes in the presence of a mitotic inhibitor was also tested. Cells cultivated in the presence of DBcAMP for 16 h were treated with 10^{-7} M Colcemid. Proc-

TABLE I

Effect of Inhibitors of Mitosis and Protein Synthesis on Cell Process Formation

Treatment	Percent of cells with processes ≥20 μm					
	3–6 h	18 h	24 h	30 h	48 h	72 h
DBcAMP (1×10^{-3} M)	20.12 ± 4.7	34.4 ± 7.2			32 ± 13	17 ± 6.7
Cycloheximide (2×10^{-6} M)	−1.9 ± 1.6	−7.6 ± 1.2		−8.9 ± 47	−6.2 ± 3.4	
DBcAMP & cycloheximide	3.2 ± 1.5	6.9 ± 3.1		−0.8 ± 1.6	−3.0 ± 2.0	
Colcemid (10^{-7} M)	−6.05 ± 2.4	−8.7 ± 3.0		−6.3 ± 2.9	−3.1 ± 3.1	
DBcAMP & Colcemid	−3.4 ± 4.1	1.3 ± 0.0		−0.1 ± 2.1	0.3 ± 2.0	
Vinblastine sulfate (10^{-8} M)	−8.9 ± 1.2	−7.6 + 1.0	−4.4 ± 0.9			
DBcAMP & vinblastine sulfate	−6.6 ± 0.5	−1.05 ± 1.0	3.25 ± 1.0			
Lumicolchicine (10^{-7} M)	1.0 ± 1.0	2.5 ± 1.9		0.5 ± 1.0	4.1 ± 3.0	
DBcAMP & lumicolchicine	25.5 ± 5.0	39.0 ± 6.0		29.1 ± 3.1	30.0 ± 2.8	

Greene melanoma cells were plated at 5.0×10^5 cells per flask (25 cm^2 area); additions were made 24 h later. Medium was changed every other day. All cells exposed to cycloheximide were pretreated with this drug for 3 h. Cells with processes greater than or equal to 20 μm were scored. Each datum represents the mean of five measurements. 300–500 cells were counted for each measurement. Concentrations and standard deviations are shown. Values are expressed as percent more or less than control, which was adjusted to 0.

TABLE II

Effect of DBcAMP Related Compounds on Process Formation

Treatment	Percent of cells with processes ≥20 μm					
	15 h	24 h	39 h	48 h	63 h	72 h
AMP (1 mM)	1.8 ± 1.8	−0.7 ± 1.5	−0.6 ± 2.8		−1.2 ± 1.6	
DBcAMP (1 mM) + theophylline (6 × 10^{-4} M)	21.8 ± 2.5	26.2 ± 0.7	12.4 ± 1.4		9.2 ± 0.8	
Butyrate (2 mM)	5.7 ± 1.7	7.3 ± 1.6	6.7 ± 2.7		9.3 ± 1.1	
cAMP (1 mM) + theophylline (6 × 10^{-4} M)		19.0 ± 1.6		8.6 ± 1.6		6.2 ± 2.1
Theophylline (6 × 10^{-4} M)		5.4 ± 0.5		1.8 ± 2.2		5.5 ± 1.5
DBcAMP	19.2 ± 3.0	28.3 ± 2.0	10.5 ± 2.0		8.0 ± 1.0	
cAMP		10.1 ± 1.0		4.3 ± 1.0		2.0 ± 0.0

Conditions were the same as for Table I.

TABLE III

Effect of Cholera Toxin and Crude Racemized MSH on Process Formation

Treatment	Percent of cells with processes ≥20 μm				
	6 h	10 h	17 h	24 h	41 h
DBcAMP (1 mM)	12.0 ± 2.0	17.4 ± 1.6	34.0 ± 3.3	25.6 ± 3.1	19.6 ± 1.5
Cholera toxin (1.25 μg/ml)	12.3 ± 1.5	19.6 ± 3.0	34.2 ± 5.6	27.0 ± 3.0	19.1 ± 2.0
MSH (2 × 10^{-8} M)	2.4 ± 0.7	1.7 ± 1.0	1.7 − 0.5	5.5 ± 2.1	10.5 ± 1.0

Conditions were the same as for Table I.

TABLE IV

Effect of Cholera Toxin and Crude Racemized MSH on Intracellular Cyclic AMP Levels

	cAMP/dish	cAMP/mg protein	cAMP/10^6 cells	Increase above control
	pmol	*pmol*	*pmol*	
Control	3.6 ± 0.6	9.4 ± 1.6	2.4 ± 0.4	–
Theophylline (6 × 10^{-4} M)	5.2 ± 0.2	13.5 ± 0.5	3.5 ± 0.1	1.5×
MSH (2 × 10^{-8} M)	9.1 ± 0.9	23.7 ± 2.3	6.1 ± 0.6	2.5×
Cholera toxin (1.25 μg/ml)	37.8 ± 2.4	98.4 ± 6.3	25.2 ± 1.6	10.5×
MSH + theophylline	15.0 ± 3.0	39.1 ± 7.8	10.0 ± 2.0	4.2×
Cholera toxin + theophylline	42.3 ± 8.0	110.2 ± 20.8	28.2 ± 5.3	11.8×

Intracellular cyclic AMP content after a 1-h exposure to cholera toxin (1.25 μg/ml) or to crude racemized MSH (2 × 10^{-8} M). Cells were seeded into 80-mm diameter Falcon plastic tissue culture dishes (1.5 × 10^6 cells/dish) for 24 h. Cholera toxin or MSH was then added for 1 h, the medium was poured off, and cyclic AMP assays were performed.

esses disappeared within 1 h, and the cells acquired a polygonal morphology, despite the continued presence of DBcAMP (Table V). 10^{-7} M colchicine had the same effect. These results suggest that microtubules are necessary for both the formation and maintenance of processes in Greene melanoma cells.

Effect of Inhibitors of Protein Synthesis on DBcAMP-Induced Process Formation

In order to learn whether protein synthesis is necessary for the induction of long cell processes by DBcAMP, cycloheximide was added to the cultures. Incorporation of [^{3}H]leucine was inhibited by exposure to cycloheximide (2 × 10^{-6} M) for 24 h, regardless of the presence or absence of DBcAMP (Table VI). Table I shows that, in the presence of cycloheximide, DBcAMP increased process formation to only 6.9 ± 3.1% above the control level after 24 h; cycloheximide alone reduced it to 7.6 ± 1.2% below control level. The difference between cells exposed to cycloheximide alone and cells exposed to both cycloheximide and DBcAMP at what is usually

TABLE V

Effect of Colcemid on DBcAMP-Induced Processes

Treatment	Percent of cells with processes ≥20 μm			
	Time after addition of Colcemid			
	0 h	1 h	5 h	10 h
DBcAMP (1 mM) + theophylline (6×10^{-4} M)	27.2 ± 3.3	21.6 ± 1.5	27.8 ± 3.2	29.5 ± 5.4
Colcemid (10^{-7} M) + DBcAMP (1 mM) + theophylline (6×10^{-4} M)	27.2 ± 3.3	8.2 ± 1.8	5.8 ± 1.0	5.4 ± 1.1

Conditions were the same as for Table I. 24 h after seeding, flasks were treated with 1 mM DBcAMP and 6×10^{-4} M theophylline. After 16 h, the medium was changed, and 10^{-7} M Colcemid was added to half the flasks.

TABLE VI

[³H]Leucine Incorporation into TCA-Insoluble Protein

Additions to medium	Incorporation cpm/10³ cells	Inhibition
		%
None	178 ± 31	–
DBcAMP (1 mM) + theophylline (6×10^{-4} M)	190 ± 34.4	−9.3
Cycloheximide (2×10^{-6} M)	22.9 ± 0.8	88
DBcAMP (1 mM) + theophyline (6×10^{-4} M) + cycloheximide (2×10^{-6} M)	24 ± 0.03	88

Cells were plated at 5×10^5 per flask (25 cm² area). After 24 h, appropriate flasks received a 3-h exposure to 2×10^{-6} M cycloheximide. 3 h later, fresh medium with the indicated additions was added. The medium contained 1 μc/ml [³H]leucine. After 24 h, cells were removed from culture flasks with CMF-EDTA Tyrode's solution and counted in a haemocytometer. Pellets were assayed for [³H]leucine incorporation according to the method of Mans and Novelli (34). Each value represents the mean of two flasks.

the time of maximum process formation is significant (t = 8.9803, df = 8).

There was no significant difference in the rate of protein synthesis between control and DBcAMP-treated cells during the 24-h period when process formation reached a maximum; however, after 48 h, DBcAMP-treated cells incorporate 60% more [³H]leucine per cell than control cells, probably because DBcAMP arrests cell division.

Estimation of Amounts of Microtubules by Electron Microscopy

Large numbers of microtubules were revealed in processes induced by a 16-h exposure to DBcAMP (Fig. 2). In contrast, tubules were far less abundant in the cytoplasm of untreated cells. The average total length of tubules per unit area in thin sections of untreated Greene melanoma cells was only 47.2% of that observed in cells treated for 16 h with DBcAMP (processes of DBcAMP-treated cells vs. cell bodies of untreated cells). DBcAMP-treated and untreated cells had 19.5 ± 14.7 and 9.2 ± 6.3 cm of tubules per square centimeter, respectively. The difference is significant at the 0.05 level (t = 3.39069, df = 74).

Measurement of Total Tubulin

COLCHICINE BINDING ASSAY: In order to determine the amount of colchicine-binding protein per cell or per mg of cellular protein in DBcAMP-treated and control cultures of Greene melanoma cells, the quantitative colchicine binding assay of Borisy (4) was used. The technique depends on: (*a*) the specific affinity of colchicine for tubulin, and (*b*) the strong adsorption of microtubular protein to DEAE ion exchangers at neutral pH.

The validity of the binding measurements was tested by the following controls. (*a*) The binding of colchicine to bovine serum albumin or to the 100,000 *g* supernate heated to 70°C for 20 min, or incubated at 0°C rather than 37°C, was not significantly greater than background (incubation in the absence of protein). (*b*) Binding activity of [³H]lumicolchicine was only 5% that of [³H]colchicine and did not increase with increasing amounts of protein. (*c*) The amount of bound colchicine was directly proportional to the amount of cellular protein in the reaction mixture over a range of 0.1–1.5 mg protein. Accordingly, this range of protein concentration was used in the measurement of the amount of colchicine-binding protein from decay curves.

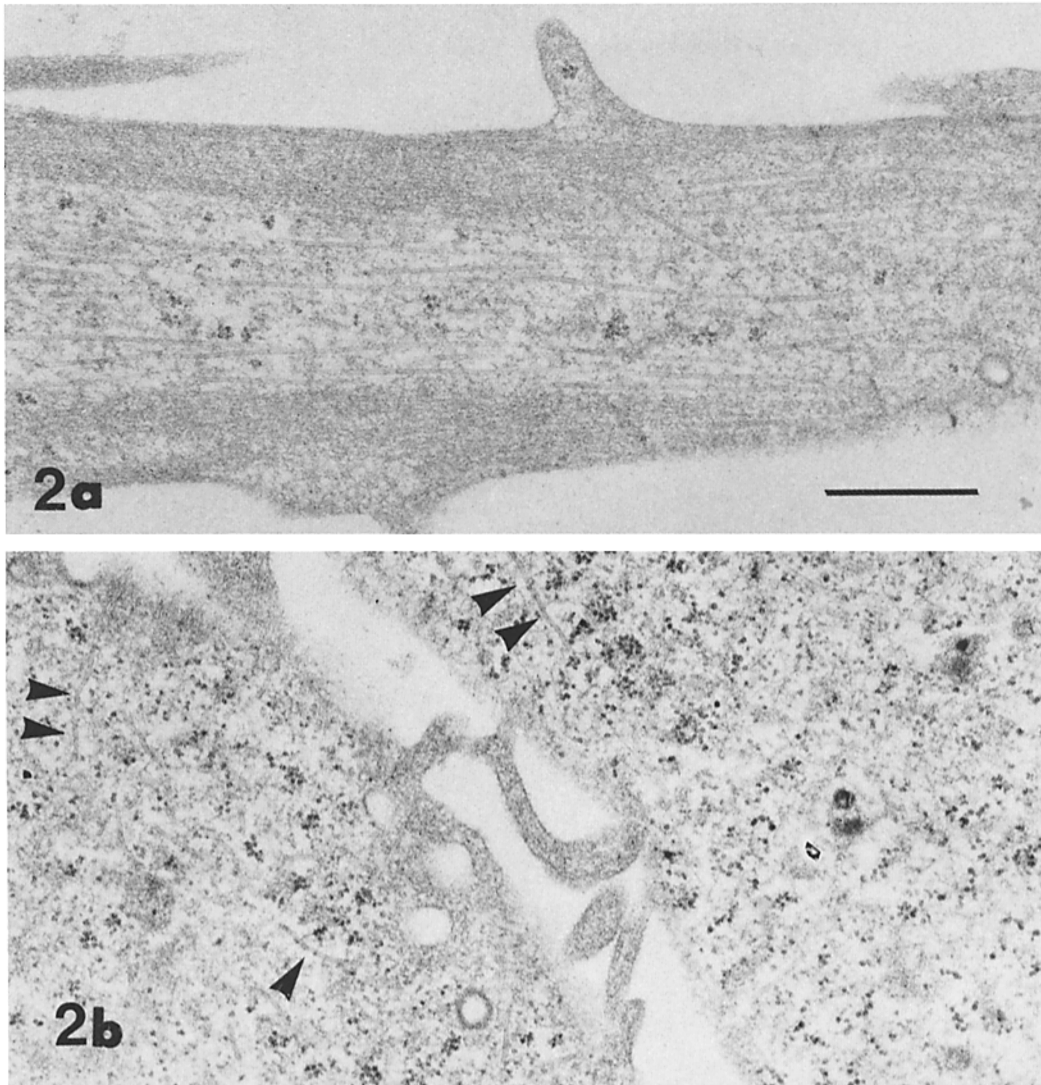

FIGURE 2 Electron micrographs of (*a*) DBcAMP-treated (1 mM, 16 h) and (*b*) untreated Greene melanoma cells. Note that the cell bodies of untreated cells have microtubules (arrows) but the tubules are more abundant and longer in the cell processes of DBcAMP treated cells. Bar represents 2.5 μm.

To compare the specific colchicine-binding activity of DBcAMP-treated, cholera toxin-treated, and untreated cells, it was necessary to determine the decay rates of colchicine binding and to extrapolate to zero incubation time (55). Fig. 3 shows the decay rates for these various treatments. It is clear in all cases that (*a*) the decay rates in treated and untreated cells are similar in both the pellets and the supernates, (*b*) vinblastine significantly stabilizes the binding activity (55), and (*c*) extrapolation to 0 incubation time gives similar specific binding activities. The specific binding activity per cell of DBcAMP-treated and cholera-toxin treated cells at the time of maximal process formation is, respectively, 100% and 90% that of control cells (Table VII).

The solid Greene tumor has significantly less binding activity (3,400 ± 200 cpm/mg protein and 1,800 ± 400 cpm/mg protein in the supernate and pellet, respectively) than both the mouse brain

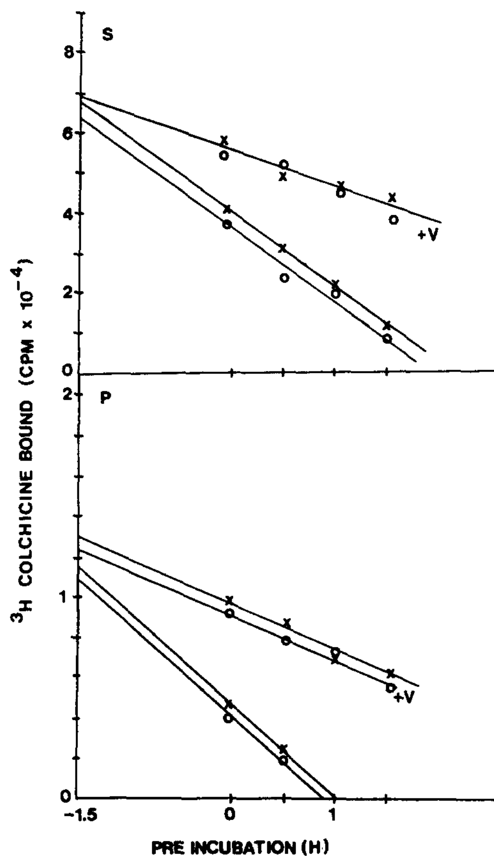

FIGURE 3 Colchicine binding decay curves of colchicine binding protein from untreated (x) and cholera toxin-treated (O) cells. S represents 100,000 *g* supernate, P pellet. All samples were incubated with [^{3}H]colchicine for 1.5 h; only the time of incubation at 30°C before the addition of [^{3}H]colchicine (preincubation) varied. Curves for protein from cells treated with DBcAMP (10^{-3} M) were similar to those of cells treated with cholera toxin but are eliminated from the figure for clarity. Samples contained the following amounts of protein: cholera toxin-treated cells (S = 0.965 mg/150 λ, P = 0.481 mg/150 λ); untreated cells (S = 0.975 mg/150 λ, P = 0.469 mg/150 λ). Curves denoted by +v represent decay in the presence of 5×10^{-4} M vinblastine sulfate.

(390,000 ± 1,500 cpm/mg protein and 201,000 ± 1,000 cpm/mg protein in the supernate and pellet, respectively) and the cultivated unstimulated Greene melanoma cells.

ACRYLAMIDE GEL ELECTROPHORESIS: Densitometric scans of SDS-acrylamide gels of the 100,000 *g* supernate of Greene melanoma cells (Fig. 4) show a tubulin band at 55,000 mol wt in cholera toxin-treated and untreated samples. This protein coelectrophoreses with guinea pig brain tubulin. Integrated optical densities at 645 nm obtained from gel scans were directly proportional to the amount of tubulin over a concentration range of 2–40 μg tubulin per gel. The [^{3}H]colchicine-binding data (Table VIII) shows that the amount of tubulin (in the soluble protein fraction) per cell is roughly the same in both cholera toxin-treated (16 h) and untreated Greene melanoma cells (roughly 10.4 μg/10^6 cells for controls; 10.5 μg/10^6 cells for cholera toxin-treated cells). Also, the colchicine-binding activity of the tubulin in the soluble protein fraction is not altered by cholera toxin-treatment for 16 h (roughly 1,154 cpm/μg tubulin for controls; 1,066 cpm/μg tubulin for cholera toxin-treated cells). Similar results were obtained with DBcAMP.

It is possible that some of the protein in the 55,000 mol wt band is not tubulin. This would invalidate any calculation of the specific colchicine-binding activity of tubulin in treated and untreated cells. Two considerations justify our estimates: first, our estimates of specific colchicine-binding activity (cpm/μg tubulin) agree well with those estimated by others (37) using purified neuroblastoma or porcine brain tubulin; second, the ratio of the heights of the 55,000 mol wt band of the treated and untreated cells with respect to other major bands on the gels is remarkably similar. If DBcAMP effected changes in the amount of tubulin, there would have to be a reciprocal drop in the nontubulin 55,000 mol wt protein for the total amount of 55,000 mol wt protein to be the same. This is improbable.

Measurement of the Ratio of Unpolymerized to Polymerized Tubulin

A buffer containing glycerol and DMSO was used to stabilize existing microtubules so that they could be separated from free tubulin by centrifugation (41). Using the colchicine-binding assay of Sherline et al. (49), the ratio of assembled to free tubulin was determined in cholera toxin-treated and untreated cells (Table IX, Fig. 5). Consistent with our ultrastructural observations, the amount of assembled tubulin is significantly greater in cells treated with cholera toxin.

The ratio of assembled to free tubulin must be accepted with reservations. Ideally, the cells should be sonicated at 37°C to avoid depolymerization of tubules. No colchicine-binding activity

TABLE VII

Specific Binding Activity of DBcAMP-Treated and Cholera-Toxin Treated Cells

Treatment	Fraction	mg protein/10⁶ cells	Median cell volume	cpm/mg protein	cpm/10⁶ cells
Control	Supernate	0.1480	1,300	71,432	10,572
	Pellet	0.1043		28,916	3,016
Cholera toxin 1 μg/ml, 16 h	Supernate	0.1500	1,415	64,280	9,642
	Pellet	0.1090		23,900	2,600
DBcAMP 1 mM, 16 h	Supernate	0.1520	1,420	68,300	10,382
	Pellet	0.1085		26,500	2,875

[³H]Colchicine binding cpm/mg protein was obtained by extrapolation to zero incubation time using the decay curves shown in Fig. 3.

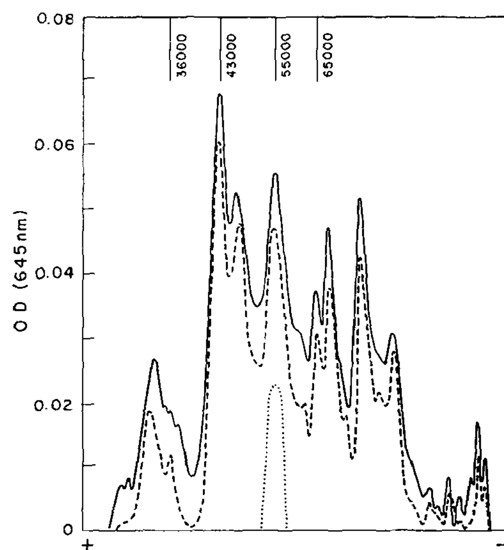

FIGURE 4 Densitometric scans (645 nm) of fast green-stained SDS acrylamide gels. Greene melanoma cells were cultured with and without 1.25 μg/ml cholera toxin. The 100,000 *g* supernates of the cells were brought to 1% in SDS and 2.5% in mercaptoethanol and applied to gels. When samples were heated to 50°C for 15 min before application to the gels, two bands rather than one were seen at the 55,000 mol wt weight region. (——) 66 μg of protein from control cells; (---) 55 μg of protein from cells exposed to cholera toxin; (····) 2.8 μg of tubulin from guinea pig brain. Molecular weights were determined from gels of standards of known molecular weight.

was found in cells sonicated at 37°C, so 25°C was chosen. Because lower temperatures reduce the stability of tubules, the difference in the percentage of free tubulin between cholera toxin-treated and untreated cells obtained at 25°C is probably a minimum. In fact, when cells were sonicated at 0°C in MTS buffer, the percentages of free tubulin in cholera toxin-treated and untreated cells were 37% and 47% respectively, as compared to 26% and 41%, respectively, at 25°C.

DISCUSSION

Role of Cyclic AMP in Process Formation

Cyclic AMP induces the formation of long, narrow processes in cultivated Greene melanoma cells. This conclusion is supported by several observations. First, experiments with compounds re-

TABLE VIII

Colchicine Binding Data and Acrylamide Gel Electrophoresis Data from a Single Experiment

	Control	Cholera toxin (16 h)
No of cells equivalent to 10 λ of 100,000*g* supernate	0.720 × 10⁶	0.696 × 10⁶
Median cell volume	1,415 μm³	1,550 μm³
μg soluble protein/10⁶ cells	139.0	143.7
μg protein/10 λ of 100,000*g* supernate	100.1	100.0
[³H]colchicine binding cpm/10⁶ cells	12,000 ± 840	11,200 ± 180
[³H]colchicine binding (cpm/mg soluble protein)	86,300 ± 840	77,900 ± 180
μg protein applied to gel	66.0	55.0
Estimated μg protein as tubulin (determined from standard curve of fast green-stained tubulin)	4.95	4.01
μg tubulin in soluble protein fraction/10⁶ cells	10.4	10.5
Percent of tubulin in 100,000 × *g* supernate	7.5	7.3
[³H]colchicine binding cpm/μg tubulin	1,154	1,066

Quantitation of tubulin by [³H]colchicine binding and from SDS acrylamide gel electrophoresis obtained on the same sample (see Fig. 4). Amount of tubulin on gels was estimated from a standard curve of absorbancy at 645 nm vs. known quantities of fast-green-stained tubulin.

TABLE IX
Ratio of Assembled to Free Tubulin in Cholera Toxin-Treated and Untreated Cells

	Combined length of proceses per 100 cells (μm) measured at 16 h	[^{3}H]colchicine bound				
		SN1	SN2	P2	(SN2)/(SN1)	Total
		cpm/10^6 cells				
Cholera toxin	1,120 ± 445	2,177	5,924	238	2.26	8,339
Control	450 ± 270	3,420	4,680	161	1.36	8,261

Cells were seeded 24 h before the start of the experiment at 2 × 10^6 cells/75 cm^2 flask. At zero time, cholera toxin (1 μg/ml) was added to one set of flasks, and, at 16 h, the combined lengths of all the cell processes were determined in both cholera toxin-treated and untreated flasks. 1,500 cells in each group were counted; the processes were measured from distal tip to the nearest edge of the nucleus. Cells were then removed from the flasks with CMF-EDTA-Tyrode's solution (37°C). An aliquot was taken to measure cell numbers, and the cell suspension was centrifuged. The sedimented cells were resuspended in MTS buffer, and the [^{3}H]colchicine binding of the various fractions was measured as described in Materials and Methods.

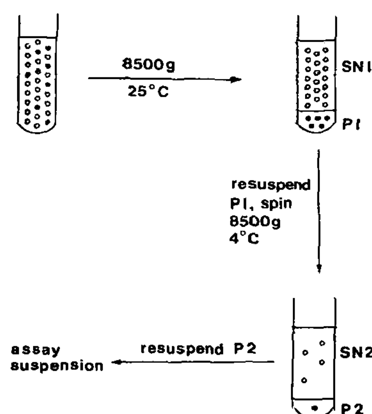

FIGURE 5 Flow diagram for estimation of free and assembled tubulin in undisrupted cells. Homogenized cells in MTS buffer (upper left corner) were centrifuged at 8,500 g, 10 min, 25°C. Unassembled tubulin (open circles) remained in the supernate (SN1). Assembled tubulin (black circles) was sedimented (P1). P1 was resuspended in TS buffer at 4°C and centrifuged at 8,500 g, 10 min, 4°C. Disassembled tubulin (open circles) remained in the supernate (SN2). The pellet from this centrifugation (P2), containing residual tubulin, was resuspended in TS buffer. This suspension, as well as SN1 and SN2, were assayed for colchicine-binding activity.

lated to DBcAMP suggest that process formation is caused primarily by cyclic AMP. The relatively low levels of cyclic AMP that accumulate in Greene melanoma cells exposed to theophylline (Table IV) suggest a low basal adenylate cyclase activity and are probably not sufficient to induce process formation. The butyrate moiety stimulates process formation but significantly less than either DBcAMP or 3',5'-cAMP. Although Prasad and Hsie (43) found that 1 mM sodium butyrate did not induce neurite formation in cultured neuroblastoma, Wright (58) reported that sodium butyrate, like DBcAMP, slowed the growth rate of CHO cells and caused them to become more spindly. But this morphological effect was noticeable only after 3 days, whereas the effect of DBcAMP was maximal after 1 day. Second, cholera toxin stimulated process formation in Greene melanoma cells. The toxin is known to bind to receptors on the cell surface, stimulating adenylate cyclase and increasing levels of cyclic AMP in Greene melanoma cells (Table IV). Cholera toxin, unlike DBcAMP, does not produce the multiplicity of N^6-substituted adenines (26, 38) that could perhaps influence process formation (29). Although DBcAMP yields a variety of related compounds in CHO cells, it also results in increased concentrations of cAMP (26, 38). Exposure to cholera toxin results in an increase in intracellular cAMP within 1 h. When Cloudman melanoma is exposed to MSH, cAMP is maximal at 30 min and then falls in spite of the continued presence of MSH. The increase in tyrosinase activity resulting from MSH does not occur until several hours after the major elevation of cAMP (40). It may also be the case in Greene melanoma that the morphologic changes occur later than the highest cAMP concentration; however, the time-course of cAMP stimulation has not been measured.

MSH, which mimics the effects of dibutyryl cyclic AMP in Cloudman melanoma, is a poor stimulator of process formation in Greene melanoma cells. This observation is consistent with the observation that the hormone does not raise intracellular cyclic AMP as much as cholera toxin.

Varga (personal communication, this department) has found that, although ^{125}I-MSH binds to Greene melanoma cells in culture, the binding cannot be blocked by increasing concentrations of unlabeled MSH. Thus, either the MSH binding is not specific or there are so few specific sites that they are undetectable. These considerations are consistent with the conclusion obtained from studies of Cloudman melanoma cells—namely, that cholera toxin and MSH do not bind to the same receptors. Neither [^{125}I]MSH (36) nor fluorescein-labeled MSH (Varga, DiPasquale, and McGuire, unpublished results) binding to Cloudman melanoma cells is inhibited by high concentrations of cholera toxin.

Role of Microtubules in Cell Process Formation

Microtubules are cytoskeletal elements believed to maintain cellular configuration in vitro and in vivo (7, 14, 17, 30, 51, 52, 59). Our results support this conclusion and suggest that microtubules are necessary for both the formation and maintenance of processes in Greene melanoma cells *in vitro*. The formation and maintenance of these processes are sensitive to tubulin-binding compounds and insensitive to lumicolchicine. In thin sections of long, narrow DBcAMP-induced processes of Greene melanoma cells there are numerous microtubules oriented parallel to the axis of the cell processes.

The induction of cell processes by DBcAMP is reduced by cycloheximide (see also reference 28). This does not mean that process formation requires the *de novo* synthesis of tubulin. The formation of cell processes may require the addition of new surface material at the leading edge (1, 11, 20, 22, 27) and adhesion to the substratum (8, 21, 23) as well as microtubules. If the ability of a cell to insert new surface material at the leading edge and to adhere to the substratum depend on continued protein synthesis, then the extension of the process would be restricted despite an adequate supply of pre-existing tubulin.

Role of Cyclic AMP in Assembly of Microtubules

Of the three postulated mechanisms whereby cyclic AMP effects a morphological change in cultivated Greene melanoma cells, our results favor a stimulation of microtubule assembly. Microtubules are more abundant in cells exposed to DBcAMP. Similar results with Chinese hamster ovary cells have been reported (5, 42). Colchicine-binding activity per cell and per milligram protein in both DBcAMP- and cholera toxin-treated cells is not significantly different from that in untreated cells, even at a time when in treated cells there is a significant increase in the number of cell processes 20 μm or longer. Finally, the ratio of assembled to free tubulin in cells exposed to cholera toxin is significantly greater than that in untreated cells. We conclude that cyclic AMP stimulates the assembly of microtubules from existing tubulin.

Other workers have reported that the amount of tubulin in polygonal and dendritic cells is the same. Mouse neuroblastoma cells are polygonal at optimal growth conditions but extend long processes when deprived of serum. Despite this change in shape, the amount of tubulin per cell is unchanged (35). Hier et al. (24) found that DBcAMP stimulates process formation in chick embryo sensory ganglia without increasing the colchicine-binding activity per ganglion; however, this observation was not verified by measuring decay rates of colchicine binding in the presence and absence of DBcAMP.

Two possible mechanisms through which cAMP might influence tubule assembly are: (*a*) the stimulation of a protein kinase with subsequent phosphorylation of a factor that promotes assembly; (*b*) reduction of calcium concentration in the cytosol with corresponding increase in assembly. Greengard and Kuo (19) and Goodman et al. (15) have shown that cyclic AMP in cell-free systems activates a protein kinase, which in turn phosphorylates serine residues in microtubular protein. Li et al. (32) have shown an in vivo activation of protein kinase activity in Chinese hamster ovary cells by DBcAMP. Phosphorylation might enhance the affinity of tubulin monomers for each other and thus shift the equilibrium toward assembly. More recently, Sloboda et al. (50) showed that, in an in vivo system, cyclic AMP stimulates the phosphorylation of a high molecular weight protein that co-purified with tubulin. However, this phosphorylation was apparently unnecessary for in vitro assembly, since tubules assembled in the absence of the high molecular weight protein. DBcAMP might exert its effect on assembly by altering membrane permeability to calcium or the cell membrane's capacity to bind calcium. Microtubules are unstable in calcium-containing solutions (54). If cAMP reduced intracellular calcium

levels by altering the membrane's permeability to calcium or capacity to bind calcium, then it might stimulate the assembly of microtubules.

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