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Two growth factors and two hormones regulate initiation of DNA synthesis in cultured mouse cells through different pathways of events

(prostaglandin $F_{2\alpha}$/epidermal growth factor/insulin/lag phase/regulatory steps)

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ABSTRACT **Epidermal growth factor (EGF) as well as prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), when added to quiescent, confluent Swiss 3T3 cells, stimulate the initiation of DNA synthesis, which occurs with apparent first-order kinetics after a lag phase of 14–15 hr. These two growth factors appear to stimulate similar events; insulin enhances and hydrocortisone can inhibit the stimulatory effect of either. Here we show that the addition of EGF and $PGF_{2\alpha}$ together, however, results in a synergistic effect seen at the end of the lag phase, but only when EGF and $PGF_{2\alpha}$ are added within 6 hr of each other. Addition of one growth factor 10 or 15 hr after the other delayed the synergy for 15 hr after the addition of the second growth factor. Insulin further increased the rate of entry into the S phase stimulated by EGF and $PGF_{2\alpha}$ together, whereas hydrocortisone inhibited the stimulatory effect observed with either EGF or $PGF_{2\alpha}$ alone. These results suggest that, in spite of the common events responsible for the interactions with the two hormones, EGF and $PGF_{2\alpha}$ must have differences in their sequences of events that initiate DNA synthesis.**

Normal animal cells in an environment containing many different growth factors and hormones are able to regulate their rate of proliferation by a variety of poorly understood mechanisms (1–3). One approach to the understanding of the regulatory mechanisms requires the elucidation of the signals and molecular events that are delivered by growth factors and lead to chromosomal DNA replication (4–6). Serum (7) and growth factors such as epidermal growth factor (EGF) (8, 9), fibroblast growth factor (10, 11), and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (12) stimulate the initiation of DNA synthesis in cultures of quiescent, confluent Swiss mouse 3T3 cells by regulating two different phenomena: the progression through the prereplicative period (lag phase; ≈15 hr) and the rate of entry into the S phase (6, 12–15). These two phenomena, studied with defined growth factors, have been interpreted as requiring the existence of two signals: signal 1 and signal 2 (6, 12, 15). The lag phase (induced by signal 1) has been defined as the time between the initial addition of a growth factor and the initiation of DNA synthesis; it appears to be independent of the concentration of the growth factor above a minimal essential concentration. The rate of initiation of DNA synthesis (given by signal 2), however, increases with the growth factor concentration up to a saturating level and can be changed by addition of the same growth factor later during the lag phase and by addition of nonmitogenic compounds (12, 15). The resulting rate follows apparent first-order kinetics and can be quantified by a rate constant k (6, 7, 12–15).

There is evidence that in Swiss mouse 3T3 cells growth factors and hormones such as insulin and hydrocortisone regulate the rate constant through different sequences of cellular events (6, 12, 15). Although a growth factor alone can induce the initiation process, insulin, hydrocortisone, or other compounds, which alone do not initiate DNA synthesis *in these cells*, can modulate the stimulatory effect of the growth factor and, hence, alter the rate of entry into the S phase (15, 16). It has also been shown that the interaction of insulin or hydrocortisone (or both) with cells stimulated by EGF, fibroblast growth factor, or $PGF_{2\alpha}$ is dependent on the growth factor and the time of addition during the lag phase. This has suggested that there is a time-dependent sequence of regulatory events occurring during the lag phase that regulate the rate at which the cell population initiates DNA synthesis (6, 15). Similar conclusions have been drawn from results on the interaction of platelet growth factor with plasma (17).

A central problem that has remained unresolved is whether two different growth factors act by a common sequence of molecular events or whether they act by different sequences that could converge to regulate the initiation of DNA synthesis. Here we show that EGF and $PGF_{2\alpha}$ added together or one added 6 hr after the other interact in a synergistic manner to initiate DNA synthesis after the lag phase of 15 hr. When one growth factor is added 10 or 15 hr after the other, the length of the lag phase remains constant and the synergistic effect is delayed for 15 hr after the addition of the second growth factor. These results suggest that EGF and $PGF_{2\alpha}$ operate by different sequences of cellular events to induce progression through the lag phase and to regulate the rate of entry into the S phase.

MATERIALS AND METHODS

Cultures. Swiss mouse 3T3 cells (18) were maintained in Dulbecco's modified Eagle's medium containing 100 units of penicillin per ml, 100 μg of streptomycin per ml, and 10% (vol/vol) fetal calf serum. Subconfluent cultures were grown in 90-mm petri dishes at 37°C equilibrated with 10% CO_2 in air and were routinely monitored for the absence of mycoplasma contamination (12).

Assay for Initiation of DNA Synthesis and Determination of Rate Constant for Entry into S Phase. Cells (1.5×10^5) were plated in 30-mm dishes and allowed to become confluent and quiescent in Dulbecco's modified Eagle's medium supplemented with low molecular weight nutrients and 6% fetal calf serum as described (12). Cultures were radioactively labeled for autoradiography by exposing them to 1 μM [*methyl*-^{3}H]thymidine (3 μCi/ml; 1 Ci = 3.7×10^{10} becquerels) from the time of addition of the factors or hormones until the times indicated in each experiment. Pairs of cultures were then processed for au-

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Abbreviations: PGE_1, prostaglandin E_1; PGE_2, prostaglandin E_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; EGF, epidermal growth factor.

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toradiography as described (12). For determination of the rate constant k, the percentage of unlabeled cells (y) in a given time (t) was plotted as log Y against t in hours. Straight lines given by log $Y = a - bt$ fit these data well (12). Apparent first-order rate constants (k) were calculated from the slope of the straight lines (b) because $k = \ln 10b$. The maximal and minimal values of k varied within 10–15%, and the lag phase was estimated within 1 hr.

Transport Determinations. For measurements of uptake of 2-deoxy-D-[1-^{3}H]glucose, the cells were plated under culture conditions similar to those for determination of DNA synthesis. Uptake was determined as described (19).

Materials. Prostaglandin E_1 (PGE_1), prostaglandin E_2 (PGE_2), and $PGF_{2\alpha}$ were the generous gift of J. Pike of the Upjohn Company. EGF was obtained from Collaborative Research. Crystalline insulin and fatty acids were purchased from Sigma. [*methyl*-^{3}H]Thymidine (18 Ci/mmol) and 2-deoxy-D-[1-^{3}H]glucose (22 Ci/mmol) were obtained from the Radiochemical Centre (Amersham, England).

RESULTS

Effect of EGF, $PGF_{2\alpha}$, and Insulin on Initiation of DNA Synthesis. The dose–response curves of EGF and $PGF_{2\alpha}$ for the initiation of DNA synthesis within 28 hr in confluent, quiescent Swiss 3T3 cells are shown in Fig. 1. EGF (0.5–20 ng/ml) increased the fraction of labeled nuclei, which reached a plateau of 15% at 10 ng of EGF per ml. Insulin (50 ng/ml), which does not initiate DNA synthesis in 3T3 cells (Table 1) (12), had a synergistic effect with EGF; it increased the value of the labeling index from 15% to 39% and shifted the saturating concentration of EGF to 6 ng/ml (Fig. 1*A*). Addition of EGF with $PGF_{2\alpha}$ alone or together with insulin increased the labeling index even more (to 58% and 78%, respectively), and the saturating concentration of EGF was reduced from 10 ng/ml to 2 ng/ml. The dose–response curves of $PGF_{2\alpha}$ with EGF or insulin (or both) are similar (Fig. 1*B*). EGF and insulin decreased the saturating concentration of $PGF_{2\alpha}$ from 100 ng/ml to 10 ng/ml (Fig. 1*B*). EGF and $PGF_{2\alpha}$, when added at subsaturating concentrations together without or with insulin, gave almost the same synergistic effect as at saturating concentrations (Table 1).

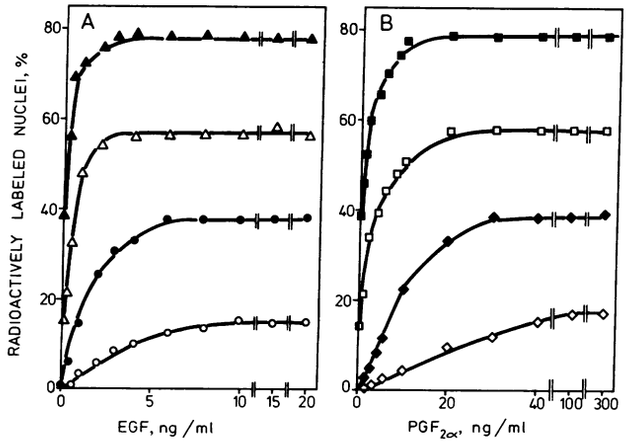

FIG. 1. Synergy among EGF, $PGF_{2\alpha}$, and insulin on the initiation of DNA synthesis. (*A*) ○, EGF; ●, EGF plus insulin (50 ng/ml); △, EGF plus $PGF_{2\alpha}$ (300 ng/ml); ▲, EGF plus insulin (50 ng/ml) and $PGF_{2\alpha}$ (300 ng/ml). (*B*) ◇, $PGF_{2\alpha}$; ◆, $PGF_{2\alpha}$ plus insulin (50 ng/ml); □, $PGF_{2\alpha}$ plus EGF (20 ng/ml); ■, $PGF_{2\alpha}$ plus insulin (50 ng/ml) and EGF (20 ng/ml). Cultures were exposed to [*methyl*-^{3}H]thymidine 0–28 hr after additions and were then processed for autoradiography.

Table 1. Effect of prostaglandins, fatty acids, and insulin on labeling index in quiescent 3T3 cells

Additions	Labeling index, % Without insulin	With insulin
None	0.5	0.8
PGE_1 (300 ng/ml)	0.5	5.3
PGE_2 (30 ng/ml)	0.4	1.6
PGE_2 (300 ng/ml)	1.0	20.0
$PGF_{2\alpha}$ (30 ng/ml)	9.9	45.0
$PGF_{2\alpha}$ (300 ng/ml)	15.0	47.4
EGF (20 ng/ml)	13.2	45.0
+ Arachidonic acid (300 ng/ml)	13.3	46.6
+ Linoleic acid (300 ng/ml)	13.8	43.9
+ Oleic acid (300 ng/ml)	13.5	44.0
+ PGE_1 (30 ng/ml)	15.3	—
+ PGE_1 (300 ng/ml)	22.2	48.0
+ PGE_2 (30 ng/ml)	13.7	50.3
+ PGE_2 (300 ng/ml)	33.4	68.6
+ $PGF_{2\alpha}$ (30 ng/ml)	55.0	79.0
+ $PGF_{2\alpha}$ (300 ng/ml)	56.0	79.5
EGF (2 ng/ml)	5.0	26.0
+ $PGF_{2\alpha}$ (30 ng/ml)	49.0	73.5
Serum (10%)	92.0	98.1

Labeling index was determined as in Fig. 1. Cultures were exposed to [*methyl*-^{3}H]thymidine 0–28 hr after additions and then processed for autoradiography. Insulin was added at 50 ng/ml. Fatty acids were dissolved in absolute ethanol and diluted so that the final concentration of ethanol in the culture medium was 0.01%.

Among the prostaglandins and fatty acids tested with EGF (Table 1), the synergistic effect was maximal for $PGF_{2\alpha}$. The biosynthetic precursors of prostaglandins [arachidonic and linoleic acids (20)] as well as oleic acid added with EGF or with EGF and insulin had no effect on the labeling index. PGE_1 (which at 300 ng/ml had only a marginal stimulatory effect with insulin), when added with EGF or with EGF and insulin, enhanced the labeling index only slightly (Table 1). PGE_2, which is similar in structure to $PGF_{2\alpha}$, had some stimulatory effect at 300 ng/ml in the presence of insulin. When added with EGF or with EGF and insulin, PGE_2 at 30 ng/ml did not significantly enhance the labeling index; however, at 300 ng/ml it had a synergistic effect, though to a lesser extent than $PGF_{2\alpha}$. Only $PGF_{2\alpha}$, which initiates DNA synthesis alone, gave the maximal enhancement at 30 ng/ml.

Interactions of EGF, $PGF_{2\alpha}$, and Hormones During Lag Phase. How do EGF and $PGF_{2\alpha}$ change the kinetics of the initiation of DNA synthesis when added at different times during the lag phase? EGF (20 ng/ml) or $PGF_{2\alpha}$ (200 ng/ml) added alone increased k from a basal level of 0.06×10^{-2} hr^{-1} to 1.1×10^{-2} or 1.3×10^{-2} hr^{-1}, respectively, after a lag phase of 14.5 hr (Fig. 2*A*). Addition of EGF and $PGF_{2\alpha}$ together increased the rate constant to 6.1×10^{-2} hr^{-1} without changing the length of the lag phase. For comparison, k for serum was 24.3×10^{-2} hr^{-1} (Fig. 2*A*). $PGF_{2\alpha}$ added 6 hr after EGF also had a synergistic effect, which was observed immediately upon completion of the lag phase, but k was decreased to 3.1×10^{-2} hr^{-1} (Fig. 2*A*). However, when $PGF_{2\alpha}$ was added 10 or 15 hr after EGF, the synergistic effect was delayed by 15 hr after the addition of $PGF_{2\alpha}$; i.e., up to 25 or 30 hr after stimulation by EGF, k was that of EGF alone before it increased from 1.1×10^{-2} to 3.4×10^{-2} or 3.3×10^{-2} hr^{-1}, respectively.

The interaction of EGF at different times during the lag phase set by $PGF_{2\alpha}$ followed a similar pattern (Fig. 2*B*). Addition of EGF 6 hr after $PGF_{2\alpha}$ had a synergistic effect at the end of the lag phase, with k (4.3×10^{-2} hr^{-1}) lower than when

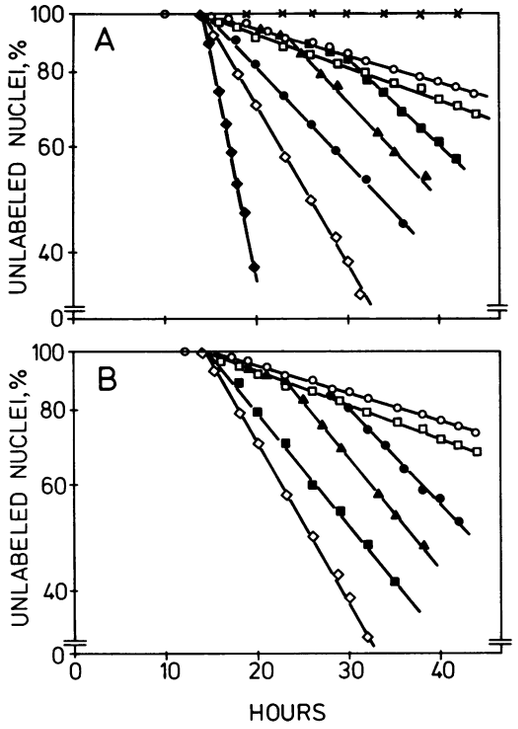

FIG. 2. Fraction of cells that remains unlabeled after synchronous or nonsynchronous addition of EGF (20 ng/ml), $PGF_{2\alpha}$ (300 ng/ml), and 10% fetal calf serum. (*A*) **X**, No additions; ○, EGF; □, $PGF_{2\alpha}$; ◆, serum. EGF with $PGF_{2\alpha}$ added at: ◇, 0 hr; ●, 6 hr; ▲, 10 hr; ■, 15 hr. (*B*) □, $PGF_{2\alpha}$; ○, EGF. $PGF_{2\alpha}$ with EGF added at: ◇, 0 hr; ■, 6 hr; ▲, 10 hr; ●, 15 hr. [*methyl*-^{3}H]Thymidine was present from 0 hr until the times indicated. Value of *k* for no addition was determined over a period of 7 days.

EGF and $PGF_{2\alpha}$ were added together (Fig. 2*B*). EGF added 10 or 15 hr after the beginning of the lag phase likewise delayed the synergistic effect by 15 hr, resulting in rate constants of 4.3 $\times 10^{-2}$ hr^{-1} and 3.7 $\times 10^{-2}$ hr^{-1} at 25 and 30 hr, respectively.

How does insulin affect the interaction of EGF and $PGF_{2\alpha}$

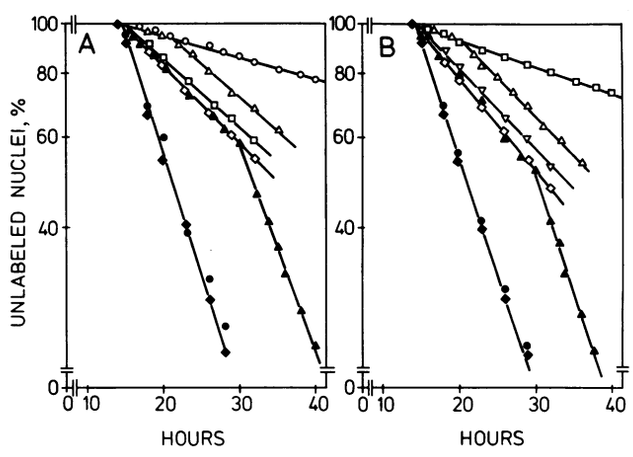

FIG. 3. Fraction of cells that remains unlabeled after synchronous or nonsynchronous addition of EGF (20 ng/ml), $PGF_{2\alpha}$ (300 ng/ml), and insulin (50 ng/ml). (*A*) ○, EGF. EGF with insulin added at: ◇, 0 hr; □, 6 hr; △, 15 hr. EGF plus insulin with $PGF_{2\alpha}$ added at: ◆, 0 hr; ●, 6 hr; ▲, 15 hr. (*B*) □, $PGF_{2\alpha}$. $PGF_{2\alpha}$ with insulin added at: ◇, 0 hr; ▽, 6 hr; △, 15 hr. $PGF_{2\alpha}$ plus insulin with EGF added at: ◆, 0 hr; ●, 6 hr; ▲, 15 hr. [*methyl*-^{3}H]Thymidine was present from 0 hr until the times indicated.

during the lag phase? Addition of insulin to EGF increased *k* from 1.0 $\times 10^{-2}$ hr^{-1} to 3.5 $\times 10^{-2}$ hr^{-1}. In the presence of $PGF_{2\alpha}$, this value was further increased to 10.9 $\times 10^{-2}$ hr^{-1} without a change in the lag phase (Fig. 3*A*). When $PGF_{2\alpha}$ was added 6 hr after EGF and insulin, the synergistic effect was the same as if $PGF_{2\alpha}$ had been added at the beginning. This is in contrast to the lower synergistic effect of $PFG_{2\alpha}$ added 6 hr after EGF without insulin (Fig. 2*A*). However, as in the absence of insulin, $PGF_{2\alpha}$ added 15 hr after EGF and insulin caused a delay in the synergy of 15 hr. The value of *k* was that of EGF and insulin (3.5 $\times 10^{-2}$ hr^{-1}) until 30 hr, when it abruptly increased to 9.3 $\times 10^{-2}$ hr^{-1}.

The interaction of EGF with $PGF_{2\alpha}$ and insulin followed a similar pattern (Fig. 3*B*). Addition of EGF 6 hr after $PGF_{2\alpha}$ and insulin also resulted in the same synergistic effect as if EGF had

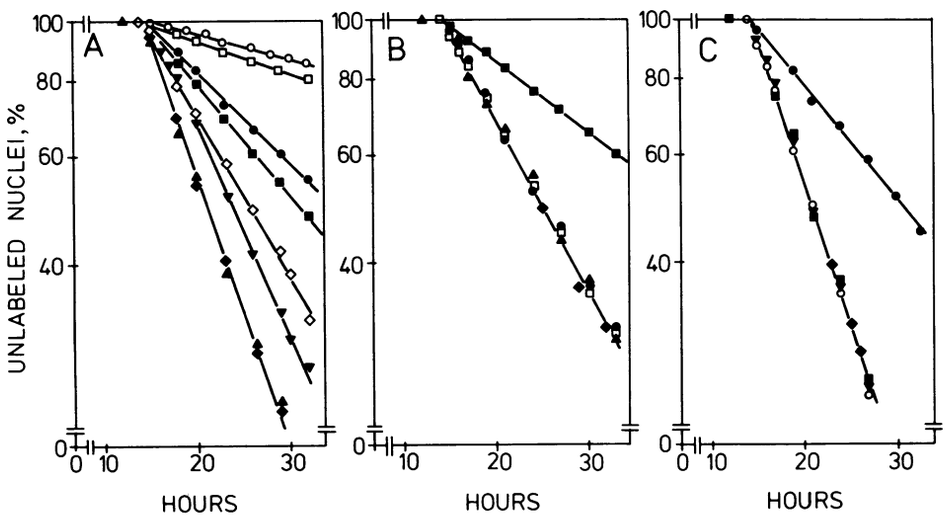

FIG. 4. Fraction of cells remaining unlabeled after addition of EGF (20 ng/ml) and $PGF_{2\alpha}$ (300 ng/ml) with or without insulin (50 ng/ml) or hydrocortisone (30 ng/ml) or both. (*A*) Addition of insulin at different times after EGF plus $PGF_{2\alpha}$. ○, EGF; □, $PGF_{2\alpha}$; ●, EGF plus insulin; ■, $PGF_{2\alpha}$ plus insulin; ◇, EGF plus $PGF_{2\alpha}$. EGF plus $PGF_{2\alpha}$ with insulin added at: ◆, 0 hr; ▲, 6 hr; ▼, 15 hr. (*B*) Addition of hydrocortisone at different times after EGF and $PGF_{2\alpha}$. □, EGF plus $PGF_{2\alpha}$. EGF plus $PGF_{2\alpha}$ with hydrocortisone added at: ■, 0 hr; ▲, 9 hr; ●, 15 hr; ◆, 21 hr. (*C*) Addition of hydrocortisone at different times after EGF, $PGF_{2\alpha}$, and insulin. ○, EGF plus $PGF_{2\alpha}$ and insulin. EGF plus $PGF_{2\alpha}$ and insulin, with hydrocortisone added at: ●, 0 hr; ▼, 9 hr; ■, 15 hr; ◆, 21 hr. [*methyl*-^{3}H]Thymidine was present from 0 hr until the times indicated.

been added at the beginning. Furthermore, EGF added 15 hr after $PGF_{2\alpha}$ and insulin resulted in a delay of 15 hr before the rate constant changed abruptly at 30 hr from that of $PGF_{2\alpha}$ and insulin (4.2×10^{-2} hr^{-1}) to that reflecting the full synergistic effect (10.2×10^{-2} hr^{-1}). Thus, the basic pattern of interaction between EGF and $PGF_{2\alpha}$ is not altered by the presence of insulin.

The pattern of interaction between EGF and $PGF_{2\alpha}$ is basically different from that of insulin with either EGF or $PGF_{2\alpha}$ (Fig. 3). Insulin added 6 hr after EGF or $PGF_{2\alpha}$ resulted in a slight loss of the synergistic effect, from 3.5×10^{-2} to 3.1×10^{-2} hr^{-1} for EGF (Fig. 3*A*) or from 4.2×10^{-2} to 3.6×10^{-2} hr^{-1} for $PGF_{2\alpha}$ (Fig. 3*B*). However, when insulin was added 15 hr after either growth factor, there was a delay of the synergistic effect of only 6 hr; i.e., the rate constant changed abruptly at about 21 hr from 1.0×10^{-2} to 3.1×10^{-2} hr^{-1} for EGF (Fig. 3*A*) or from 1.2×10^{-2} to 3.6×10^{-2} hr^{-1} for $PGF_{2\alpha}$ (Fig. 3*B*).

The effect of two different hormones added at different times during the lag phase on the synergy between EGF and $PGF_{2\alpha}$ is shown in Fig. 4. As already described (Fig. 3), insulin added together with EGF and $PGF_{2\alpha}$ had a synergistic effect with k of 10.2×10^{-2} hr^{-1} (Fig. 4*A*). Insulin added 6 hr after EGF and $PGF_{2\alpha}$ resulted in the same rate constant as if it had been added at the beginning, in contrast to the slight loss of synergy when interacting with only one growth factor (Figs. 3 and 4*A*). However, as with the interaction with either EGF or $PGF_{2\alpha}$ alone, insulin added 15 hr after EGF and $PGF_{2\alpha}$ resulted in a delay of the synergistic effect of 5 hr; i.e., k increased abruptly at about 20 hr from 6.1×10^{-2} to 8.1×10^{-2} hr^{-1} (Fig. 4*A*).

Hydrocortisone (30 ng/ml) inhibited the stimulatory effect of either EGF or $PGF_{2\alpha}$ alone or with insulin only when added within the first 6 hr of the lag phase (12, 15). When hydrocortisone was added together with EGF and $PGF_{2\alpha}$, it also inhibited the synergistic effect between the two growth factors by reducing k from 6.5×10^{-2} to 2.7×10^{-2} hr^{-1} (Fig. 4*B*). However, addition of hydrocortisone 9, 15, or 21 hr after EGF and $PGF_{2\alpha}$ had no effect on the initiation of DNA synthesis (Fig. 4*B*). The same interaction of hydrocortisone occurred with EGF, $PGF_{2\alpha}$, and insulin. Only when the glucocorticoid was added at the beginning of the lag phase was k reduced from 10.8×10^{-2} to 4.3×10^{-2} hr^{-1}. Later additions at 9, 15, or 21 hr had no inhibitory effect. Thus, insulin and hydrocortisone interact with EGF and $PGF_{2\alpha}$ together in the same way as they do with each of these growth factors separately.

Table 2. Effect of EGF, $PGF_{2\alpha}$, insulin, and serum on 2-deoxyglucose uptake and cell number

Additions	2-Deoxyglucose uptake, pmol/min per mg of protein		No. of cells $\times 10^{-5}$
	1 hr	6 hr	
None	92	64	5.6
Cycloheximide	108	48	—
Insulin	132	132	5.7
EGF	128	188	6.5
$PGF_{2\alpha}$	119	232	6.7
EGF and insulin	148	380	10.8
$PGF_{2\alpha}$ and insulin	136	445	11.5
EGF and $PGF_{2\alpha}$	156	736	15.6
EGF, $PGF_{2\alpha}$, and cycloheximide	136	112	—
EGF, $PGF_{2\alpha}$, and insulin	176	900	18.1
EGF, $PGF_{2\alpha}$, insulin, and cycloheximide	136	152	—
Serum	184	1025	20.8

For measurement of 2-deoxyglucose uptake, cells were plated in 30-mm dishes as for determination of DNA synthesis. At 1 and 6 hr after additions were made, cells were labeled for 10 min with 2.5 μCi of 2-[^{3}H]deoxyglucose (50 μM). For determination of the cell number, cells were plated at 1.5×10^5 per 50-mm dishes under the same conditions as above. Additions were made when no mitotic figures were present; 54 hr later, the cells were suspended with 0.05% trypsin and counted in isotonic buffer with a Coulter Counter. Concentrations: insulin, 50 ng/ml; EGF, 20 ng/ml; $PGF_{2\alpha}$, 300 ng/ml; cycloheximide, 10 μg/ml; and fetal calf serum, 10%. The average variation of experimental values was 10% for 2-deoxyglucose uptake and 5% for the cell number.

The synergistic effects of EGF, $PGF_{2\alpha}$, and insulin on DNA synthesis were also reflected by 2-deoxyglucose uptake (6, 19). Although insulin and EGF alone each stimulates 2-deoxyglucose uptake only marginally and $PGF_{2\alpha}$ stimulates uptake about 2.5-fold within 6 hr, insulin with either EGF or $PGF_{2\alpha}$ had a synergistic effect (Table 2). However, this effect increased dramatically when EGF and $PGF_{2\alpha}$ were added together, and in the presence of insulin these two growth factors stimulated 2-deoxyglucose uptake like serum. Similar results were obtained with 3-*O*-methylglucose, which cannot be phosphorylated (not shown), suggesting that the uptake was not due to intracellular trapping of the sugar by phosphorylation (6). The synergistic effect on 2-deoxyglucose uptake was abolished by cycloheximide, indicating that this phenomenon requires protein synthesis (Table 2).

The stimulation of DNA synthesis is reflected in an increase in cell number after addition of EGF, $PGF_{2\alpha}$, and insulin. EGF and $PGF_{2\alpha}$, which stimulate a low rate of initiation of DNA synthesis, increased the cell number only about 20% within 54 hr. However, insulin with EGF or $PGF_{2\alpha}$ resulted in a doubling of the quiescent cell number (Table 2). More dramatically, EGF and $PGF_{2\alpha}$ together increased the number of dividing cells 3-fold, and in the presence of insulin the cell number reached almost the same value as with serum. Thus, although each growth factor alone stimulates only a small fraction of the population to initiate DNA synthesis and subsequent division within a given time, together, when their effect is synergistic on the value of k, the cell number after 54 hr is more than the sum of the cell numbers obtained with either EGF or $PGF_{2\alpha}$ alone.

DISCUSSION

A basic question in growth regulation is whether each growth factor stimulates the initiation of DNA synthesis by the same pathway or whether each acts by a unique mechanism. This question includes the problem of whether cooperative effects exist among the multitude of growth factors that can be present in the culture medium or in the interstitial fluid of a cell responding to them. The results presented here show that EGF and $PGF_{2\alpha}$ together have a synergistic effect on the rate of initiation of DNA synthesis without changing the length of the prereplicative period. This suggests that some signals or events regulating the rate of entry into the S phase are different for these two growth factors. Also, insulin and hydrocortisone interact with cells stimulated by EGF and $PGF_{2\alpha}$ together at the same times during the lag phase as they do with cells stimulated with either growth factor alone. Therefore, EGF and $PGF_{2\alpha}$ may have those events in common that are enhanced by insulin and inhibited by hydrocortisone.

We postulate that each growth factor triggers a different sequence of events which must, nevertheless, have certain steps in common to enable them to cooperate with each other in increasing the rate of initiation of DNA synthesis. The full synergistic effect occurs only when the events of both sequences can interact simultaneously, as is the case when both growth

factors are added together. When a second growth factor is added at 6 hr after the beginning of the lag phase, there may be regulatory events in common that allow cooperation between the events triggered by each growth factor. Yet it appears that there are fewer in common events than when the growth factors are added together inasmuch as the synergistic effect is less. When the second growth factor is added at 10 or 15 hr, its events can no longer be integrated with those of the first growth factor. The cell has then possibly passed the regulatory event(s) occurring before or around 6 hr that allow the events induced by the second growth factor to be integrated during the lag phase set by the first growth factor. However, some biochemical effects of the first growth factor can still interact with regulatory events triggered by the second growth factor because there is a synergistic effect expressed after completion of the second sequence of events.

The long delay in the synergistic effect is in contrast to previous results in which a low, subsaturating concentration of $PGF_{2\alpha}$ (60 ng/ml) was supplemented with a saturating concentration of $PGF_{2\alpha}$ (240 ng/ml) at 15 hr. In this case, the delay in reaching the final value of k is only 4–6 hr (6, 12). However, when a subsaturating concentration of $PFG_{2\alpha}$ (60 ng/ml) is supplemented with EGF either at subsaturating (2 ng/ml) or saturating (20 ng/ml) concentrations at 15 hr, then the value of k given by $PGF_{2\alpha}$ does not increase until 15 hr after the addition of EGF (unpublished data). This also indicates that some regulatory events during the lag phase are different for EGF and $PGF_{2\alpha}$ because they cannot complement each other at any time of the lag phase as the identical growth factor can.

The synergistic effect among EGF, $PGF_{2\alpha}$, and insulin agrees with the view of Holley (5) that growth factors, hormones, and nutrients affect different cellular events leading to the initiation of DNA synthesis. The pattern of interactions of the two growth factors supports the concept that each growth factor delivers two different signals, one to set the lag phase with a specific sequence of events and another to regulate the rate of initiation of DNA synthesis (6, 12, 15). We do not know whether both signals are different or only one of the signals is different for the two growth factors. Our results are difficult to interpret by other models of the cell cycle. In particular, it remains to be seen how these results and the concept of the two signals are compatible with the model of two random transitions proposed by Brooks *et al.* (14).

Is the synergy between EGF and $PGF_{2\alpha}$ also expressed in any of the biochemical events occurring prior to the initiation of DNA synthesis? The interaction between EGF and $PGF_{2\alpha}$ within the first 6 hr is reflected in the synergistic effect on 2-deoxyglucose uptake. This synergy is observed only on the protein synthesis-dependent phase, not on the early, protein synthesis-independent phase. It could thus be postulated that integration of the events triggered by the two growth factors requires protein synthesis. Indeed, other evidence suggests that the appearance of specific proteins is correlated with the initiation of DNA synthesis (21). It has been shown that, upon stimulation of quiescent 3T3 cells by $PGF_{2\alpha}$ and insulin, a nuclear, nonhistone protein (M_r 33,000) appears at about 15 hr. However, when DNA synthesis is inhibited by addition of hydrocortisone within 5 hr after $PGF_{2\alpha}$ and insulin, this protein is markedly decreased (6). These results imply that events occurring early during the lag phase may regulate the activity of a putative initiator at the end of the lag phase. Other levels of control may also be involved. Other biochemical events correlating with the mitogenic response need to be found to enable us to identify the regulatory steps leading to the initiation of DNA synthesis.

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