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Cytoskeleton-disrupting drugs enhance effect of growth factors and hormones on initiation of DNA synthesis

(prostaglandin $F_{2\alpha}$/fibroblastic growth factor/lag phase/colchicine/mouse 3T3 cells)

ANGELA M. OTTO, ALBERT ZUMBÉ, LORNA GIBSON*, ANNE-MARIE KUBLER, AND LUIS JIMENEZ DE ASUA

Friedrich-Miescher-Institut, P.O. Box 273, CH-4002 Basel, Switzerland

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ABSTRACT **Addition of growth factors, such as prostaglandin $F_{2\alpha}$ or fibroblastic growth factor, to quiescent Swiss mouse 3T3 cells resulted in an abrupt increase in the rate of initiation of DNA synthesis after a lag phase of 13–15 hr. This increase could be quantified by a rate constant k. Addition of colchicine, Colcemid, or vinblastine had a synergistic effect on the initiation of DNA synthesis triggered by $PGF_{2\alpha}$ or FGF by increasing the value of k. These drugs alone had no effect. Colchicine had a synergistic effect only if added within 8 hr of the $PGF_{2\alpha}$ or FGF addition. Also, colchicine exerted its full effect when it was present only for the first 5 hr with either growth factor. These results suggest that an intact cytoskeleton is not required for the initiation of DNA synthesis. Furthermore, cytoskeleton-disrupting drugs enhance the stimulatory effect of the growth factors.**

The stimulation of quiescent animal cells to divide, both *in vivo* and *in vitro*, is accomplished through the orderly expression of a reproducible program of signals and events that precede DNA synthesis (1–5). In cultured mammalian cells, under defined growth conditions, the regulatory events leading to the initiation of DNA synthesis and cell division are controlled by growth factors, hormones, other defined macromolecular components, ions, and nutrients (3, 6, 7). Addition of serum (8, 9) or growth factors (5, 10, 11) to quiescent fibroblastic cells is followed by a constant lag phase, before an abrupt increase in the rate of initiation of DNA synthesis is observed. The latter process follows first-order kinetics and can be quantified by a rate constant k (4, 8, 12).

Growth factors initially interact with receptors of the plasma membrane to deliver the mitogenic signals required for cell proliferation (13–15). Recently, it has been suggested that in chicken embryo and Swiss mouse 3T3 cells the association of microtubules (MT) and microfilaments with proteins of the plasma membrane may play a regulatory role in conveying the signals delivered by the growth factors from the cell surface to their specific intracellular targets (16, 17).

Here we show the effect of different cytoskeleton-disrupting drugs at various times after the addition of prostaglandin $F_{2\alpha}$ ($PGF_{22\alpha}$) (18) or fibroblastic growth factor (FGF) (19), alone or in combination with hormones, on the initiation of DNA synthesis and cell division in confluent quiescent Swiss 3T3 cells.† In our system, colchicine, Colcemid, and vinblastine, which disrupt MT (20, 21), do not prevent the progression through the lag phase. In fact, these drugs increase the rate of initiation of DNA synthesis stimulated by growth factors.

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MATERIALS AND METHODS

Cultures. Swiss mouse 3T3 cells (22) were maintained as described (4).

Assay for the Initiation of DNA Synthesis and Determination of Rate Constant for Entry into S Phase. Cells were plated in experimental culture conditions as described (4). For determination of rate constant k the percentage of resting cells in G_1 (4) that remained unlabeled (y) in a given time (t) was calculated from the labeling index (4). The results were plotted as the $\log_{10} y$ against t in hours. Straight lines given by $\log_{10} y = a - bt$ fit these data well (4). First-order rate constants (k) were then calculated by geometrical methods from the slope of the curves (b), because $k = \log_e 10 \times b$.

Materials. $PGF_{2\alpha}$ was a generous gift of J. Pike of the Upjohn Co. FGF was obtained from Collaborative Research. Crystalline insulin, colchicine, Colcemid, and vinblastine were purchased from Sigma. [*methyl*-^{3}H]Thymidine was from the Radiochemical Centre, Amersham, England.

RESULTS

Effect of cytoskeleton-disrupting drugs on the initiation of DNA synthesis stimulated by $PGF_{2\alpha}$ or FGF and hormones

$PGF_{2\alpha}$ (300 ng/ml) added to confluent quiescent Swiss 3T3 cells stimulated about 20% of the cells to initiate DNA synthesis within 28 hr. Synchronous addition of colchicine or Colcemid in a concentration range of 0.02–100 μM with $PGF_{2\alpha}$ increased the fraction of labeled nuclei; the fraction reached a plateau of 40% at 0.4 μM. Insulin at the physiological concentration of 50 ng/ml, which does not initiate DNA synthesis in 3T3 cells (4–6), had a synergistic effect with $PGF_{2\alpha}$ (Fig. 1) and its combination with colchicine or Colcemid further increased the stimulatory effect of $PGF_{2\alpha}$ up to 75% labeled nuclei (Fig. 1 *A* and *B*). Vinblastine likewise enhanced the stimulation of $PGF_{2\alpha}$ alone or $PGF_{2\alpha}$ plus insulin and also exerted its maximal effect at 0.4 μM (Fig. 1*C*). Vinblastine at concentrations above 10 μM caused detachment of cells from the substratum.

Similarly, colchicine, Colcemid, or vinblastine increased the stimulatory effect of FGF (50 ng/ml) on the initiation of DNA synthesis from 30 to 80% over 28 hr. The maximal effect of colchicine or Colcemid was at 0.4 μM (Fig. 1 *D* and *E*), whereas

Abbreviations: $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; FGF, fibroblastic growth factor; MT, microtubules.

* Present address: Basel Institute of Immunology, CH-4058 Basel, Switzerland.

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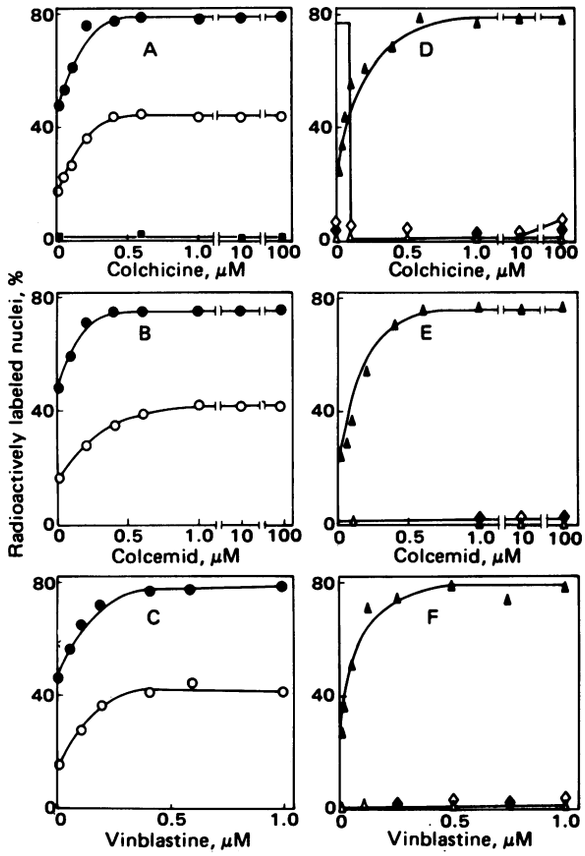

FIG. 1. Effect of different concentrations of cytoskeleton-disrupting drugs on the percentage of cells synthesizing DNA stimulated by $PGF_{2\alpha}$ (300 ng/ml) or by FGF (50 ng/ml) without or with insulin (50 ng/ml). Cultures were exposed to [*methyl*-^{3}H]thymidine from 0 to 28 hr after additions and then processed for autoradiography. (*A*) ○, $PGF_{2\alpha}$ + colchicine; ●, $PGF_{2\alpha}$ + insulin + colchicine; ■, colchicine. (*B*) ○, $PGF_{2\alpha}$ + Colcemid; ●, $PGF_{2\alpha}$ + insulin + Colcemid. (*C*) ○, $PGF_{2\alpha}$ + vinblastine; ●, $PGF_{2\alpha}$ + insulin + vinblastine. (*D*) ▲, FGF + colchicine; △, colchicine; ◇, insulin + colchicine; ◆, insulin + hydrocortisone (100 ng/ml) + colchicine. The empty bar on the left shows FGF + insulin + hydrocortisone (100 ng/ml). (*E*) ▲, FGF + Colcemid; △, Colcemid; ◆, insulin + hydrocortisone (100 ng/ml) + Colcemid; ◇, insulin + Colcemid. (*F*) ▲, FGF + vinblastine; △, vinblastine; ◇, hydrocortisone (100 ng/ml) + vinblastine; ◆, insulin + hydrocortisone (100 ng/ml) + vinblastine.

the effect of vinblastine reached a plateau at 0.2 μM (Fig. 1*F*). Insulin (50 ng/ml) and hydrocortisone (100 ng/ml), which have a synergistic effect with FGF (5, 6, 11) (Figs. 1*D* and 2*B*), produced an increase in labeled nuclei similar to that of the combination of any one of these drugs with FGF alone (Fig. 1 *D*, *E*, and *F*). The two hormones added separately or together did not have any stimulatory effect in the absence of FGF (5, 6, 11). Colchicine, Colcemid, or vinblastine alone or in combination with the hormones did not stimulate the initiation of DNA synthesis (Fig. 1). Only at high concentrations (100 μM) of colchicine and in the presence of insulin could a marginal effect (3.5%) on the labeling index be observed. Lumicolchicine, a photoinactivated analogue of colchicine (21), which does not bind to tubulin, had no effect on the initiation of DNA synthesis alone or with $PGF_{2\alpha}$ (Table 1).

The effect of colchicine on the dose–response curves of $PGF_{2\alpha}$ or FGF for the initiation of DNA synthesis within 28 hr is shown in Fig. 2. Colchicine added at 2 μM had a synergistic effect with $PGF_{2\alpha}$ in shifting the dose–response curve of $PGF_{2\alpha}$ to lower concentrations (Fig. 2*A*). The addition of colchicine markedly increased the value of the labeling index from 20%

Table 1. Effect of $PGF_{2\alpha}$, insulin, and cytoskeleton-disrupting drugs on the labeling index and cell division in quiescent 3T3 cells

Addition	Labeling index	Cells
None	0.5	490,000
$PGF_{2\alpha}$	21.9	640,000
+ colchicine	48.2	350,000
+ lumicholchicine	20.8	630,000
+ colchicine 0–5 hr	49.0	740,000
$PGF_{2\alpha}$ + insulin	49.5	980,000
+ colchicine 0–5 hr	80.2	1,220,000
Lumicolchicine	0.8	500,000
10% serum	93.1	1,800,000

Labeling index was determined as in Fig. 1. For determination of cell number, Swiss 3T3 cells were plated at 1.5×10^5 cells in 60-mm dishes in culture conditions similar to those for determination of DNA synthesis (4, 5). $PGF_{2\alpha}$ was added at 300 ng/ml with or without insulin (50 ng/ml). Colchicine and lumicolchicine were added at 2 μM and 1 μM, respectively. Removal of colchicine and readdition of $PGF_{2\alpha}$ and insulin were done as for Fig. 3. After 48 hr of additions, cells were removed from the dish with trypsin solution (0.05%), resuspended in isotonic buffer, and counted in a Coulter Counter.

to 40% and decreased the level of $PGF_{2\alpha}$ required for maximum effect to about 1/10 (Fig. 2*A*). Insulin, which had an effect similar to that of colchicine with $PGF_{2\alpha}$, when added together with colchicine showed a further synergistic effect with $PGF_{2\alpha}$ by increasing the fraction of labeled nuclei up to 80% (Fig. 2*A*). Similarly, colchicine interacted synergistically with FGF (Fig. (Fig. 2*B*). Colchicine added at 2 μM with FGF also increased the labeling index from 30 to 80% within 28 hr. Hydrocortisone and insulin, which independently interacted synergistically with FGF, did not produce a further increase. All these combinations with the growth factor also decreased the level of FGF required for its maximal effect on DNA synthesis (Fig. 2*B*).

Interaction of colchicine and $PGF_{2\alpha}$ or FGF and hormones during the lag phase

Does colchicine change the length of the lag phase or the rate of initiation of DNA synthesis or both? When colchicine (2 μM)

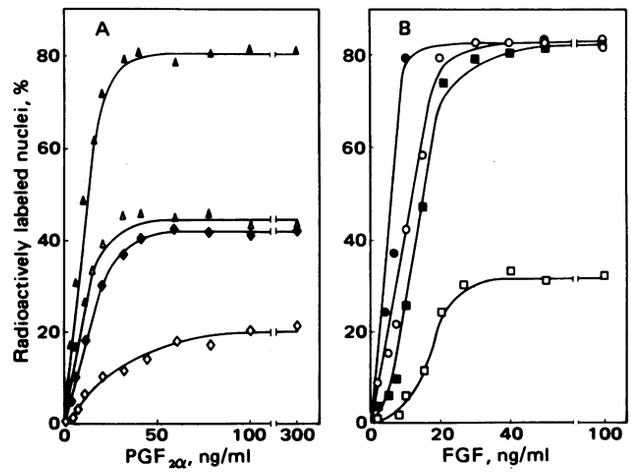

FIG. 2. Effect of different concentrations of $PGF_{2\alpha}$ or FGF in combination with insulin (50 ng/ml), hydrocortisone (100 ng/ml), or both, with or without colchicine (2 μM), on the labeling index after 28 hr. [*methyl*-^{3}H]Thymidine was added to the culture medium as in Fig. 1. (*A*) ◇, $PGF_{2\alpha}$; ◆, $PGF_{2\alpha}$ + colchicine; △, $PGF_{2\alpha}$ + insulin; ▲, $PGF_{2\alpha}$ + insulin + colchicine. (*B*) □, FGF; ■, FGF + colchicine; ○, FGF + insulin + hydrocortisone; ●, FGF + insulin + hydrocortisone + colchicine.

was added with $PGF_{2\alpha}$ or $PGF_{2\alpha}$ plus insulin, an increase in the value of k was observed after a constant lag phase of 14–15 hr. The values of k obtained in the presence of $PGF_{2\alpha}$ or $PGF_{2\alpha}$ plus colchicine were 0.018 and 0.055/hr, respectively. $PGF_{2\alpha}$ and insulin increased the value of k to 0.056/hr, similar to that of $PGF_{2\alpha}$ plus colchicine. $PGF_{2\alpha}$, insulin and colchicine further increased k to 0.112/hr (Fig. 3*A*). When colchicine was added 8 or 15 hr after $PGF_{2\alpha}$ or $PGF_{2\alpha}$ and insulin, no increase in the value of k was observed (Fig. 3*B*). The values of k obtained when colchicine was added 8 hr after $PGF_{2\alpha}$ or 8 and 15 hr after $PGF_{2\alpha}$ plus insulin were 0.019 and 0.055/hr, respectively (Fig. 3*B*).

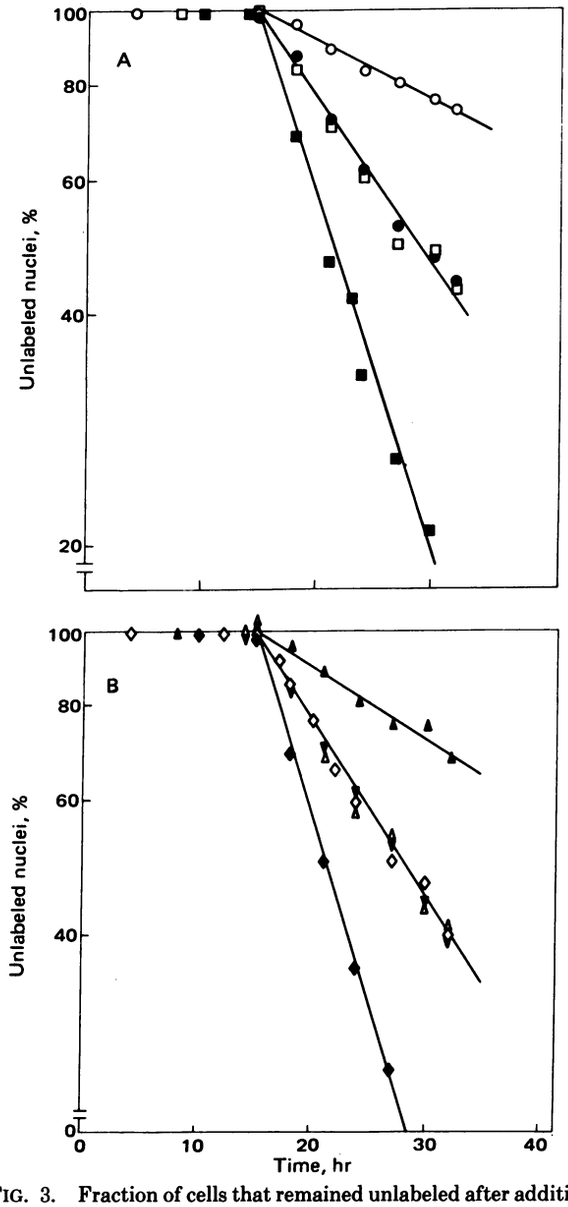

FIG. 3. Fraction of cells that remained unlabeled after addition of $PGF_{2\alpha}$ (300 ng/ml), alone or with insulin (50 ng/ml), in the presence or absence of colchicine (2 μM). Colchicine was removed from the culture medium by aspiration and cells were washed twice with Dulbecco's medium prewarmed at 37°C. Conditioned medium (2 ml) retrieved from parallel cultures was added to the treated culture with $PGF_{2\alpha}$ or $PGF_{2\alpha}$ + insulin at the same concentrations as at the start. [*methyl*-^{3}H]Thymidine was added from 5 hr until the times indicated. (*A*) Effect of synchronous addition of $PGF_{2\alpha}$, insulin, and colchicine: ○, $PGF_{2\alpha}$; ●, $PGF_{2\alpha}$ + colchicine; □, $PGF_{2\alpha}$ + insulin; ■, $PGF_{2\alpha}$ + insulin + colchicine. (*B*) Effect of nonsynchronous addition of colchicine to cells stimulated by $PGF_{2\alpha}$ + insulin: ▲, $PGF_{2\alpha}$ + colchicine at 8 hr; ▼, $PGF_{2\alpha}$ + insulin was added at 0 hr and colchicine was added at 8 hr, or (△) at 15 hr; ◇, $PGF_{2\alpha}$ + colchicine added from 0 to 5 hr; ◆, $PGF_{2\alpha}$ + insulin + colchicine added from 0 to 5 hr.

Table 2. Effect of FGF, insulin, hydrocortisone, and colchicine on the rate constant k

Addition	Time of addition, hr	Rate constant k, hr^{-1}
None	–	0.0005
FGF	0	0.0184
+ colchicine	0	0.1331
+ colchicine	0–5	0.1311
+ colchicine	8	0.0187
+ colchicine	15	0.0186
FGF + insulin + hydrocortisone	0	0.132
FGF + insulin + colchicine	0	0.134
Insulin + hydrocortisone + colchicine	0	0.0005
Serum	0	0.2501
Serum + colchicine	0	0.2521

The duration of the lag phase was 13 hr. Rate constants and duration of lag phase were calculated for groups that had at least eight data points per line. The value for no addition was taken over a period of 7 days (4, 5). FGF was added at 50 ng/ml, insulin at 50 ng/ml, hydrocortisone at 100 ng/ml, and colchicine at 2 μM. Removal of colchicine at 5 hr and replacement with conditioned medium and FGF (50 ng/ml) were done similarly as for $PGF_{2\alpha}$ as indicated in Fig. 3.

How long must colchicine be present to increase the value of k? When colchicine was added with $PGF_{2\alpha}$ or $PGF_{2\alpha}$ and insulin and then removed at 5 hr by changing the culture medium, while $PGF_{2\alpha}$ or $PGF_{2\alpha}$ plus insulin was returned, the value of k was increased (k = 0.054 and 0.111/hr, respectively) as if colchicine were continuously present (Fig. 3*B*). Also, preincubation with colchicine for 1 hr and its removal prior to addition of $PGF_{2\alpha}$ alone or $PGF_{2\alpha}$ and insulin give the same results (unpublished results). Similarly, colchicine increased the value of k if added synchronously with FGF, but not when added 8 or 15 hr later (Table 2). The full effect of colchicine with FGF was also observed when colchicine was removed after 5 hr (Table 2). The value of k obtained with FGF and colchicine was identical to that observed for FGF with hydrocortisone and insulin with or without colchicine. Addition of colchicine (2 μM) to serum-stimulated cultures had no effect on the value of k (Table 2).

The presence of colchicine with $PGF_{2\alpha}$ or $PGF_{2\alpha}$ plus insulin from 0 to 5 hr did not affect mitosis, because the increase in cell number correlated approximately with the labeling index. Continuous exposure of the stimulated cells to colchicine decreased the cell number, whereas the presence of lumicolchicine did not have any effect (Table 1).

DISCUSSION

Growth factors such as $PGF_{2\alpha}$ or FGF stimulate the initiation of DNA synthesis in cultured mouse cells by regulating two different parameters: the length of the lag phase and the rate of initiation of DNA synthesis (4, 5, 11).

Our results show that MT-disrupting drugs such as colchicine, Colcemid, or vinblastine added with a growth factor, alone or in combination with hormones, to quiescent Swiss 3T3 cells increase the rate of initiation of DNA synthesis without changing the length of the lag phase (Fig. 3 and Table 2).

Insulin and colchicine increase the stimulatory effect of $PGF_{2\alpha}$ upon DNA synthesis by different mechanisms for three reasons: First, in combination with $PGF_{2\alpha}$ and insulin, colchi-

cine exerts an additional synergistic effect. Second, insulin acts at any time during the lag phase and must then remain in the culture (5), whereas colchicine exerts its effect only within the first 8 hr after the addition of the growth factor and need not be continuously present. Third, insulin, with or without $PGF_{2\alpha}$, does not disrupt the apparent assembly of the MT (not shown). Furthermore, these results show that colchicine must act on an early event, which is only monitored at the end of the lag phase.

Colchicine interacts in a different manner with $PGF_{2\alpha}$ than with FGF, because it enhances the effect of $PGF_{2\alpha}$ about 2-fold, whereas colchicine with FGF results in about 7-fold stimulation.

Other results with cytochalasin B, added from 0 to 5 hr to cells stimulated by $PGF_{2\alpha}$ or $PGF_{2\alpha}$ plus insulin, indicate that alternation of the microfilaments also enhances the stimulatory effect (unpublished data). Thus the enhancement cannot be due to a disruption of MT alone, but suggests the involvement of microfilaments. Also, other cytoskeleton components, such as intermediate filaments or centrioles, cannot be excluded.

Where and how do these cytoskeleton-disrupting drugs act to enhance initiation of DNA synthesis stimulated by growth factors and the hormones? The findings that cytoskeleton-disrupting drugs do not prevent the stimulation of quiescent Swiss 3T3 cells by $PGF_{2\alpha}$, FGF, or serum rule out the possibility that intact cytoplasmic MT are an absolute requirement for the initiation of DNA synthesis (16, 17). Yet the change in the degree of polymerization or other organizational parameters of cytoskeleton components may play a regulatory role in modulating the value of k. Theoretically, one can envisage at least three levels in the cell at which the cytoskeleton-disrupting drugs may act. The first possibility is the plasma membrane, where these drugs may indirectly loosen the restriction on the membrane fluidity for receptor interactions (13, 16) or change the internalization of the receptors into the cell (14, 15). The second possibility is at the level of protein synthesis. Colchicine could release ribosomes attached to cytoskeleton components and may thereby affect the rate of total or specific protein synthesis, which would be reflected in an increase in the value of k. In fact, an increased rate of protein synthesis has been correlated with changes in the rate of initiation of DNA synthesis in 3T3 cells (8). Furthermore, an increase in the synthetic rate of two nuclear nonhistone proteins has been observed during the lag phase upon stimulation by $PGF_{2\alpha}$ and insulin (5). The third possibility is that colchicine may act at the chromosomal level by facilitating transcription of messenger RNA of specific proteins required for increasing the rate of initiation of DNA synthesis. Our results offer an experimental framework for investigating the possible relationship between cytoskeleton components and the regulation of initiation of DNA synthesis in animal cells.

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