Acta Pharmacologica Sinica 1983 Sep; **4** (3) : 205–210

SYNERGISTIC EFFECT BETWEEN ANTITUBULIN AGENTS AND CELLULAR cAMP IN STIMULATING DNA SYNTHESIS BY 3T3 CELLS

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ABSTRACT Incorporation of [^{3}H]TdR into 3T3 cells stimulated by insulin and cholera toxin or 8Br-cAMP was markedly enhanced by colchicine, demecolcine, vinblastine or nocodazole. These antitubulin agents enhanced DNA synthesis in a dose-dependent manner. In the presence of colchicine and insulin, cholera toxin or 8Br-cAMP stimuleted DNA synthesis in a concentration-dependent fashion. When insulin was replaced by other growth factors, e. g. vasopressin, EGF, FDGF or PDGF, addition of colchicine also enhanced DNA synthesis stimulated by cholera toxin or 8Br-cAMP. The effect of colchicine on DNA synthesis was also demonstrated by increased autoradiographic labeling of nuclei. Our findings show that depolymerization of cytoplasmic microtubules acts synergistically with cAMP-elevating agents and peptide hormones to initiate DNA synthesis.

KEY WORDS cytoplasmic microtubule; antitubulin agents; cAMP; DNA synthesis; 3T3

Received 1982 Aug 23
This is a portion of the work presented at XIII International Cancer Congress in Seattle, Washington, USA, 1982 Sep 8–15 (Abstract No. 03091).

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cells; growth factor; [^{3}H]TdR incorporation; autoradiography

Quiescent cultures of 3T3 cells can be stimulated to reinitiate DNA synthesis and cell division by serum or growth-promoting factors[1]. Several studies suggest that the microtubular network may play a role in the regulation of the transition of cells from G_0/G_1 into 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[2-6]. In addition, microtubule stabilization by taxol inhibits initiation of DNA synthesis by EGF[7]. These findings suggest that organized microtubules impose a constraint in the action of a variety of mitogenic agents in fibroblastic cells. Recent work from this laboratory has shown that an increase in the intracellular concentration 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[8,9]. These findings suggest that a sustained increase in the intracellular level of cAMP constitutes a mitogenic signal for Swiss 3T3 cells.

Cyclic nucleotides and microtubules interact in modulating a variety of cellular properties[10,11]. Since both cAMP-elevating agents and antimicrotubule drugs influence events leading the initiation of DNA synthesis in 3T3 cells, it was of importance to elucidate whether these agents could have opposite,synergistic or common steps in their 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 were propagated in 90 mm Nunc Petric dishes in Dulbecco's modified Eagle's medium (DME). 10% fetal bovine serum, 100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin in a humidified atmosphere of 10% CO_2 + 90% air at 37℃. Cells were subcultured at 5×10^4 in 30 mm Nunc petric dishes with the same medium and whithin 8 d from the time of initial plating the cultures became confluent and quiescent at G_0/G_1. Such quiescent cultures were washed twice with DME at 37℃ to removed residual serum immediately preceding experiments with growth factors.

INCORPORATION OF [^{3}H]TdR INTO DNA Confluent and quiescent cultures of Swiss 3T3 cells in 30 mm Nunc Petric dishes were washed twice with DME at 37℃. Then the cultures were incubated in 2 ml of DME medium containing [^{3}H]TdR(1 μM, 1 μCi ml^{-1}) and various agents as indicated. After 40 h at 37℃, [^{3}H]TdR incorporation into acid-insoluble pool was assayed as follows: cultures were washed twice with ice-cold phosphate-buffered saline (0.15 M NaCl in 0.01 M K_2PO_4 buffer, pH 7.4) and acid-soluble radioactivity was removed by a 20-min exposure to ice-cold 5% trichloroacetic acid at 4℃. The acid-insoluble material remaining on the dishes was washed twice in ethanol, and then solubilized by a 30-min incubation with 0.1M NaOH and 2% Na_2CO_3, and the radioactivity was assayed.

AUTORADIOGRAPHY After incubation with 10 μl per 30 mm dish of undiluted [^{3}H]TdR (1 mCi ml^{-1}) for more than 40 h, the cells were fixed in formal saline and 5% TCA, dried with ethanol, coated by chrome alum overnight, and then coated again with Kodak autoradiographic film AR 10 for exposure in the dark room for at least 1 week, developed and stained with Giemsa stain,and then counted the percentage of nuclei labelled cells.

MATERIALS Bovine insulin (25.5 international units mg^{-1}), cholera toxin, 8 Br-cAMP, colchicine, demecolcine, vinblastine sulfate and nocodazole were purchased from Sigma Chemical Co. 3-isobutyl-1-methyl-xanthine (IBMX) was purchased from Aldrich Chemical Co. [^{3}H]TdR (20 Ci $mmol^{-1}$) was provided by new England Nuclear.

RESULTS

To determine the interaction between the intracellular levels of cAMP and the state of organisation of the microtubules in modulating the initiation of DNA synthesis in Swiss 3T3 cells, quiescent cultures of these cells were transferred to medium supplemented with insulin, cholera toxin or both and exposed to various concentrations of the anti-tubulin agents colchicine, demecolcine, vinblastine and nocodazole. Fig 1 shows that these microtubular disrupting agents cause a marked and dose-dependent enhancement of the incorporation of [^{3}H]TdR into acid-insoluble material in those cultures exposed to cholera toxin and insulin. Therefore, antimicrotubular drugs should also enhance DNA synthesis stimulated by 8Br-cAMP instead of cholera toxin. Fig 2 shows that this is indeed the case; colchicine, demecolcine, vinblastine and nocodazole markedly enhance the stimulation of [^{3}H]TdR incorporation caused by 8Br-cAMP and insulin. These findings suggest that disassembly of microtubules potentiates the mitogenic effects of cAMP and insulin.

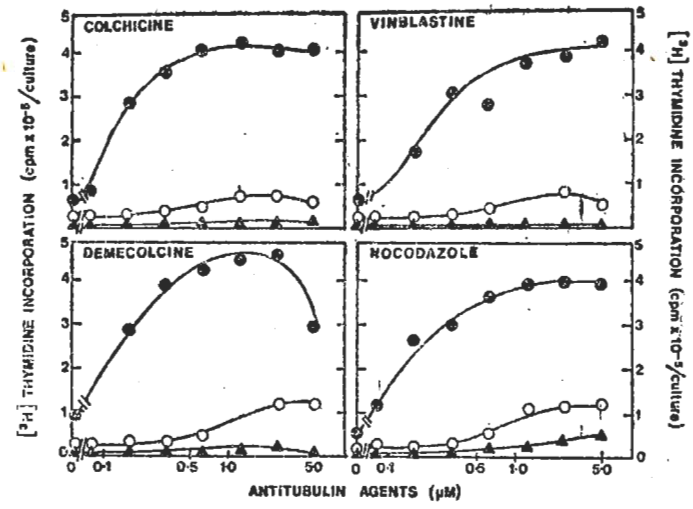

Fig 1. Dose-response curves for enhancing effects of antitulin agents on [^{3}H]TdR incorporation stimulated with cholera toxin plus insulin in Swiss 3T3 cells. ● Insulin + Cholera toxin, ○ Insulin 1 μg ml^{-1}, ▲ Cholera toxin 0.1 μg ml^{-1}.

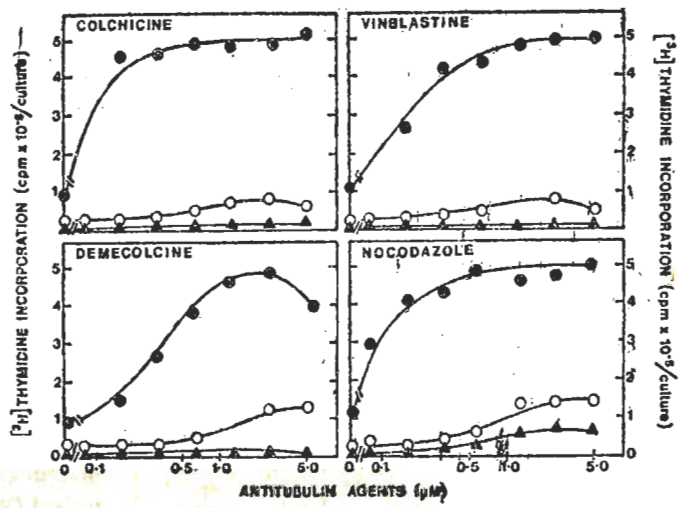

Fig 2. Dose-response curves for enhancing effects of antitubulin agents on [^{3}H]TdR incorporation in Swiss 3T3 cells stimulated by 8Br-cAMP and insulin. ● Insulin + 8Br-cAMP, ○ Insulin 1 μg ml^{-1}, ▲ 8Br-cAMP 2 mM.

The above conclusion is further substantiated by the experiments shown in Fig 3. Quiescent 3T3 cells were exposed to insulin, colchicine and to various concentrations of either cholera toxin(Fig 3, left) or 8Br-cAMP(Fig 3, right). The results show that an increase in cAMP, either endogenously generated in cholera toxin treated cells or exogenously supplied as 8Br-cAMP causes a marked increase in [^{3}H]TdR incorporation in those cultures treated with colchicine and insulin.

It was important to assess whether cAMP-elevating agents and antimicrotubule drugs interact synergistically in eliciting a DNA synthetic response rather than in changing the specific activity of the [^{3}H]thymidine precursor pool. Quiescent cultures of 3T3 cells were treated with various combinations of insulin, cholera toxin, 8Br-cAMP, IBMX and colchicine and incorporatiion of [^{3}H]TdR into DNA was

quantified by autoradiography of labelled nuclei. As can be seen in Fig 4, colchicine enhances the labelling index of cultures treated with cAMP-elevating agent and insulin either in the absence or in the presence of IBMX. The results indicate that colchicine and cAMP-elevating agents enhance the proportion of cells that enter into DNA synthesis.

In the experiments described above, the interaction between cAMP-elevating agents and anitubulin drugs was studied in the presence of insulin, It was, therefore, of interest to determine whether insulin can be replaced by other growth-promoting factors. The experiment described in Fig 5 shows that a marked synergistic interaction between colchicine and cAMP-elevating agents can be demonstrated when vasopressin, fibroblast-derived growth factor (FDGF), platelete-derived growth factor (PDGF) or epidermal growth factor (EGF) are added instead of insulin.

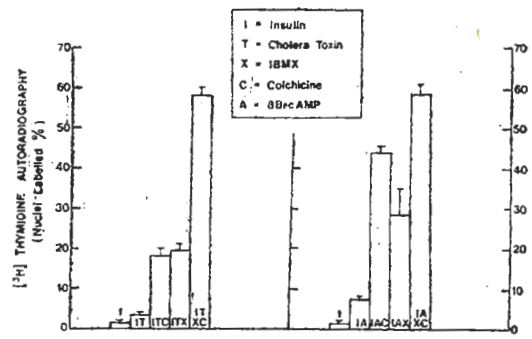

Fig 4. Effect of colchicine on DNA synthesis assessed by autoradiography after a 40 h exposure to [^{3}H]TdR in cultures of Swiss 3T3 cells exposed to insulin and cholera (left) or to insulin and 8Br-cAMP (right) in the absence or in the presence of IBMX. The concentrations were insulin 1 μg ml^{-1}, cholera toxin 100 ng ml^{-1}, 8Br-cAMP 2 mM, IBMX 10 μM, and colchicine 1 μM.

Also, the good dose-response could be obtained in Balb 3T3 cells as well. Fig 6 shows

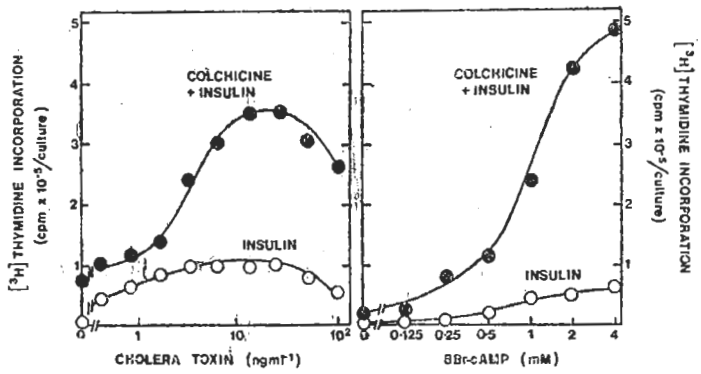

Fig 3. Dose response curves for the effect of cholera toxin (left) or 8Br-cAMP (right) on [^{3}H]TdR incorporation into Swiss 3T3 cells in the presence of insulin or insulin + colchicine. The final concentrations of insulin & colchicine was 1 μg ml^{-1} and 10^{-6} M, respectively.

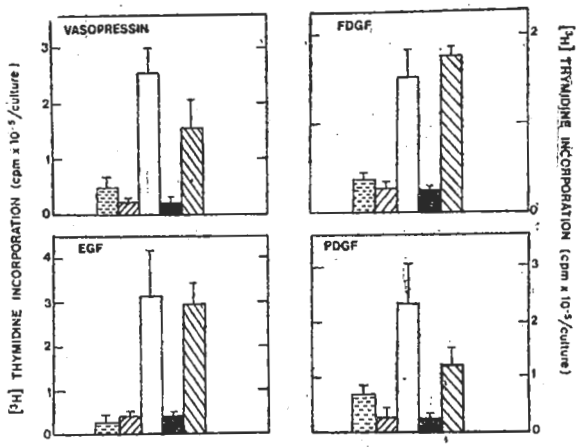

Fig 5. Synergistic effects of colchicine and cAMP-elevating agents in stimulating DNA synthesis in the presence of growth factors. The concentrations of mitogens were vasopressin 20 ng ml^{-1}, EGF 5 ng ml^{-1}, FDGF 5 μl ml^{-1}, and PDGF 1.5 μl ml^{-1}.

Growth factor (GF) + Colchicine,

GF + Cholera toxin

GF + Cholera toxin + Colchicine,

GF + 8Br-cAMP

GF + 8Br-cAMP + Colchicine

a marked synergistic interaction between colchicine and cAMP-elevating agents in a good dose-dependent manner enhancing DNA synthesis in Balb 3T3 cells.

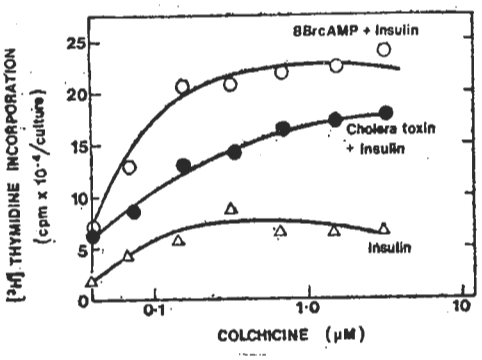

Fig 6. Dose-response curves for enhancing effects of colchicine on [^{3}H]TdR incorporation stimulated with insulin 1 μg ml^{-1}, insulin plus cholera toxin 100 ng ml^{-1} or insulin plus 8Br-cAMP 2 mM in Balb 3T3 cells.

DISCUSSION

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. 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. The involvement of cAMP was revealed by increasing its endogenous production by cholera toxin or by treatment with 8Br-cAMP. The enhancing effect of antimicrotubule drugs and cAMP-elevating agents could be demonstrated by incorporation of [^{3}H]TdR into acid-insoluble materials and autoradiography of labelled nuclei. 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, PDGF, EGF or FDGF.

The interplay between cyclic nucleotides and microtubule organization in cell regulation has been the subject of considerable literature. Thus, increased intracellular levels of cAMP changes the arrangement and length of microtubules[12] and cAMP-dependent phosphorylation of microtubule-associated proteins modulated microtubule assembly and disassembly "*in vitro*" [13]. Further, antimicrotubule drugs block a variety of cellular effects of cAMP[14]. It is, therefore, plausible that some effects of cAMP are mediated by a change in the state of organization of the microtubule network. Conversely, since disruption of the microtubules has been shown to potentiated the effect of various hormones in increasing cAMP levels in certain cell types[15]; it is plausible that certain effects of antimicrotubule drugs could be exerted via cAMP. Moreover, the results presenetd here systematically indicate the important role of cytoplasmic microtubules organisation in the regulation of 3T3 cell growth initiated by peptide growth factors. And a plausible model is that a disrupted microtubule network and increased levels of cAMP regulate DNA synthesis in a synergistic manner.

ACKNOWIEDGMENT This work was collaborated with Dr E Rozengurt, Head of Laboratory of Membrane physiology & Growth Regulation of Imperial Cancer Research Fund Laboratories, London.

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