

Interplay of Cyclic AMP and Microtubules in Modulating the Initiation of DNA Synthesis in 3T3 Cells

ZU-WU WANG and ENRIQUE ROZENGURT

Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, United Kingdom

ABSTRACT The results presented here show that disruption of the microtubule network acts synergistically with cAMP-elevating agents to stimulate the entry into DNA synthesis of 3T3 cells. Antimicrotubule agents and increased cAMP levels require an additional growth-promoting factor for inducing initiation of DNA synthesis; such requirement can be furnished by insulin, vasopressin, epidermal growth factor, platelet-derived growth factor, or fibroblast-derived growth factor. The involvement of the microtubules is indicated by the fact that enhancement of the DNA synthetic response was demonstrated with the chemically diverse agents colchicine, nocodazole, vinblastine, or demecolcine, all of which elicited the response in a dose-dependent manner. We verified that colchicine and nocodazole, at the doses used in this study, induced microtubule disassembly in the absence as well as in the presence of cAMP-elevating agents as judged by measurement of [³H]colchicine binding of total and pelletable tubulin. The involvement of cAMP was revealed by increasing its endogenous production by cholera toxin or by treatment with 8BrcAMP. The enhancing effects of antimicrotubule drugs and cAMP-elevating agents could be demonstrated by incorporation of [³H]-thymidine into acid-insoluble material, autoradiography of labeled nuclei, or flow cytometric analysis. The addition of antimicrotubule drugs does not increase the intracellular level of cAMP nor does addition of cAMP-elevating agents promote disassembly of microtubules (as judged by measuring [³H]colchicine binding of total and pelletable tubulin) in 3T3 cells. In view of these findings and the striking synergistic effects between these agents in stimulating DNA synthesis in the presence of a peptide growth factor, we conclude that increased cAMP levels and a disrupted microtubule network regulate independent pathways involved in proliferative response.

Quiescent 3T3 cells resting in the G₀/G₁ phase of the cell cycle can be stimulated to reinitiate DNA synthesis when combinations of growth-promoting factors that act synergistically are added to cultures maintained in serum-free medium (31, 32). Understanding the mechanisms whereby extracellular factors modulate cell proliferation requires identification of the intracellular signals important for initiating a mitogenic response. Recent work from this laboratory has shown that an increase in the cellular level of cAMP acts synergistically with insulin and other growth-promoting agents to stimulate quiescent cultures of 3T3 cells to reinitiate DNA synthesis and cell division (33–36). The elevated cAMP levels were induced by either increasing its endogenous production by cholera toxin (36), adenosine agonists, (34) or prostaglandin E₁ (33) or by exogenous addition of cAMP derivatives, such as 8BrcAMP (35). The mitogenic effectiveness of these compounds was markedly potentiated by inhibitors of cyclic nucleotide phosphodiesterase

(33–36). These findings suggest that a sustained increase in the intracellular level of cAMP acts as one of the mitogenic signals for Swiss 3T3 cells.

Several other studies suggest that changes in the state of organization of the cytoplasmic microtubules may act as a signal in the regulation of the transition of cells from G₀/G₁ phase to DNA synthesis. In cultures of 3T3 cells, disruption of the microtubular network with colchicine or other antitubulin agents markedly enhances the DNA synthetic response to various growth-promoting factors including epidermal growth factor (EGF), and insulin (13–15, 24, 26, 39), fibroblast-derived growth factor (FDGF, 13, 15), platelet-derived growth factor (PDGF, 8), phorbol esters (7), and vasopressin (7). In addition, microtubule stabilization by taxol inhibits initiation of DNA synthesis by EGF (5). Thus, disassembly of cytoplasmic microtubules can enhance the stimulatory effect of a variety of mitogenic agents in fibroblastic cells.

The interplay between cyclic nucleotides and microtubule organization in cell regulation has been the subject of considerable literature. Because increased levels of cAMP can change the arrangement and length of microtubules (3, 9, 21, 27, 38, 42) and since antimicrotubule drugs block a variety of cellular effects of cAMP (16, 18, 19, 22, 28, 29), it is plausible that a change (increase or decrease) in the state of organization of the microtubules may play a necessary role in modulating or mediating several cellular actions of cAMP. Conversely, the fact that disruption of microtubules markedly potentiates the effect of various hormones in increasing cAMP levels in a variety of cell types (17, 20, 23, 30, 37) raises the possibility that certain cellular effects of antimicrotubule drugs could be mediated by cAMP. Thus, microtubules may play a role in mediating certain actions of cAMP and, conversely, certain effects of antimicrotubule drugs could be exerted via cAMP.

Since both cAMP-elevating agents and antimicrotubule drugs stimulate events leading to initiation of DNA synthesis in 3T3 cells (see above), it was of importance to determine whether these agents could have opposite, synergistic, or common steps in their mechanism of mitogenic action. The experiments presented here were designed to examine the interaction of microtubules and cAMP in modulating the stimulation of DNA synthesis in 3T3 cells.

MATERIALS AND METHODS

Cell Culture: Swiss 3T3 mouse cells (41) were propagated in 90-mm Nunc Petri dishes in Dulbecco's modified Eagle's medium (DME), 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin in a humidified atmosphere of 10% CO₂ and 90% air at 37°C. Cells were subcultured at 10⁵ in 30-mm Nunc Petri dishes with DME containing 10% fetal bovine serum. After 6 d, the cultures became confluent and quiescent at G₀/G₁ phase. Such quiescent cultures were washed twice with DME at 37°C to remove residual serum immediately preceding experiments with growth factors.

Incorporation of [³H]Thymidine into DNA: Confluent and quiescent cultures of Swiss 3T3 cells in 30-mm Nunc Petri dishes were washed twice with DME at 37°C. Then the cultures were incubated in 2 ml of a 1:1 mixture of DME and Waymouth medium (25) containing [³H]thymidine (TdR:1 µM, 1 µCi/ml) and various agents as indicated. After 40 h at 37°C, [³H]TdR incorporation into acid-insoluble pools was assayed as follows: cultures were washed twice with phosphate-buffered saline (PBS) (0.15 M NaCl in 0.01 M K₂PO₄ buffer, pH 7.4), and acid-soluble radioactivity was removed by a 20-min exposure to 5% trichloroacetic acid (TCA) at 4°C. The acid-insoluble material remaining on the dishes was washed twice in alcohol, and then solubilized by a 30-min incubation with 0.1 M NaOH/2% Na₂CO₃, and the radioactivity was assayed (7).

Flow Cytofluorometric Assay: Cells were detached by minimal treatment with trypsin (0.025%) and 0.4 mM EDTA, suspended in DME containing 10% bovine serum, centrifuged at 1,000 rpm for 2 min, washed twice with PBS, suspended in 1 ml of saline, and rapidly squirted through a 25-gauge needle into 3 ml at a 70:30 ratio of ethanol/saline. This suspension could be stored at 4°C for 1–2 wk. Immediately before a cytofluorometric assay, the ethanolic suspension was subjected to centrifugation at 1,000 rpm for 2 min and the cell pellet was resuspended in 0.5 ml of a mixture containing 50 µg/ml of mithramycin in a 25:75 ratio of ethanol/water plus 15 mM MgCl₂ (40). After 30 min, DNA histograms were obtained with a Fluorescence Activated Cell Sorter (Becton-Dickinson & Co., Oxnard, CA).

cAMP Radioimmunoassay: Confluent and quiescent cultures of Swiss 3T3 cells in 30-mm dishes were washed twice with DME at 37°C and then incubated in 2 ml of DME/Waymouth medium in the presence of various additions and for various times. At the end of the specified incubation period, the medium was rapidly removed and cAMP was extracted with 200 µl of 0.1 M HCl (4). After 20 min, the HCl solution was neutralized and the precipitated protein remaining on the dish was dissolved in 1 ml of 0.1 M NaOH/2% Na₂CO₃. Appropriate dilutions of the neutralized HCl extract were acetylated and cAMP was determined in duplicate by radioimmunoassay.

Tubulin Assay: The fraction of the total intracellular pool of tubulin present as polymerized microtubules was measured as described by Eichhorn and

Peterkofsky (10, 11). Briefly, the medium was rapidly removed and replaced by 0.2 ml of microtubule-stabilizing medium (MTM) (12) consisting of 50% glycerol, 10% dimethylsulfoxide, 5 mM sodium phosphate, 5 mM MgCl₂, pH 6.9, at 22°C. The cells were harvested by scraping, four dishes were pooled, and the suspension was sonicated at room temperature. The sonicate was centrifuged at 100,000 g for 60 min at 22°C to pellet the intact microtubules. The supernate was removed and the recovered pellet was resuspended in 100 µl of ice-cold PM buffer (10 mM sodium phosphate, 10 mM MgCl₂, pH 6.9) with 1 mM guanosine triphosphate and 0.5% Triton X-100 and then resonicated at 0°C to ensure total disruption of the microtubules (10). Tubulin in this solution was measured by the [³H]colchicine binding assay using DEAE-cellulose filters as described by Borisy (1). For determination of the total cell tubulin pool, duplicate dishes were treated as described above, except that MTM buffer was replaced by PM and sonication was performed at 0°C to insure that all tubulin would be soluble. These values and those obtained from the MTM-treated samples were used to obtain the percentage of total tubulin polymerized into microtubules.

Indirect Immunofluorescent Staining: Swiss 3T3 cells were grown on 10-mm glass coverslips. After the cells were incubated with drug for the desired time, the coverslips were washed three times in PBS and fixed in 2.5% (wt/vol) glutaraldehyde at 37°C for 20 min. The coverslips were washed with PBS, treated with 0.1 M glycine for 10 min, and then with 0.1% Triton X-100 for 2 min. The coverslips were covered with 15 µl of rabbit antiserum to tubulin (kindly provided by Dr. R. Brooks, Imperial Cancer Research Fund) that was diluted 1:10 in PBS. After 60 min at 37°C in a humidified atmosphere, the coverslips were extensively washed with PBS, drained, and covered with 15 µl of rhodamine-conjugated antiserum to rabbit immunoglobulin G for 60 min at 37°C, rinsed with PBS, mounted on glass slides in a drop of PBS/glycerol (1:9), and examined with a Zeiss photomicroscope.

Materials: Bovine insulin (25.5 international U/mg), cholera toxin, 8BrcAMP, colchicine, demecolcine, vinblastine sulfate, and nocodazole were purchased from Sigma Chemical Co., St. Louis, MO. 3-Isobutyl-1-methyl-xanthine (IBMX) was obtained from Aldrich Chemical Co., Inc., Milwaukee, WI. PDGF was partially purified (700-fold) by sulfadex gel chromatography and heating as described by Deuel et al. (6). FDGF was partially purified as previously described (2). [³H]thymidine (20 Ci/mmole), [³H]colchicine (37.2 Ci/mmole), and antigens and antibodies for radioimmunoassay of cAMP were obtained from New England Nuclear (Boston, MA).

RESULTS

Effect of cAMP-elevating Agents, Antimicrotubule Drugs, and Peptide Factors on [³H]TdR Incorporation by 3T3 Cells

To determine the interaction between the intracellular levels of cAMP and the state of organization of the microtubules in modulating the initiation of DNA synthesis in Swiss 3T3 cells, confluent and quiescent cultures of these cells were transferred to medium supplemented with insulin, cholera toxin, or both and exposed to various concentrations of the antitubulin agents colchicine, vinblastine, demecolcine, and nocodazole. As shown in Fig. 1, addition of antimicrotubule drugs in the absence or presence of cholera toxin was not sufficient to elicit a proliferative response in cultures maintained in serum-free medium. However, microtubule-disrupting agents caused a marked and dose-dependent enhancement of the incorporation of [³H]TdR into acid-insoluble material when added to cultures stimulated by both cholera toxin and insulin. Results identical to those shown in Fig. 1 were obtained when 8BrcAMP at 2 mM was added instead of cholera toxin (results not shown).

In the experiments described in Fig. 1, the interaction between cAMP-elevating agents and antitubulin drugs was studied in the presence of insulin. Therefore, it was of interest to determine whether insulin could be replaced by other growth-promoting factors. The experiment described in Fig. 2 shows that a marked synergistic interaction between colchicine and cAMP-elevating agents can be demonstrated when vasopressin, FDGF, PDGF, or EGF are added instead of insulin.

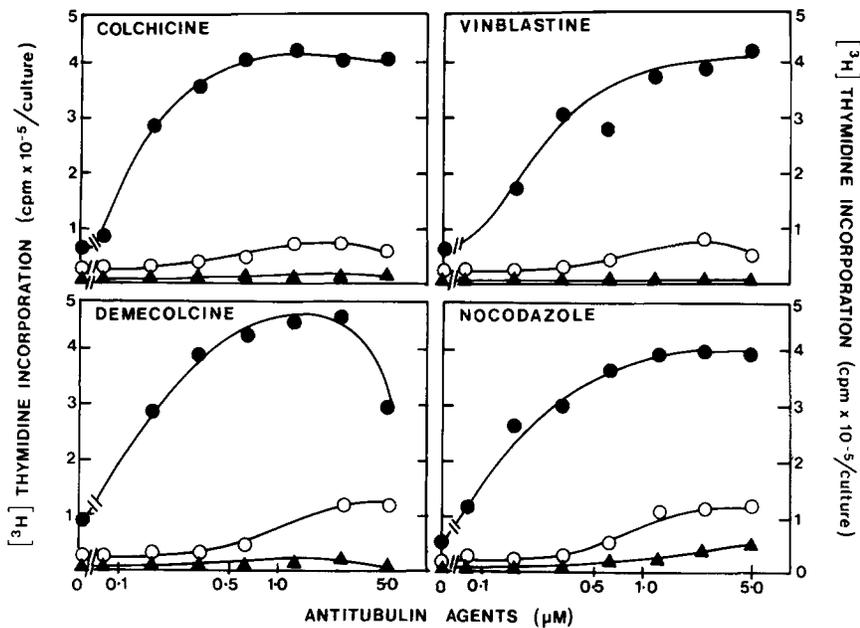


FIGURE 1 Effect of colchicine, demecolcine, vinblastine, and nocodazole on $[^3\text{H}]$ TdR incorporation into acid-precipitable material in the presence of cholera toxin, insulin, or both in Swiss 3T3 cells. Measurement of $[^3\text{H}]$ TdR incorporation into acid-insoluble material after a 40-h incubation was done as described in Materials and Methods. The final concentration of the antitubulin agents was varied from 8×10^{-8} to 5×10^{-6} M as indicated. \blacktriangle , 100 ng/ml of cholera toxin; \circ , 1 $\mu\text{g}/\text{ml}$ of insulin; \bullet , 100 ng/ml of cholera toxin plus 1 $\mu\text{g}/\text{ml}$ of insulin.

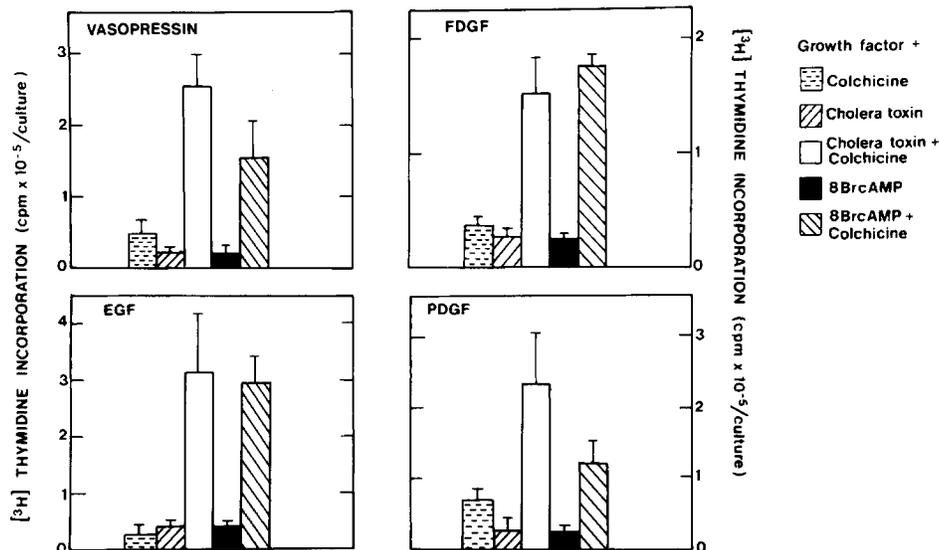


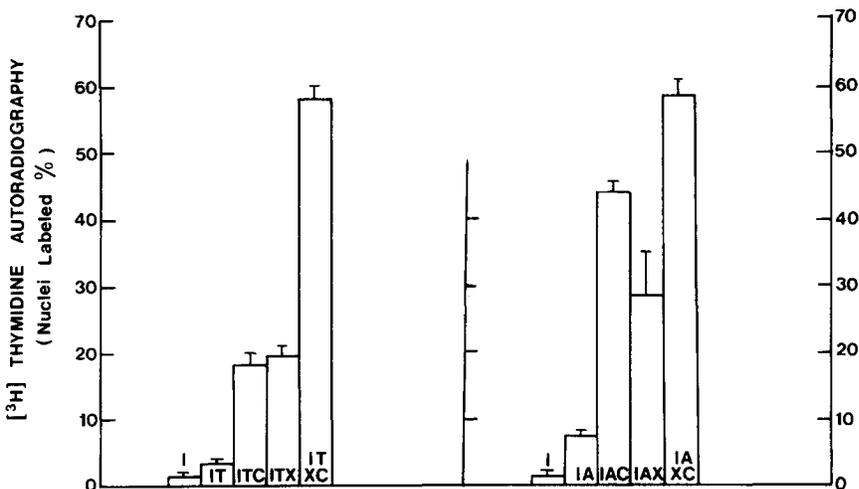
FIGURE 2 Synergistic effects of colchicine and cAMP-elevating agents in stimulating DNA synthesis in the presence of vasoressin, EGF, FDGF, or PDGF. The concentrations of the mitogens were 20 ng/ml, 5 ng/ml, 5 $\mu\text{g}/\text{ml}$, and 3.5 $\mu\text{g}/\text{ml}$ for vasoressin, EGF, FDGF, and PDGF, respectively. Measurement of $[^3\text{H}]$ TdR incorporation into acid-insoluble material after a 40-h incubation was done as described in Materials and Methods.

Stimulation of 3T3 Cells as Revealed by Autoradiography and by Flow Cytofluorometric Analysis

It was important to assess whether cAMP-elevating agents, antimicrotubule drugs, and insulin interact synergistically in eliciting initiation of DNA replication rather than in changing the specific activity of the $[^3\text{H}]$ TdR precursor pool. Quiescent cultures of 3T3 cells were treated with various combinations of insulin, cholera toxin, 8BrcAMP, IBMX, and colchicine and incorporation of $[^3\text{H}]$ TdR into DNA was quantified by autoradiography of labeled nuclei. As can be seen in Fig. 3, colchicine enhances the labeling index of cultures treated with cAMP-elevating agent and insulin either in the absence or in the presence of IBMX. Since colchicine added without other growth-promoting agents does not induce any significant DNA synthesis in Swiss 3T3 cells, the interactions between cAMP-elevating agents and antimicrotubule drugs in the presence of insulin shown in Fig. 3 were clearly synergistic rather than

additive. Similar results were obtained when the concentration of insulin was increased 10-fold (to 10 $\mu\text{g}/\text{ml}$). For example, in the presence of 10 $\mu\text{g}/\text{ml}$ insulin, the percentages of labeled nuclei were 29 to 31% in cultures treated with cholera toxin plus IBMX or 8BrcAMP plus IBMX, respectively; these values increased to 62 and 65%, respectively, when parallel cultures (also treated with cAMP-elevating agents and the hormone) received 2.5 μM colchicine. Thus, colchicine and cAMP-elevating agents synergistically enhance the proportion of cells that enter into DNA synthesis. This conclusion is further substantiated by experiments in which the cell cycle distribution of the population was determined by flow cytofluorometric analysis. In these experiments we exploited the fact that colchicine is not effective in enhancing DNA synthesis once S phase has been initiated but can block cell cycle traverse at mitosis (14). Initiation of S phase in cultures of 3T3 cells stimulated by cAMP-elevating agents and insulin takes place after a lag of 17 h (35). As shown in Fig. 4, colchicine added simultaneously with 8BrcAMP plus IBMX or cholera toxin plus IBMX to cultures incubated with insulin causes a marked

FIGURE 3 Effect of colchicine on DNA synthesis assessed by autoradiography after a 40-h incubation in cultures of Swiss 3T3 cells exposed to insulin and cholera toxin (left) or to insulin and 8BrcAMP (right) in the absence or in the presence of IBMX (*X*). The concentrations of the mitogens were 1 $\mu\text{g}/\text{ml}$, 100 ng/ml, 2 mM, 10 μM , and 1 μM for insulin (*I*), cholera toxin (*T*), 8BrcAMP (*A*), IBMX (*X*), and colchicine (*C*), respectively.



enhancement in the proportion of cells in S plus G₂ plus M as compared with the cultures that received colchicine 20 h after the addition of cAMP-elevating agents and insulin. These findings are in agreement with the results shown in Fig. 3 and indicate that antimicrotubule drugs, cAMP-elevating agents, and insulin, added at saturating concentrations, act synergistically to stimulate entry into DNA synthesis to 3T3 cells.

Effect of Colchicine on the Intracellular Content of cAMP in Swiss 3T3 Cells

Since disruption of microtubules could increase cAMP levels and potentiate cholera toxin-induced accumulation of cAMP as reported in a variety of other cell types (17, 20, 23, 30, 37), it is plausible that certain effects of these drugs are exerted via cAMP. To test this hypothesis, we have measured cAMP levels in quiescent 3T3 cells exposed to cholera toxin with or without IBMX in the absence or presence of colchicine. As shown in Table I, addition of cholera toxin (100 ng/ml) caused a fivefold increase in cAMP levels after a 3-h incubation; addition of colchicine did not alter the content of cAMP cultures incubated with cholera toxin either in the absence or in the presence of IBMX. Similar results were obtained when the cultures were incubated for 1 or 24 h instead of 3 h (results not shown). These results rule against the proposition that antimicrotubule drugs act in 3T3 cells by increasing the production of cAMP.

Effect of cAMP-elevating Agents, Antimicrotubule Drugs, or Both on Cytoplasmic Microtubules

The interpretation of the foregoing experiments relies on the important assumption that the antitubulin agents dissociate the microtubular network in cells treated with cAMP-elevating agents. To test this hypothesis, we determined the degree of organization of the microtubular network by applying a quantitative biochemical procedure for recovering and measuring intact sedimentable microtubules. Polymerized and depolymerized states of tubulin in cellular homogenates were preserved in a stabilizing medium (12), separated by centrifugation and quantified with a [³H]colchicine binding assay (1, 10, 11). As shown in Fig. 5 (left), exposure of 3T3 cells to various concentrations of nocodazole leads to a dose-dependent decrease of the fraction of cellular tubulin present in the polymerized form (microtubules). The half-maximal concentration derived from

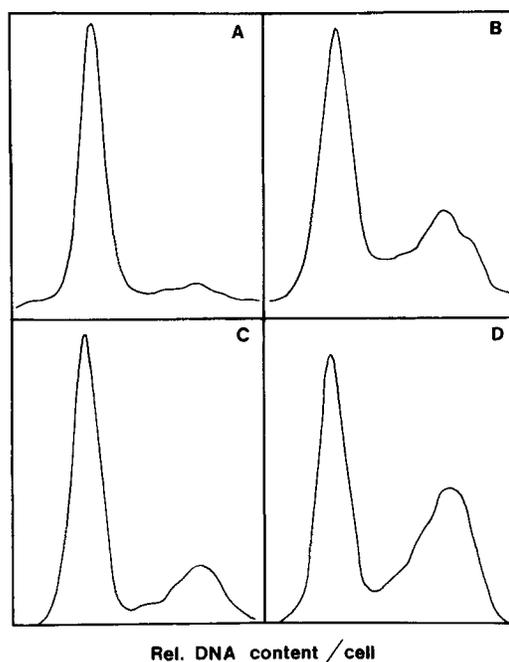


FIGURE 4 Flow cytometric DNA histograms showing the effect of 2.5 μM colchicine on the progress from G₁ to S phase in Swiss 3T3 cells stimulated for 40 h simultaneously by 2 mM 8BrcAMP with 1 $\mu\text{g}/\text{ml}$ insulin and 20 μM IBMX (*A + B*) or 100 ng/ml of cholera toxin with insulin and IBMX (*C + D*). Colchicine was added 20 h after the other agents as a control (*A and C*) or at the start of the 40-h incubation (*B and D*). Flow cytometry was carried out as described in Materials and Methods. In each case, the DNA in the right-hand peak (G₂ phase) is equivalent to twice the DNA in the G₁-phase to the left. Values for S + G₂ + M (percentages) were: (*A*) 13.2 \pm 4.2, (*B*) 41.5 \pm 5.8, (*C*) 23.5 \pm 2.9, and (*D*) 40.4 \pm 0.4.

Fig. 5 (0.1 μM) is in reasonable agreement with the half-maximal concentration of nocodazole that caused enhancement of DNA synthesis stimulated by cholera toxin and insulin (0.15 μM , Fig. 1) or by 8BrcAMP and insulin (0.1 μM , results not shown).

To check whether the antitubulin agents are able to induce microtubule disassembly in cells treated with cAMP-elevating agents, quiescent cultures of Swiss 3T3 cells were treated with cAMP-elevating agents for 24 h and then exposed to 0.5 μM nocodazole for 1 h. As shown in Fig. 5 (right), cAMP-elevating

agents cause a small increase rather than a decrease in the state of polymerization of cellular tubulin. Similar results were obtained when the cells were treated with cAMP-elevating agents for 1 or 18 h instead of 24 h. Addition of nocodazole markedly reduced the proportion of organized tubulin either in the absence or in the presence of cAMP-elevating agents. A similar result was obtained when microtubules were visualized by immunofluorescence with a specific antibody directed against tubulin (Fig. 6). The immunofluorescent staining pattern of 3T3 cells shows an abundant array of microtubules both in the absence (Fig. 6, upper) and in the presence (Fig. 6, middle) of cholera toxin, and is virtually identical to that published by Brinkley et al. (3). When cholera toxin-treated 3T3 cells were exposed to nocodazole (Fig. 6, lower), the filaments essentially disappeared and the cells lost their fibroblastic shape. Thus, nocodazole reduced the proportion of organized tubulin in either the absence or presence of cAMP-elevating agents, as judged by measuring [³H]colchicine binding of total and pelletable tubulin as well as by indirect immunofluorescence.

Effect of Removal or Delayed Addition of Nocodazole on DNA Synthesis Stimulated by Cholera Toxin and Insulin

In an attempt to determine which stage during the transition of cells from G₀/G₁ to S phase was enhanced by disruption of

TABLE I
Effect of Colchicine, Cholera Toxin, and IBMX on cAMP Content in 3T3 Cells

Additions	cAMP content pmol/mg protein
—	9.4 ± 1.5 (12)
Colchicine	4.6 ± 0.9 (12)
Cholera toxin	46.6 ± 3.9 (9)
Cholera toxin + colchicine	44.3 ± 8.1 (12)
Cholera toxin + IBMX	79.7 ± 6.2 (12)
Cholera toxin + IBMX + colchicine	77.6 ± 5.6 (12)

Quiescent cultures of Swiss 3T3 cells were exposed to colchicine (2.5 μM), cholera toxin (100 ng/ml), IBMX (10 μM), or their combinations for 3 h. The experimental conditions and the measurements of cAMP were as described in Materials and Methods.

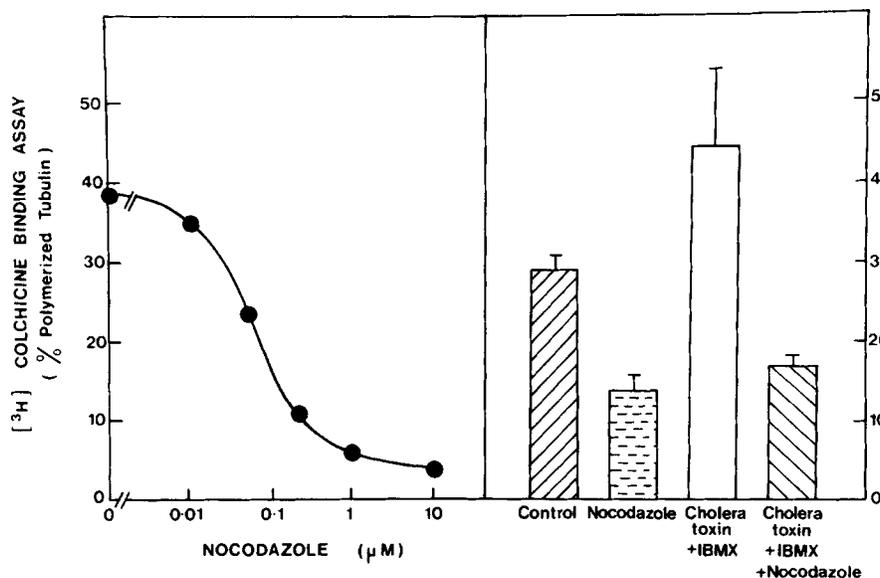


FIGURE 5 Depolymerization of cytoplasmic microtubules by various concentrations of nocodazole (left) or by nocodazole in the absence or presence of cholera toxin plus IBMX (right). Cultures of quiescent Swiss 3T3 cells were washed and transferred to medium containing various concentrations of nocodazole for 2 h (left). In a separate experiment, the cultures were incubated with 100 ng/ml of cholera toxin plus 20 μM IBMX for 24 h and then exposed to 0.5 μM nocodazole for an additional 1 h (right). The fraction of total cellular tubulin in 3T3 cells present as sedimentable microtubules was measured as described under Materials and Methods.

microtubules, quiescent 3T3 cells were incubated with cholera toxin plus insulin for 48 h, but transiently exposed to nocodazole for various periods of time from the onset of stimulation by the toxin and the hormone. The cultures were exposed to [³H]TdR (present from time zero and added again with cholera toxin and insulin after the wash to remove the nocodazole) throughout the 48-h incubation. Nocodazole (rather than colchicine) was used in these experiments because this drug can be more completely removed by washing (15, 43), which offers obvious advantages in analyzing the temporal relations of its effects. As shown in Fig. 7 (▲), a 4-h exposure of the cells to nocodazole followed by washing of the cells was not effective in enhancing DNA synthesis. In contrast, an 8-h exposure to nocodazole produced a substantial enhancement of DNA synthesis. These findings suggest that disruption of microtubules is effective in stimulating DNA synthesis by cholera toxin and insulin when induced in mid G₁ phase. This conclusion is further substantiated by experiments in which nocodazole was added at various times after cholera toxin and insulin. Fig. 7 (Δ) shows that the addition of nocodazole can be delayed up to 4 h after addition of toxin and hormone to quiescent 3T3 cells without any diminution of enhancement of DNA synthesis. These findings suggest that disruption of microtubular network is required several hours after the initial interaction of the mitogenic agents with the cell.

DISCUSSION

Recently, it has been demonstrated that both an increase in the intracellular level of cAMP (33–36) and a disruption of the microtubular network (7, 8, 13–15, 24, 26) markedly potentiate the DNA synthetic response of 3T3 cells to various growth-promoting factors. Because cyclic nucleotide generation and microtubule assembly-disassembly are interrelated in a variety of cell types, it was of importance to examine whether microtubules and cAMP modulate the initiation of DNA synthesis in 3T3 cells by common or separated pathways.

In theory, cAMP and microtubules may share common steps in their mechanism of mitogenic action. Since disruption of cytoplasmic microtubules potentiates the accumulation of cAMP in response to hormones and cholera toxin in a variety of cell types (17, 20, 23, 30, 37), it appeared feasible that the mitogenic effects of antimicrotubule agents in 3T3 cells could

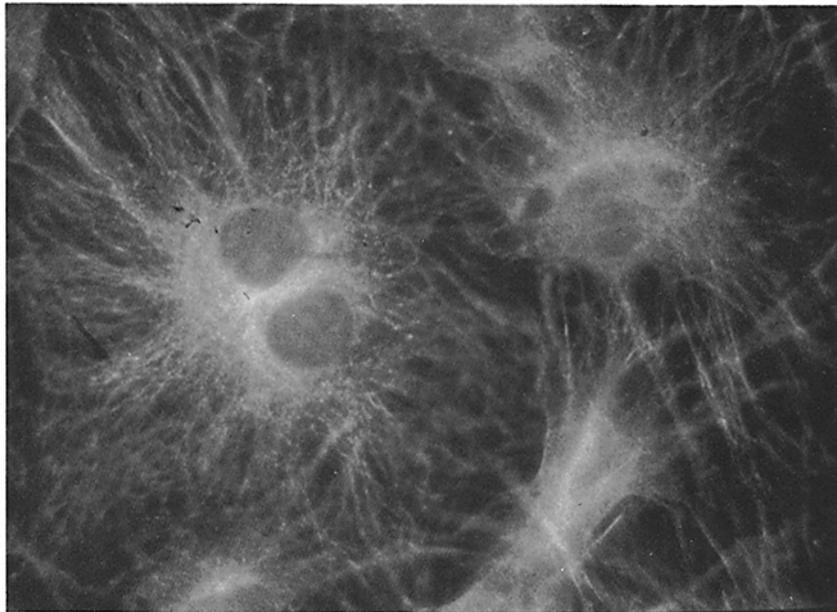
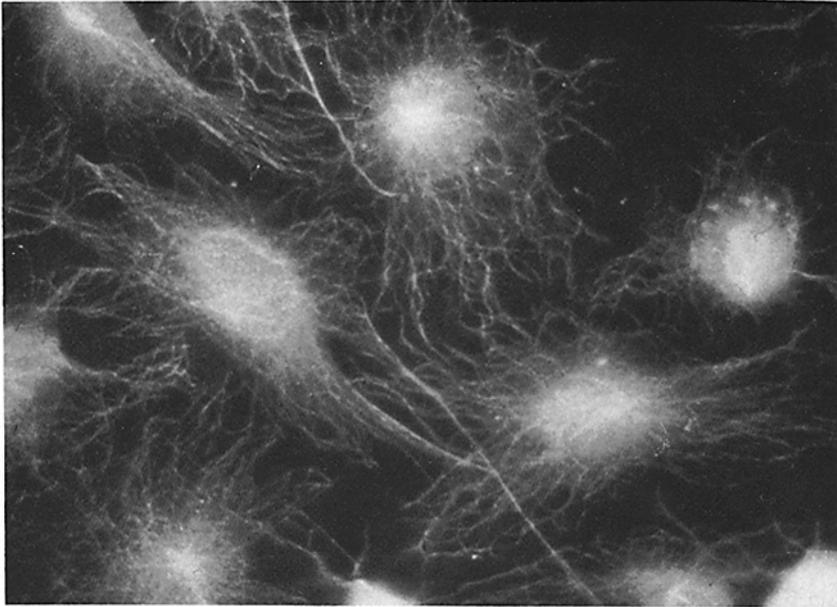


FIGURE 6 Indirect immunofluorescence of Swiss 3T3 cells using antibodies directed against tubulin. *Upper*, control cell; *middle*, Swiss 3T3 cells treated with 100 ng/ml of cholera toxin plus 20 μM IBMX for 24 h; *lower*, cultures incubated with cholera toxin and IBMX for 24 h and then exposed to 2.5 μM nocodazole for an additional 1 h. \times 600.

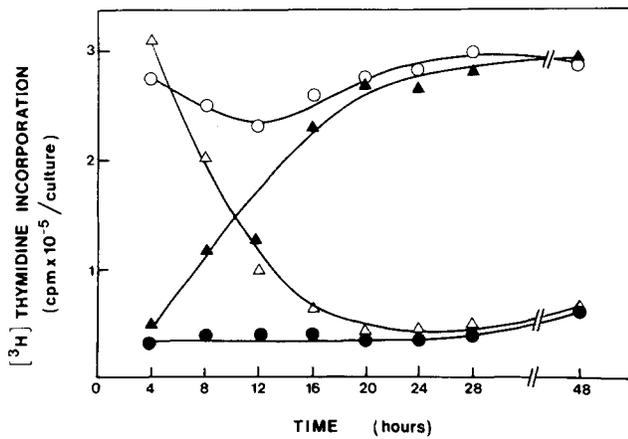


FIGURE 7 Effect of addition or removal of nocodazole at various times on its ability to enhance $[^3\text{H}]$ thymidine incorporation in 3T3 cells continuously exposed to insulin and cholera toxin. Quiescent Swiss 3T3 cells were transferred to insulin (200 ng/ml) and cholera toxin (30 ng/ml; $t = 0$). Then, nocodazole (2.5 μM) was added at the time shown (Δ). Other cultures of 3T3 cells were exposed to nocodazole, insulin, and cholera toxin for the time shown, washed with 1:1 DME/Waymouth medium, and then incubated again with insulin and cholera toxin but without nocodazole (\blacktriangle). As controls, parallel cultures incubated with insulin and cholera toxin with (\circ) or without (\bullet) nocodazole were subjected to the same washing procedure at the time shown, but after this, they received an identical medium. All the incubations were terminated at 48 h, $[^3\text{H}]\text{TdR}$ was present throughout. The 48-h value was obtained with continuous exposure without any wash.

be exerted via cAMP. However, this hypothesis is not supported by the fact that colchicine failed to increase cAMP levels in 3T3 cells when added in either the absence or presence of cholera toxin alone or with IBMX (Table I). In addition, antimicrotubule drugs enhanced mitogenesis induced by 8Br-cAMP; this provides further support to the proposal that microtubule disassembly acts at a step distal to cAMP generation.

An alternative possibility that would be consistent with the hypothesis that cAMP and microtubules share common steps in their mitogenic action is that cAMP, like the antimicrotubule drugs, promotes dissolution of the cytoplasmic microtubules (38). This interpretation was not substantiated by direct biochemical measurements of polymerized tubulin in cultures of 3T3 cells. We found that cAMP-elevating agents cause a small increase rather than a decrease in the state of polymerization of tubulin in 3T3 cells. This observation is in line with the findings of Eichhorn and Peterkofsky (10) with virus-transformed 3T3 cells. A similar conclusion was obtained when the microtubules were visualized by indirect immunofluorescence. All these results are not consistent with the hypothesis that cAMP and disassembly of microtubules enhance mitogenesis in 3T3 cells via a common or overlapping mechanism; The antimicrotubule drugs do not increase the production of cAMP nor do the cAMP-elevating agents promote disassembly of microtubules in these cells.

An alternative hypothesis is that cAMP and microtubule disassembly regulate separate (rather than common) events that signal initiation of DNA synthesis in the presence of a peptide factor. Evidence in favor of this interpretation comes from experiments in which disruption of the microtubule network, cAMP-elevating agents, and peptide factors act synergistically to stimulate the entry into DNA synthesis of 3T3 cells. These synergistic effects could be demonstrated by incorporation of $[^3\text{H}]\text{TdR}$ into acid-insoluble material, autoradiography

of labeled nuclei, or flow cytofluorometric analysis.

The involvement of the microtubules in the enhancement of the DNA synthetic response was demonstrated with the chemically diverse agents colchicine, nocodazole, vinblastine, or demecolcine, all of which were found to elicit the response in a dose-dependent manner. We verified that at the doses used in this study nocodazole induced microtubule disassembly in either the absence or presence of cAMP-elevating agents as judged by measuring $[^3\text{H}]\text{colchicine}$ binding of total and pelletable tubulin or by indirect immunofluorescence. The fact that cAMP was active in synergistically stimulating mitogenesis in 3T3 cells without polymerized tubulin further suggests that the action of the cyclic nucleotide is not mediated by a change in the state of organization of the microtubules. Additional support for the hypothesis that cAMP and antimicrotubule drugs control different events leading to initiation of DNA synthesis comes from the fact that cAMP and disassembly of microtubules appear to act at different points of the G_1 phase; cAMP-elevating agents were demonstrated to be required in early G_1 phase (35), whereas antimicrotubule drugs are still effective in enhancing DNA synthesis when added several hours after the initial interaction of the mitogenic agents in the cell, i.e., in mid- G_1 phase.

Understanding the mechanisms whereby extracellular agents modulate cell proliferation requires identification of the intracellular signals important for initiating a mitogenic response. We conclude that cAMP and microtubule disassembly constitute separate mitogenic signals that are elicited by different sets of extracellular agents. In confluent and quiescent Swiss 3T3 cells, neither of these signals is sufficient to induce a proliferative response in serum-free medium. However, when these signals are elicited in the presence of other growth-promoting factors, they act synergistically to stimulate exit from G_0 phase and entry into DNA synthesis in 3T3 cells. The multiple control of initiation of DNA synthesis in 3T3 cells by identifiable internal signals provides a model for understanding the mechanisms by which extracellular factors may regulate cell proliferation.

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