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Initiation of cell proliferation in cultured mouse fibroblasts by prostaglandin $F_{2\alpha}$

(serum/insulin/cyclic nucleotides/DNA synthesis)

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ABSTRACT **Prostaglandin (PG) $F_{2\alpha}$ added to quiescent Swiss mouse 3T3 cell cultures initiates DNA synthesis and cell proliferation in a small proportion of the cells. Insulin, which markedly potentiates the effect of $PGF_{2\alpha}$ on the initiation of cell division when added with $PGF_{2\alpha}$, causes a simultaneous reduction in intracellular cyclic AMP and an increase in cyclic GMP concentrations similar to those observed after serum addition. Prostaglandins E_1 and E_2 are less effective than $PGF_{2\alpha}$ in initiating DNA synthesis and this effect is only observed at higher concentrations. Therefore, some prostaglandins can act as extracellular factors to regulate cell proliferation.**

Untransformed fibroblasts in tissue culture may exhibit two extreme physiological growth states: quiescence where the majority of the cells are in the $G_0 - G_1$ phase of the cell cycle, or active proliferation (1, 2). Transition from the quiescent to the rapidly proliferating state can be regulated by the concentration of essential nutrients (3), serum (4), or other growth-promoting factors such as insulin present in the medium (5, 6). Most of these factors seem to act on the cell surface (3), and hence the continuation of the stimuli which triggers growth reinitiation within the cell probably requires the generation of intracellular signals in the vicinity of the plasma membrane. Changes in the intracellular concentrations of cyclic AMP, cyclic GMP, Ca^{++} ions, and in the influx of essential nutrients into the cell have been suggested as such regulatory signals (7–12).

Recent reports have suggested the possibility that some of the fatty acids, precursors in the biosynthesis of prostaglandins and phospholipids of the plasma membrane, could control the proliferative capacity of cells (13–15). For instance, the growth in tissue culture of Swiss 3T3, SV3T3 (13), and mouse myeloma cells (14) in a lipid-depleted medium is dependent upon the addition of a variety of unsaturated fatty acids. Furthermore, transformed fibroblasts and cancer cells (16–20) as well as untransformed fibroblasts before the mitotic period (13) have markedly increased the synthesis of some prostaglandins, and this raises the possibility that such substances can act as both negative (7) and as positive extracellular factors to regulate cell proliferation.

Here we have shown that in a series of structurally closely related prostaglandins (PGs) only $PGF_{2\alpha}$ at very low concentrations can promote the initiation of DNA synthesis and cell division in 3T3 fibroblasts and we correlate this growth initiation with changes in the intracellular concentrations of cyclic AMP and cyclic GMP.

MATERIALS AND METHODS

Cell Cultures. Swiss mouse 3T3 fibroblasts (1) were maintained in Dulbecco's modified Eagle's medium (DEM) containing 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 10% fetal calf serum. Subconfluent cultures were grown in 90 mm Nunc petri dishes at 37° under 10% CO_2 pressure and routinely monitored for the absence of mycoplasma contamination.

Cell Growth. Subconfluent cultures were trypsinized and the cells were usually resuspended in DEM supplemented with 5% fetal calf serum unless otherwise indicated. Cells were counted in a Coulter counter and were seeded at 1.2 or 2.0×10^5 cells per 50 mm dish in 5 ml of medium. Usually prostaglandins were added 6 hr after plating or when the cells become quiescent.

Measurement of DNA Synthesis and Percentage of Radioactively Labeled Nuclei. Cells were plated at 0.8 to 1×10^5 cells in 30 mm dishes in 2 ml of DEM with 5% serum and were allowed to become quiescent after 3–4 days after an intermediate medium change into fresh DEM and 5% serum. Cultures were used when no mitotic cells were detected. Cells were radioactively labeled with [*methyl*-^{3}H]thymidine at 3 μCi/ml, 3 μM from 6 until 28 hr after addition of the simulating agents. The medium was removed by aspiration and the cell monolayers were consecutively rinsed with 3 ml of phosphate-buffered saline twice, then with 3 ml of 5% tricholoroacetic acid (w/v), and finally with 3 ml of absolute ethanol, and allowed to dry for 30 min. The cells were then lysed with 2 ml of 2% Na_2CO_3 in 0.2 M NaOH at 37° for 20 min. An aliquot of 0.5 ml was added to a Triton X-100–toluene scintillation fluid which contained 0.2 ml of 50% tricholoroacetic acid to neutralize the NaOH, and the radioactivity was determined in a Packard Tri-Carb scintillation counter. Autoradiography was carried out in parallel cultures labeled with [*methyl*-^{3}H]thymidine at 3 μCi/ml, 1 μM for the same period of time as above (11).

Cyclic AMP and Cyclic GMP Determination. For the determination of the intracellular levels of cyclic nucleotides, cultures were grown in 90 mm dishes in DEM containing 5% fetal calf serum. In order to obtain a confluent monolayer, the medium was changed twice, and the cells were used after 3–4 days after the last medium change as described above. The medium was removed 10 min after the additions and 1.5 ml of 5% tricholoracetic acid was added; the cultures were immediately frozen by exposure to solid CO_2. Extracts from ten 9 cm petri dishes cultures (total of 2.0×10^7 cells) were combined and cyclic [^{3}H]AMP and cyclic [^{3}H]GMP were added to monitor the recovery of both cyclic nucleotides. To test the authenticity of the product assayed, half of the samples were digested with 3′:5′-cyclic nucleotide phosphodiesterase before column chromatography, and the values obtained for the phosphodiesterase-resistant material were 0.04 ± 0.02 and 0.3 ± 0.1 pmol/mg of protein for cyclic GMP and cyclic AMP, respectively. Purification of the digested and undigested samples by Dowex formate

Abbreviations: $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PGB_1, prostaglandin B_1, etc.; DEM, Dulbecco's modified Eagle's medium.

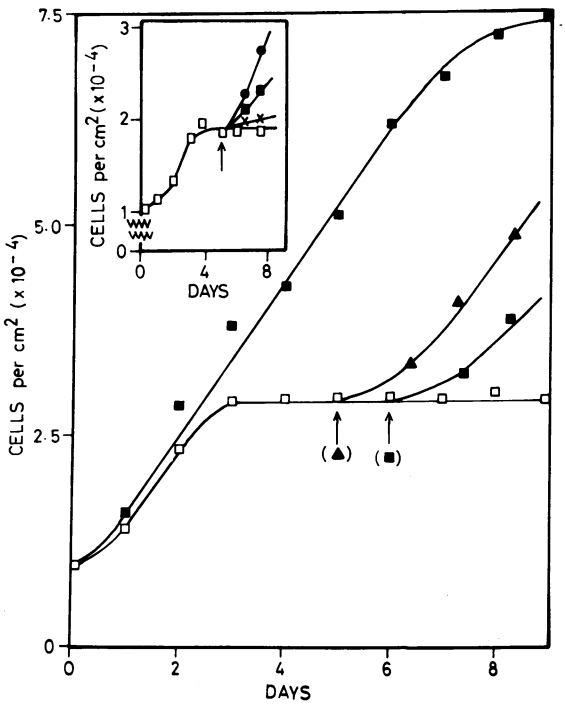

FIG. 1. Effect of $PGF_{2\alpha}$ addition on growing and resting 3T3 cells. Cells were plated at a density of 1×10^4 cells per cm^2 in DEM, containing 5% serum. After 6 hr the medium was supplemented by the addition of (□) none and (■) 75 ng/ml of $PGF_{2\alpha}$. At the time indicated nonmultiplying cultures were supplemented with (▲) 400 ng/ml and (■) 75 ng/ml of $PGF_{2\alpha}$. The insert shows a similar experiment: (x) 50 ng/ml of insulin, (■) $PGF_{2\alpha}$ 400 ng/ml, and ● $PGF_{2\alpha}$ and insulin at the same concentrations.

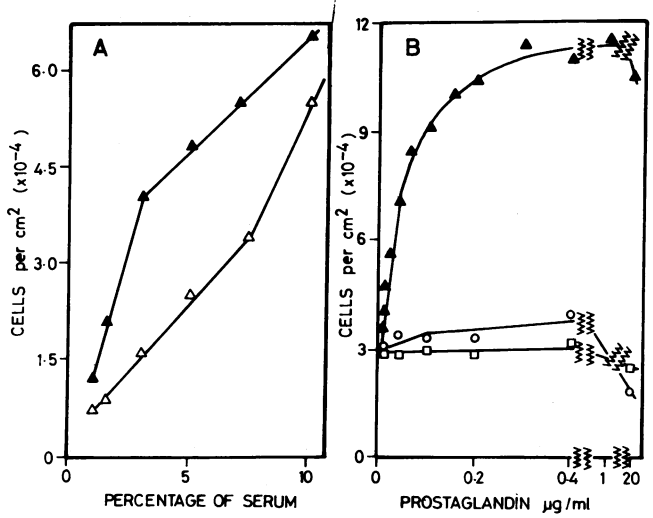

FIG. 2. (A) Effect of $PGF_{2\alpha}$ on the saturation density of 3T3 cells at different serum concentrations. The cells were plated at a density of 0.7×10^4 per cm^2 in DEM containing 1% serum. After 6 hr the medium was supplemented with different serum concentrations: (△) control, and (▲), $PGF_{2\alpha}$ 100 ng/ml. After 7 days the final cell saturation density was determined. (B) Effect of $PGF_{2\alpha}$, PGE_1, and PGB_1 concentrations on the final cell saturation density. Cells were plated at a density of 1×10^4 cells per cm^2 in DEM containing 4% serum. After 6 hr the medium was supplemented with different concentrations of (▲), $PGF_{2\alpha}$; (○), PGE_1; and (□), PGB_1. The final cell numbers were determined after 7 days of growth.

chromatography (11) and their subsequent radioimmunoassay for cyclic necleotides were carried out as previously described (21). Protein was determined by the method of Lowry *et al.* (22).

Materials. Prostaglandins $F_{2\alpha}$, E_1, E_2, and B_1 were the generous gifts of Dr. J. Pike, Upjohn Co. Polyphloretin phosphate from Leo-Hesingborg Laboratory, Sweden was kindly provided by Dr. G. D. Clarke, Imperial Cancer Research Fund; fatty acids, crystalline insulin, oxytocin, eledoisin, physalaemin, and indomethacin were obtained from Sigma. [*Methyl*-^{3}H]thymidine was purchased from the Radiochemical Centre, Amersham, and chemicals used for the radioimmunoassay were obtained from Collaborative Research Inc., U.S.A.

RESULTS

Growth stimulation by prostaglandins

The effect of $PGF_{2\alpha}$ addition on growing and resting 3T3 cells is shown in Fig. 1. Addition of 75 ng/ml of $PGF_{2\alpha}$ to medium containing 5% serum increased the final cell density by about 2.5-fold without appreciably affecting the initial rate of cell growth. When 75 or 400 ng/ml of $PGF_{2\alpha}$ was added to the medium of resting cultures, the number of cells increased by 40–50% within 72 hr of the additions, and the cell numbers obtained were dependent upon the concentration of $PGF_{2\alpha}$ added. (Fig. 1). Addition of physiological concentrations (50 ng/ml) of insulin with $PGF_{2\alpha}$ increased the number of new cells at 72 hr by a factor of 2-fold over that achieved with $PGF_{2\alpha}$ alone. Insulin alone, however caused little or no increase (insert of Fig. 1). This suggested that $PGF_{2\alpha}$ and insulin behaved synergistically to promote cell division in Swiss 3T3 cells.

The addition of $PGF_{2\alpha}$ to the culture medium greatly decreased the amount of serum required to form a confluent monolayer of 3T3 cells. Thus a fixed concentration of $PGF_{2\alpha}$ (100 ng/ml) with serum concentrations in the range of 1–5% caused a 2- to 3-fold increase in the final cell density measured after 7 days of growth, when the cells had ceased dividing. Progressively smaller increases were observed at higher serum concentrations (Fig. 2A). The growth-stimulatory effect of $PGF_{2\alpha}$ upon Swiss 3T3 cells in 4% serum was observed with as little as 10–20 ng/ml and reached a maximum of 4-fold at 300 ng/ml. Little (less than 10% of the increase of $PGF_{2\alpha}$) or no increase in cell number was observed with PGE_1 and PGB_1, respectively, in the same concentration range (Fig. 2B). High concentrations (20 µg/ml and above) of $PGF_{2\alpha}$, PGE_1, and PGB_1 decreased the final cell densities. Addition of $PGF_{2\alpha}$ (100 ng/ml) to simian-virus-40-transformed Swiss 3T3 cells growing in medium containing 0.5% serum yielded no increase in the number of cells.

Initiation of DNA synthesis

The ability of the different prostaglandins to reinitiate DNA synthesis in confluent and quiescent cultures of 3T3 cells is shown in Fig. 3. Addition of $PGF_{2\alpha}$ in a concentration range of 5 ng/ml to 5 µg/ml stimulated the incorporation of [^{3}H]thymidine into DNA reaching a maximum increase of 7-fold over control cultures at 200 ng/ml. At this concentration of $PGF_{2\alpha}$ approximately 14% of the cell population had initiated DNA synthesis by 26 hr compared with 88% after serum addition to the cultures (Table 1). PGE_2 at 100 ng/ml caused a small increase (less than 2-fold) in DNA synthesis, while at relatively high concentrations (1–5 µg/ml) PGE_1 and PGE_2 increased thymidine incorporation into DNA to 50 or 60% of the value obtained with $PGF_{2\alpha}$. PGB_1 failed to

Table 1. Initiation of DNA synthesis by prostaglandin $F_{2\alpha}$, insulin, and fatty acids

Additions	cpm per 2.5 × 10^5 cells	Radioactively labeled nuclei (%)
Control (depleted medium)	8,400	0.25
$PGF_{2\alpha}$ (30 ng/ml)	41,448	9.1
$PGF_{2\alpha}$ (200 ng/ml)	81,099	14.4
Insulin (50 ng/ml)	36,357	4.7
Insulin (50 ng/ml) + $PGF_{2\alpha}$ (30 ng/ml)	135,864	35.0
Insulin (50 ng/ml) + $PGF_{2\alpha}$ (200 ng/ml)	148,785	36.5
Arachidonic acid (400 ng/ml)	8,925	0.2
Arachidonic acid (400 ng/ml) + insulin (50 ng/ml)	34,929	4.2
Arachidonic acid (5 μg/ml)	14,106	1.8
Arachidonic acid (5 μg/ml) + indomethacin (40 μM)	2,139	0.8
Linoleic acid (5 μg/ml)	6,879	1.5
Insulin (10 μg/ml)	185,361	21.1
Insulin (10 μg/ml) + $PGF_{2\alpha}$ (200 ng/ml)	268,728	39.5
Serum (1%)	36,042	—
Serum (1%) + $PGF_{2\alpha}$ (200 ng/ml)	189,672	20.0
Serum (10%)	625,185	88.0
Serum (10%) + PGE_1 (50 μg/ml)	328,542	46.0
Serum (10%) + $PGF_{2\alpha}$ (50 μg/ml)	500,200	80.0

[*Methyl*-^{3}H]thymidine was added to the conditioned medium from 6 to 26 hr after additions. The results were expressed as cpm of [^{3}H]thymidine incorporated into DNA and as the percentage of radioactively labeled nuclei. The conditions were similar to those described in *Materials and Methods*.

stimulate DNA synthesis at any concentration used (Fig. 3). Addition of physiological concentrations of insulin (50 ng/ml) yielded a very small increase in the initiation of DNA synthesis, but when added with $PGF_{2\alpha}$ markedly enhanced the fraction of cells synthesising DNA obtained with $PGF_{2\alpha}$ alone. The value of 35% of cells labeled was greater than the sum of the values obtained with separate additions of insulin (4%) or $PGF_{2\alpha}$ (14%). At high concentrations (10 μg/ml) insulin stimulated a greater proportion of the cells to synthesize DNA (21%) and $PGF_{2\alpha}$ addition only caused an additive increase in DNA synthesis (Table 1). A synergistic effect between $PGF_{2\alpha}$ and low serum concentrations (1%) for thymidine incorporation into DNA was also observed. Pretreatment of quiescent cultures with very high concentrations of PGE_1 and $PGF_{2\alpha}$ (50 μg/ml) for 20 min before the addition

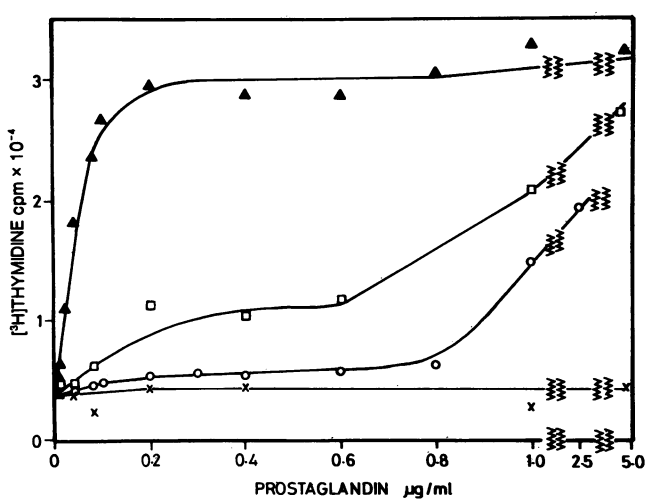

FIG. 3. Stimulation of DNA synthesis by different concentrations of prostaglandins. (▲) $PGF_{2\alpha}$, (□) PGE_2, (○) PGE_1, and (×) PGB_1 were added to the medium of quiescent cultures and after 6 hr the cells were exposed to [*methyl*-^{3}H]thymidine for 20 hr. The results were expressed in cpm of [^{3}H]thymidine incorporated into DNA per 2.5 × 10^5 cells.

of 10% serum caused reductions of 50% and 10%, respectively, in the proportion of cells synthesizing DNA. Addition of $PGF_{2\alpha}$ and insulin to cultures resting for 2 days in medium containing 250 μg/ml of bovine serum albumin and no exogenously added serum gave essentially the same results as cultures resting in DEM and 5% serum.

Effect of precursors, physiological analogues, and inhibitors of $PGF_{2\alpha}$

Arachidonic and linoleic acid are precursors in the biosynthesis of prostaglandins (23). However, these unsaturated fatty acids added at concentrations between 0.4 and 5 μg with or without insulin failed to stimulate significantly the initiation of DNA synthesis in quiescent 3T3 cells (Table 1). The very small increase in DNA synthesis observed with arachidonic acid (about 2%) was completely abolished by the addition of 40 μM indomethacin, 10 times the concentration at which it inhibits the conversion of unsaturated fatty acids to prostaglandin in the canine spleen (25). Eledoisin and physalaemin as well as carbamyl choline and acetylcholine, which have similar physiological effects to $PGF_{2\alpha}$ in renal function and the reproductive system (23, 24), failed to initiate DNA synthesis even in the presence of insulin, while the addition of oxytocin increased DNA synthesis to 16% only in the presence of insulin. Polyphloretin phosphate, an inhibitor of the prostaglandins' actions (23) at a concentration of 2.5 μg/ml reduced the capacity of $PGF_{2\alpha}$ to initiate DNA synthesis in 3T3 cells by about 60% without appreciably inhibiting the stimulation observed with serum (Table 2).

Changes in intracellular cyclic nucleotides

Cyclic AMP and cyclic GMP concentrations were determined 10 min after various additions to confluent 3T3 cultures, under conditions parallel to those described for the initiation of DNA synthesis (Table 3). Addition of serum to quiescent cultures caused a 14-fold stimulation in the intracellular levels of cyclic GMP and about a 30% decrease in the concentration of cyclic AMP as previously reported (7,

Table 2. Effect of vasoactive substances on the initiation of DNA synthesis

Additions	Radioactively labeled nuclei (%)
Control (depleted medium)	0.2
$PGF_{2\alpha}$	11.0
$PGF_{2\alpha}$ + polyphloretin phosphate	4.7
Insulin	4.3
Eledoisin	0.2
Physalaemin	0.2
Oxytocin	1.0
Insulin + $PGE_{2\alpha}$	34.2
Insulin + eledoisin	3.9
Insulin + physalaemin	4.3
Insulin + oxytocin	16.2
Serum	85.0
Serum + polyphloretin phosphate	72.5

Additions were made at the following concentrations: $PGF_{2\alpha}$, 200 ng/ml; eledoisin, 200 ng/ml; physalaemin 200 ng/ml; insulin 50 ng/ml; oxytocin, 50 ng/ml; polyphloretin phosphate 2.5 μg/ml; and fetal calf serum, 10%. Conditions were similar to Table 1.

10, 11). $PGF_{2\alpha}$ added at 300 ng/ml produced a 5-fold increase in cyclic GMP, while cyclic AMP concentrations were only slightly decreased (about 10%). Addition of insulin at 50 ng/ml produced a small increase in intracellular cyclic GMP (2-fold) and the full 30% decrease in cyclic AMP observed upon serum addition. When insulin was added back with $PGF_{2\alpha}$ the observed increase in cyclic GMP (12-fold) was greater than the increase observed with either agent alone. PGE_1 at this concentration yielded little or no change in intracellular cyclic nucleotides. At relatively high concentrations of $PGF_{2\alpha}$ (2.5 μg/ml) the changes were similar to those observed at 300 ng/ml, while the addition of PGE_1 at this concentration increased both the intracellular levels of cyclic AMP and cyclic GMP by about 2- and 4-fold, respectively.

DISCUSSION

Cyclic AMP and cyclic GMP have been implicated in the control of proliferation of untransformed cultured fibroblasts. Early transient decreases in the intracellular levels of cyclic AMP and increases in cyclic GMP were observed immediately after growth reinitiation by serum (7–11). Previous reports have suggested that those substances (oxytocin, serotonin, prostaglandin $F_{2\alpha}$) which like cholinergic agents stimulate smooth muscle contraction also increase intracellular concentrations of cyclic GMP (8), and it was, therefore, interesting to determine whether they could also affect cell growth.

The present results show that very low concentrations of $PGF_{2\alpha}$ (300 ng/ml) can initiate DNA synthesis and cell division in quiescent 3T3 fibroblasts and induce an early increase in intracellular cyclic GMP (4- to 5-fold) with a very small decrease in intracellular cyclic AMP. At the same concentrations the structurally related prostaglandins E_1, E_2, and B_1 fail to initiate significant increases in DNA synthesis. Furthermore PGE_1 also fails to increase intracellular levels of cyclic GMP; PGE_2 and B_1 were not tested for their effects on cyclic nucleotide levels. The growth-initiating effects of $PGF_{2\alpha}$ are augmented by physiological concentrations of insulin, although insulin alone at these concentrations initiates little cell growth. This effect of insulin is similar to its potentiation of fibroblast growth factor for the full mitogenic activity in 3T3 cells (6). Furthermore, addition of insulin decreases cyclic AMP levels to those observed upon serum addition to the cultures, which suggests a possible mechanism to explain the potentiation of the growth-initiating properties of $PGF_{2\alpha}$ by insulin.

Table 3. Changes in cyclic nucleotides

Additions	Cyclic nucleotides (pmol/mg) cAMP	cGMP
No additions	22.0 ± 0.8	0.18 ± 0.02
Serum (10%)	16.1 ± 1.9	2.50 ± 0.22
$PGF_{2\alpha}$, (300 ng/ml)	19.2 ± 0.8	0.90 ± 0.02
Insulin (50 ng/ml)	16.3 ± 0.9	0.32 ± 0.06
$PGF_{2\alpha}$ (300 ng/ml) + insulin (50 ng/ml)	17.0 ± 2.2	2.00 ± 0.31
PGE_1 (300 ng/ml)	21.6 ± 2.2	0.18 ± 0.02
$PGF_{2\alpha}$ (2.5 μg/ml)	17.7 ± 0.7	0.64 ± 0.10
PGE_1 (2.5 μg/ml)	47.2 ± 3.0	0.86 ± 0.21

Additions were made to quiescent cultures of 3T3 cells and ten 9 cm petri dish cultures were isolated 10 min later (*Materials and Methods*). Results are expressed as pmol/mg of total cell protein.

The correlation between the activation of DNA synthesis and changes in the concentrations of cyclic nucleotides is not complete. Thus $PGF_{2\alpha}$ and insulin when added together cause changes in cyclic AMP and cyclic GMP comparable to those induced by serum but initiate DNA synthesis in only 35% of the cells. Other effects of serum on 3T3 cells are also independent of changes in cyclic nucleotide levels. For instance, addition of high concentrations of insulin, which decreases the intracellular concentration of cyclic AMP (27) and increases cyclic GMP (8), only stimulates the early phase of the 2-deoxyglucose transport, which is usually biphasic, while the addition of insulin and 8-bromo cyclic GMP fails to stimulate the protein-synthesis-dependent second phase (26). Also the early increase in phosphate transport stimulated by serum responds to controls other than cyclic AMP (26, 27). The possibility that some of the growth-promoting effects of serum are mediated by the action of prostaglandins warrants investigation.

Prostaglandins E_1 and E_2 at moderately high concentrations (0.5–5 μg/ml) mimic the effect of $PGF_{2\alpha}$ upon the induction of DNA synthesis in quiescent 3T3 fibroblasts, although at very high concentrations (25–50 μg/ml) PGE_1 acts as an inhibitor. This growth-promoting effect is presumably not due to a generalized detergent action upon membranous enzyme systems (e.g., the guanylate cyclase) (6) because no increase was observed upon addition of similar concentrations of PGB_1.

It is possible that at high concentrations (1.0 μg–5.0 μg/ml) the differential cellular changes induced by the prostaglandins become less distinct, because these structurally closely related molecules cross-react with each others' receptors (28).

There are other examples of the involvement of prostaglandins and cell proliferation. For instance, PGE_2 and $PGF_{2\alpha}$ can stimulate DNA and RNA synthesis, respectively, in hemapoietic stem cells (29) and mouse mammary gland explants (30). Furthermore, increased amounts of prostaglandins were detected in virally transformed fibroblasts

(16), ascites cells (19), chemically induced mammary tumors (20), and in spontaneous human cancer (17, 18). Taken together these observations show that the prostaglandins can act as positive as well as negative extracellular regulators of the growth of cells, and these effects can be correlated with the changes in the intracellular levels of cyclic nucleotides.

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