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Activation of inositol phospholipid breakdown by prostaglandin $F_{2\alpha}$ without any stimulation of proliferation in quiescent NIH-3T3 fibroblasts

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Stimulation of NIH-3T3 cells with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) caused a dose- and time-dependent generation of inositol phosphates. The first detectable changes were in the levels of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4. Increases in Ins(1,3,4)P_3, InsP_2 and Ins$P$ were detected later, and only minor changes were observed in putative InsP_5 or InsP_6. The accumulation of inositol phosphates was synergistically increased by the addition of calf serum, whereas $PGF_{2\alpha}$ had no effects on cell proliferation in either the presence or the absence of calf serum. Stimulation of a different clone of NIH-3T3 cells (AmNIH-3T3) or Swiss 3T3 cells with $PGF_{2\alpha}$ resulted in both inositol phospholipid breakdown and cell proliferation. No differences were found in the characteristics of $PGF_{2\alpha}$-stimulated inositol phosphate generation between the two clones of NIH-3T3 cells, nor was there any difference in receptor number or K_d. These results question the role of inositol phospholipid breakdown in mitogenesis and demonstrate significant differences in the biochemical properties of apparently the 'same' cells.

INTRODUCTION

The stimulation of the proliferation of fibroblast cells in culture has been demonstrated to be achieved by a range of growth factors which are capable of activating inositol phospholipid turnover (Berridge, 1987). Agonist-stimulated PtdIns(4,5)P_2 breakdown generates two second-messenger molecules, *sn*-1,2-diacylglycerol and Ins(1,4,5)P_3, which activate protein kinase C and stimulate the release of Ca^{2+} from intracellular stores respectively (Downes & Michell, 1985). A rise in intracellular free Ca^{2+} concentration has been demonstrated to be one of the earliest detectable events after the stimulation of mitosis in fibroblast cells (Hesketh *et al.*, 1985) and the activation of protein kinase C leads to the phosphorylation of a range of cellular proteins, but in particular the Na^+/H^+ antiporter (Hesketh *et al.*, 1985). Activation of the antiporter results in alkalinization of the cytosol, an event which appears obligatory for growth-factor-stimulated mitosis (Schuldiner & Rozengurt, 1982).

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is an agonist which has been demonstrated to be a growth factor for, and to stimulate the breakdown of PtdIns(4,5)P_2 in, Swiss 3T3 fibroblast cells (MacPhee *et al.*, 1984). We have been examining the regulation of the proliferation of NIH-3T3 cells and the role of inositol phospholipid turnover in this process. We report here that $PGF_{2\alpha}$ stimulates inositol phospholipid breakdown in a clone of NIH-3T3 cells, but is not a mitogen. However, in another clone of NIH-3T3 cells and in Swiss 3T3 cells, the agonist stimulates not only inositol phospholipid turnover but also mitogenesis. These results place questions on the role of inositol phospholipid turnover in the stimulation of cell proliferation.

MATERIALS AND METHODS

Cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) donor calf serum in a humidified atmosphere of air/CO_2 (19:1). Cells were plated on to 24-well plates for experiments and allowed to grow to confluence. The cells were labelled for inositol phosphate experiments by changing the medium for the final 24 h to inositol-free DMEM containing 0.4 ng of EGF/ml, 0.4 μg of transferrin/ml, 0.04 μg of insulin/ml and 2 μCi of [^{3}H]inositol/ml.

On the day of experiments, the medium was aspirated and the cells were washed twice with Hanks buffered saline, pH 7.4, containing 10 mM-glucose and 2% (w/v) BSA (HBG). They were then incubated for 5 min in HBG containing 10 mM-LiCl. The cells were then stimulated as described in the Results section by the addition of agonists to fresh HBG containing 10 mM-LiCl, in a final volume of 0.25 ml. All the above procedures were performed at 37 °C. Incubations were terminated by rapid aspiration, followed by the addition of 0.05 ml of ice-cold 10% (v/v) $HClO_4$. The cells were scraped and transferred to tubes; each well was then washed with 3×0.075 ml of water, with the washings being added to the appropriate tubes. After removal of the cellular debris by centrifugation (14000 *g*, 2 min), 0.275 ml of the acid-soluble fraction was transferred to a fresh tube containing 0.075 ml of 10 mM-EDTA and neutralized by the addition of 0.185 ml of a 1:1 (v/v) mixture of Freon and tri-n-octylamine (Sharpes & McCarl, 1982). The inositol phosphate content of the neutralized samples was measured by liquid-scintillation counting after separation on Dowex (formate form) columns as previously described (Wakelam *et al.*, 1986) or by h.p.l.c.

H.p.l.c. analysis was performed on a Partisil 5 WAX

Abbreviations used: $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor.

column (Laserchrom, East Grinstead, Sussex, U.K.) by using a gradient of 1–35% (v/v) 1 M-$(NH_4)_2HPO_4$ (solution B) adjusted to pH 3.7 with H_3PO_4. The gradient was 1% B for 10 min, raised to 8% over 1 min, held at 8% for 10 min, raised to 17% over 10 min, held at this level for 10 min and then increased to 35% over 2 min and held at this level for 3 min. The flow rate was 1 ml/min, and fractions were collected every 15 s. The identity of fractions was determined by running standard ^{3}H-labelled samples of Ins(1)P, Ins(1,4)P_2, Ins(1,4,5)P_3, Ins(1,3,4)P_3 and Ins(1,3,4,5)P_4. The identities of the two later-running fractions were assumed to be InsP_5 and InsP_6, but this identification must be regarded as putative. The elution times were Ins$P$ 7 min, Ins(1,4)P_2 15.5 min, Ins(1,3,4)P_3 17.25 min, Ins(1,4,5)P_3 18.5 min, Ins-(1,3,4,5)P_4 26 min, putative InsP_5 28 min and putative InsP_6 31 min.

$PGF_{2\alpha}$ binding was determined on confluent, quiescent cells in 24-well plates (approx. 1.25×10^5 cells per well). The cells were washed twice with DMEM and incubated for 1 h in DMEM containing 1% BSA at 37 °C. The monolayers were then washed with Hanks buffered saline containing 0.2% BSA, 0.01 mM-indomethacin and 10 mM-Hepes, pH 7.3, and incubated for 15 min at 40 °C in ligand-free binding medium. The binding medium consisted of Hanks buffered saline, pH 7.3, 0.05% BSA, 10 mM-Hepes, 0.01 mM-indomethacin, 1% (v/v) cell-culture-grade physiological amino acid mixture (Gibco, Paisley, Scotland, U.K.) and 2 mM-bacitracin. This medium was then replaced with fresh binding medium containing the stated concentrations of [^{3}H]$PGF_{2\alpha}$. After a 2 h incubation on ice, the monolayers were rapidly washed four times with ice-cold Hanks buffered saline, pH 7.3, containing 0.1% BSA, 10 mM-Hepes and 0.01 mM-indomethacin. The washed cells were solubilized in 0.5 M-NaOH containing 2% (w/v) Na_2CO_3 and 1% (w/v) SDS. After neutralization with 1 M-HCl, the total cell-associated radioactivity was determined by liquid-scintillation counting. Non-specific binding was determined in the presence of 1 mM unlabelled $PGF_{2\alpha}$, and was approx. 37% of total binding. Cell number was determined on parallel wells of cells treated as above, except in the absence of radiolabelled ligand, after trypsin treatment, in an Industrial D Coulter counter.

The incorporation of [^{3}H]thymidine was determined by using confluent quiescent cells in 24-well plates. The cells were washed in DMEM and then incubated for 24 h in fresh DMEM containing 1 μCi of [^{3}H]thymidine/ml and other additions as stated in the Results section. The medium was then aspirated and the monolayers were washed with 4×1 ml of 5% (w/v) trichloroacetic acid, and the acid-insoluble radioactivity was determined by liquid-scintillation counting after solubilization in 0.3 M-NaOH and neutralization with 1.5 M-HCl.

Growth curves utilized cells cultured in 35 mm-diameter dishes. The cells were plated at a density of 10^4 cells per plate in normal medium. After 24 h the medium was removed, and the cells were washed with DMEM and incubated in fresh DMEM with the appropriate additions. At 24 h intervals cell number was determined by Coulter counting after trypsin treatment.

Tissue culture media and supplies were from Gibco, all radiochemicals were from Amersham International, Amersham, Bucks., U.K.; peptides were from Cambridge Research Biochemicals, Cambridge, U.K.; $PGF_{2\alpha}$, bacitracin, transferrin and BSA were from Sigma, Poole, Dorset, U.K.; all other regeants were of the highest grade available from previously reported sources (Wakelam *et al.*, 1986; Black & Wakelam, 1990).

RESULTS

Stimulation of [^{3}H]inositol-labelled NIH-3T3 cells with $PGF_{2\alpha}$, in the presence of 10 mM-LiCl, caused a dose-dependent accumulation of inositol phosphates (Fig. 1). A saturating response was observed at 2 μM-$PGF_{2\alpha}$, with half-maximal stimulation (EC_{50}) occurring at 0.18 μM. This half-maximal value is very similar to the K_d value obtained for [^{3}H]$PGF_{2\alpha}$-receptor binding in these cells (Black & Wakelam, 1990). At saturating concentrations we observed an 11–12-fold increase in inositol phosphate generation after a 30 min stimulation.

Analysis of the $PGF_{2\alpha}$-stimulated inositol phosphates on Dowex formate columns suggested that the initial phosphate formed was InsP_3 (results not shown). This analysis was extended by the use of separation on a Partisil 5 WAX h.p.l.c. column. Fig. 2 demonstrates that stimulation with 2.1 μM-$PGF_{2\alpha}$ induced the generation of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 within 5 s, though the magnitude of the increase in Ins(1,3,4,5)P_4 generation at 5 s (approx. 40% increase) was less than that for Ins(1,4,5)P_3 (approx. 150% increase). The stimulation of Ins(1,4,5)P_3 generation was always observed to be biphasic, as demonstrated in Fig. 2. Peak accumulation of Ins(1,4,5)P_3, in the particular experiment demonstrated, was observed at 15 s, when values were approx. 400% of control; the levels then declined, returning to basal by 30 s. In some experiments peak accumulation was not observed until after 20 s stimulation, and in some the magnitude of the increase in Ins(1,4,5)P_3 was greater, reaching over 600% of the control value. Peak ac-

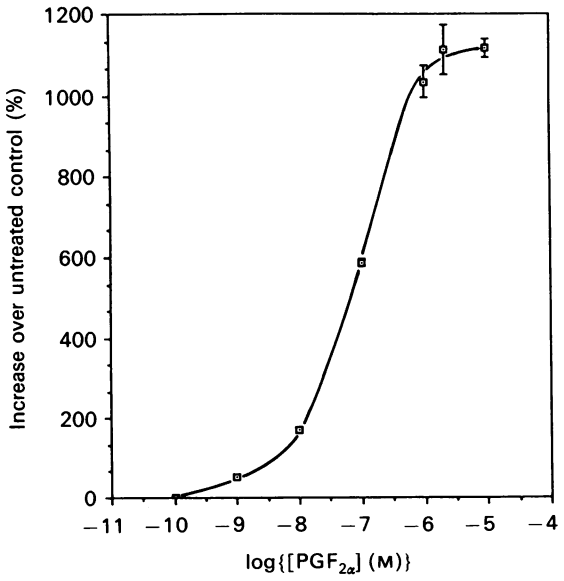

Fig. 1. Dose-dependence of $PGF_{2\alpha}$-stimulated inositol phosphates generation in NIH-3T3 cells

[^{3}H]Inositol-labelled cells were stimulated for 30 min with various doses of $PGF_{2\alpha}$ in the presence of 10 mM-LiCl. The results are expressed as mean percentage increase in the radioactivity (d.p.m.) associated with the inositol phosphates over the untreated control cells (±S.D.), and are pooled from three experiments in each of which $n = 4$.

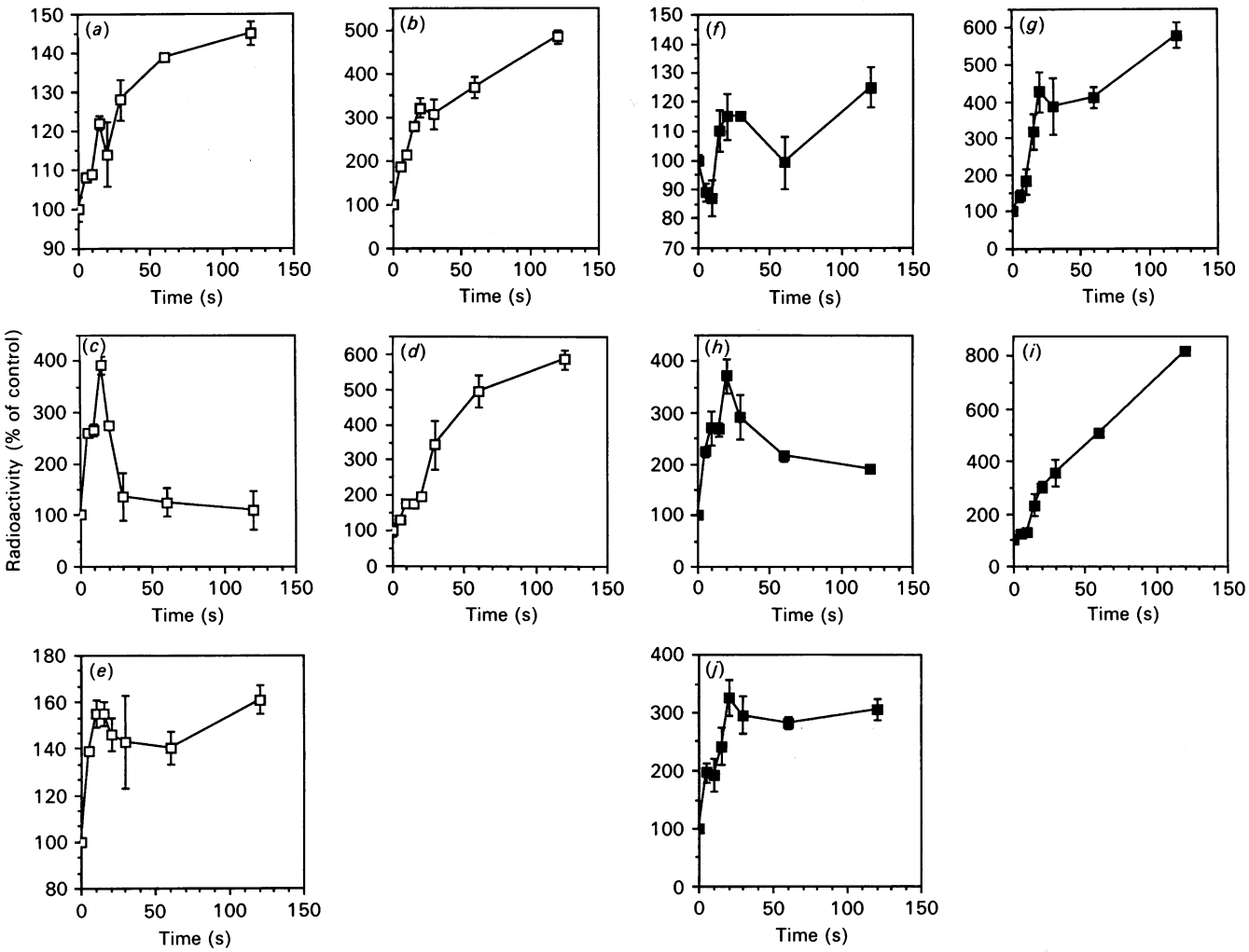

Fig. 2. Analysis of the $PGF_{2\alpha}$-stimulated changes in inositol phosphates in NIH-3T3 and Am-NIH-3T3 cells

[^{3}H]Inositol-labelled cells were stimulated with 2.1 μM-$PGF_{2\alpha}$ for various times in the presence of 10 mM-LiCl. The inositol phosphates were separated by h.p.l.c. as described in the Materials and methods section. Results are presented as the mean percentage of the radioactivity associated with the unstimulated control ($n = 3$ in each case) and the data are from single experiments typical of three or four. (*a*)–(*e*), NIH-3T3-cell inositol phosphates; (*f*)–(*j*), Am-NIH-3T3-cell inositol phosphates. The control values (d.p.m.) were: (*a*) Ins*P* 198976±6023; (*b*) InsP_2 7807±463; (*c*) Ins(1,4,5)P_3 1523±97; (*d*) Ins(1,3,4)P_3 2159±403; (*e*) Ins(1,3,4,5)P_4 3409±54; (*f*) Ins*P* 78595±3089; (*g*) InsP_2 4570±383; (*h*) Ins(1,4,5)P_3 958±55; (*i*) Ins(1,3,4)P_3 1886±306; (*j*) Ins(1,3,4,5)P_4 1005±143.

cumulation of Ins(1,3,4,5)P_4 was detected after stimulation for 10 s, but, unlike Ins(1,4,5)P_3, the level appeared to remain elevated up until 120 s, where it was 160% of that at basal. A 10 s delay was observed before accumulation of Ins(1,3,4)P_3 was detectable (Fig. 2); accumulation of this isomer was then rapid, and after 60 s the level was 500% of basal; after 60 s the rate of generation appeared to decline. Rapid accumulation of inositol mono- and bis-phosphates was also detected. Small changes were also observed in the putative inositol penta- and hexa-kisphosphate fractions, with the increases being significant after 10 s at about 120 and 130% of the control values respectively (results not shown).

Stimulation of NIH-3T3 cells with 4% calf serum induced a 4-fold increase in total inositol phosphates, whereas in the same experiments a maximal dose of $PGF_{2\alpha}$ induced a 10-fold increase (Table 1). However, when the two stimulants were added together to the cells, a 21-fold increase in inositol phosphate generation was detected (Table 1). This synergistic accumulation of inositol phosphates would be thought to be reflected in an equally synergistic effect on mitosis. However, $PGF_{2\alpha}$, at a concentration that gave a maximal effect on PtdIns(4,5)P_2 breakdown, had no effect on calf-serum-stimulated [^{3}H]thymidine incorporation into serum-starved cells (Table 2). This lack of effect was observed at all calf serum concentrations tested. Since [^{3}H]thymidine incorporation into DNA is dependent on uptake of the base into the cells, it was considered possible that in these cells the prostaglandin was inhibiting thymidine uptake while at the same time stimulating DNA synthesis; therefore mitosis was also measured by determining cell number by Coulter counting. Fig. 3 demonstrates that $PGF_{2\alpha}$ was indeed having no effect on calf-serum-stimulated cell proliferation. In addition to having no potentiating effect on calf-serum-stimulated mitosis,

Table 1. Calf-serum- and $PGF_{2\alpha}$-stimulated inositol phosphates accumulation in NIH-3T3 cells

[^{3}H]Inositol-labelled cells were stimulated for 30 min in the presence of 10 mM-LiCl, and then inositol phosphates generation was determined as described in the Materials and methods section. Results are presented as means ± S.D. pooled from two typical experiments; $n = 8$ in each case.

Stimulant	Inositol phosphates generation (% increase over untreated control)
4% calf serum	426 ± 62
2.1 μM-$PGF_{2\alpha}$	1015 ± 76
4% calf serum + 2.1 μM-$PGF_{2\alpha}$	2163 ± 113

$PGF_{2\alpha}$ induced no DNA synthesis in quiescent NIH-3T3 cells in the presence of EGF, insulin and transferrin (Table 3).

Contrary to the results presented in this paper, $PGF_{2\alpha}$ has been reported to be a potent mitogen for NIH-3T3 cells (Yu *et al.*, 1988). Consequently, NIH-3T3 cells were obtained from that group (subsequently referred to as Am-NIH-3T3 cells). In that cell line we confirmed that $PGF_{2\alpha}$ is indeed a potent mitogen (Table 3) and that it potentiated calf-serum-stimulated cell growth to a value 160% of control (Fig. 4). Table 3 also demonstrates that the inclusion of 10 mM-LiCl only had a minor effect on $PGF_{2\alpha}$-stimulated [^{3}H]thymidine incorporation in both cell lines, and that the different mitogenic responses were maintained when the ion was included. MacPhee *et al.* (1984) have reported that the prostaglandin acts as a mitogen for Swiss 3T3 cells. Table 4 shows that $PGF_{2\alpha}$ stimulates [^{3}H]thymidine incorporation in Swiss 3T3 cells in a dose-dependent manner, stimulating a 2–4-fold increase, compared with a 1.3-fold increase in response to calf serum. The prostaglandin is thus a mitogen for these cells, a result further confirmed by Coulter counting (results not shown).

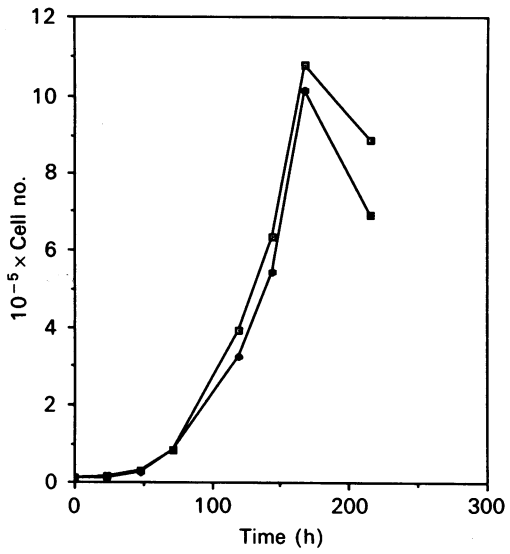

Fig. 3. NIH-3T3-cell growth curves

Cell numbers were determined by Coulter counting as described in the Materials and methods section. Six counts were made of duplicate plates, and results are expressed as means ± S.D. from a single experiment typical of two others. ⊡, Cells grown in DMEM + 10% (v/v) calf serum; ■, cells grown in DMEM + 10% calf serum + 2.1 μM-$PGF_{2\alpha}$.

Since $PGF_{2\alpha}$ stimulated DNA synthesis in Swiss 3T3 and Am-NIH-3T3 cells, but not in NIH-3T3 cells, a comparison of the stimulation of inositol phosphate generation in the cell types was made. In agreement with Sasaki (1985), the prostaglandin was found to induce a 2–3-fold increase in inositol phosphate generation in Swiss 3T3 cells, a value much less than found in NIH-3T3 cells. However, $PGF_{2\alpha}$ induced essentially an equivalent increase in inositol phosphate generation in the NIH-3T3 and the Am-NIH-3T3 cells (Table 5). Further analysis of the $PGF_{2\alpha}$-stimulated inositol phosphate response in the Am-NIH-3T3 cells revealed no major

Table 2. Effect of 2.1 μM-$PGF_{2\alpha}$ on calf-serum-stimulated [^{3}H]thymidine incorporation into growth-arrested NIH-3T3 cells

Quiescent NIH-3T3 cells were washed in DMEM and incubated for 24 h in DMEM containing 1 μCi of [^{3}H]thymidine/ml, 0.04 ng of insulin/ml, 0.4 μg of transferrin/ml, 0.4 ng of EGF/ml and the additions stated in the table. Acid-insoluble radioactivity was determined as described in the Materials and methods section. Results are expressed as means ± S.D. ($n = 4$) and are from one experiment typical of three others. The $PGF_{2\alpha}$ concentration was 2.1 μM; calf serum concentrations are stated as v/v. 'Fold increase' refers to effect of $PGF_{2\alpha}$ on stimulation by calf serum.

Additions	[^{3}H]Thymidine incorporated (d.p.m.)	Fold increase
None	11594 ± 454	
$PGF_{2\alpha}$	12337 ± 1140	1.06
0.5% calf serum	15101 ± 540	
0.5% calf serum + $PGF_{2\alpha}$	14763 ± 1459	0.98
1% calf serum	19104 ± 4588	
1% calf serum + $PGF_{2\alpha}$	18764 ± 2518	0.98
5% calf serum	18535 ± 1174	
5% calf serum + $PGF_{2\alpha}$	18879 ± 2394	1.02
10% calf serum	19136 ± 2435	
10% calf serum + $PGF_{2\alpha}$	18851 ± 1999	0.98

Table 3. Stimulation of [^{3}H]thymidine incorporation in NIH-3T3 and Am-NIH-3T3 cells

Quiescent cells were washed in DMEM and incubated for 24 h in DMEM containing 1 μCi of [^{3}H]thymidine/ml, 0.04 ng of insulin/ml, 0.4 μg of transferrin/ml, 0.4 ng of EGF/ml and the additions stated below. Acid-insoluble radioactivity was determined as described in the Materials and methods section. Results are expressed as means $\pm$ S.D. (n = 3–4) and are from one experiment typical of two others.

Cells Additions	[^{3}H]Thymidine incorporated (d.p.m.)	Fold increase over control
NIH-3T3 Serum-free control	1629 $\pm$ 173	–
10% calf serum	13228 $\pm$ 210	8.1
2.1 μM-$PGF_{2\alpha}$	1617 $\pm$ 103	1.0
Serum-free control + 10 mM-LiCl	1447 $\pm$ 40	–
10 mM-LiCl + 10% calf serum	13286 $\pm$ 324	9.1
10 mM-LiCl + 2.1 μM-$PGF_{2\alpha}$	1734 $\pm$ 174	1.2
Am-NIH-3T3 Serum-free control	1808 $\pm$ 191	–
10% calf serum	11054 $\pm$ 449	6.1
2.1 μM-$PGF_{2\alpha}$	4214 $\pm$ 210	2.3
Serum-free control + 10 mM-LiCl	2033 $\pm$ 45	–
10 mM-LiCl + 10% calf serum	15419 $\pm$ 414	7.6
10 mM-LiCl + 2.1 μM-$PGF_{2\alpha}$	3728 $\pm$ 183	1.8

differences to that in NIH-3T3 cells. Fig. 5 demonstrates that the EC_{50} for inositol phosphate generation (0.11 μM) was almost the same as in Fig. 1. Analysis of the $PGF_{2\alpha}$-stimulated changes in individual inositol phosphates in the Am-NIH-3T3 cells (Fig. 2) demonstrated a very similar pattern to that observed in NIH-3T3 cells (Fig. 2). Scatchard analysis of $PGF_{2\alpha}$-binding data generated a K_d of 0.56×10^{-6} M, with approx. 500000 receptors per cell (Fig. 6); we have obtained almost identical values for our NIH-3T3 cells (Black & Wakelam, 1990). Although no differences in $PGF_{2\alpha}$-stimulated inositol phosphate generation were detected between the two cell lines, the Am-NIH-3T3 cells were found to respond to a greater extent to bombesin and the NIH-3T3 cells to bradykinin and platelet-derived growth factor, but no differences in response to calf serum were detected (Table 5).

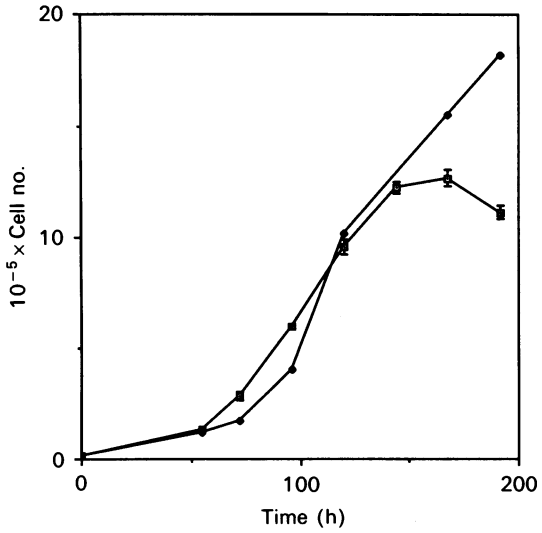

Fig. 4. Am-NIH-3T3-cell growth curves

Results are means $\pm$ S.D. of six counts on triplicate plates and are from one experiment typical of two. Other details are as in the legend to Fig. 3. ⊡, Cells grown in DMEM + 10% calf serum; ■, cells grown in DMEM + 10% calf serum + 2.1 μM-$PGF_{2\alpha}$.

DISCUSSION

$PGF_{2\alpha}$ is clearly shown in this study to stimulate the hydrolysis of PtdIns(4,5)P_2, generating Ins(1,4,5)P_3, in both clones of NIH-3T3 cells (Fig. 2). This product is metabolized in two ways; it can be dephosphorylated to generate InsP_2, or phosphorylated to generate Ins(1,3,4,5)P_4, which is in turn dephosphorylated to Ins(1,3,4)P_3 (Fig. 2). The stimulation of Ins(1,4,5)P_3 generation was biphasic, which may imply a secondary phase of PtdIns(4,5)P_2 breakdown. We have shown elsewhere that stimulation of these cells with a maximal $PGF_{2\alpha}$ concentration results in an increase in intracellular

Table 4. Stimulation of [^{3}H]thymidine incorporation in Swiss 3T3 cells

Quiescent Swiss 3T3 cells were washed in DMEM and incubated for 24 h in DMEM containing 1 μCi of [^{3}H]-thymidine/ml, 0.04 ng of insulin/ml, 0.4 μg of transferrin/ml, 0.4 ng of EGF/ml and the additions stated below. Acid-insoluble radioactivity was determined as described in the Materials and methods section. Results are expressed as means $\pm$ S.D. (n = 4) and are from one experiment typical of two others.

Addition	[^{3}H]Thymidine incorporated (d.p.m.)	Fold increase over control
Serum-free control	3257 $\pm$ 599	
10% calf serum	4142 $\pm$ 647	1.3
0.841 nM-$PGF_{2\alpha}$	6329 $\pm$ 1734	1.9
8.41 nM-$PGF_{2\alpha}$	5000 $\pm$ 981	1.5
84.1 nM-$PGF_{2\alpha}$	7658 $\pm$ 613	2.3
0.841 μM-$PGF_{2\alpha}$	7992 $\pm$ 185	2.4
2.10 μM-$PGF_{2\alpha}$	7095 $\pm$ 400	2.2

Table 5. Comparison of stimulated inositol phosphates generation between NIH-3T3 and Am-NIH-3T3 cells

[^{3}H]Inositol-labelled cells were stimulated for 30 min, in the presence of 10 mM-LiCl, with the stated agonists, and inositol phosphates generation was determined as described in the Materials and methods section. Results are means ± S.E.M., and are pooled from three separate experiments (n = 12).

Ligand	Inositol phosphates (% increase over basal) NIH-3T3 cells	Am-NIH-3T3 cells
Bombesin (2.5 μM)	9±3	193±27
Platelet-derived growth factor (1.32 μg/ml)	43±7	28±10
Bradykinin (3.2 μM)	104±9	31±3
$PGF_{2\alpha}$ (2.1 μM)	1250±78	1261±147
Calf serum (4%)	254±33	259±9

free Ca^{2+} (Lloyd *et al.*, 1989). The effects of the prostaglandin on inositol phospholipid metabolism are thus similar to those observed in response to a range of other agonists, such as bradykinin and vasopressin, in a range of cell types (see Berridge, 1987). The stimulation of inositol phosphate generation was dose-dependent, with half-maximal stimulation at 0.18 μM and a saturating response observed at 2 μM (Fig. 1). This EC_{50} is close to the K_d observed for $PGF_{2\alpha}$ receptor binding (Black & Wakelam, 1990) and similar to that reported by MacPhee *et al.* (1984) for the stimulation of inositol phospholipid metabolism in Swiss 3T3 cells.

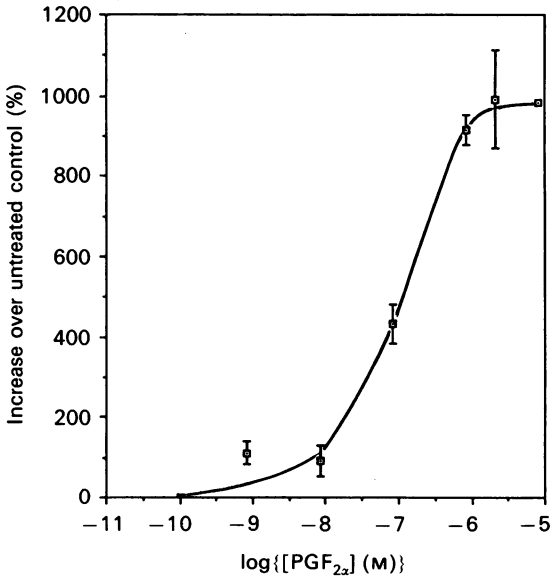

Fig. 5. Dose-dependence of $PGF_{2\alpha}$-stimulated inositol phosphates generation in Am-NIH-3T3 cells

[^{3}H]Inositol-labelled cells were stimulated for 30 min with various concentrations of $PGF_{2\alpha}$ in the presence of 10 mM-LiCl. The results are expressed as mean percentage increase in the radioactivity (d.p.m.) associated with the inositol phosphates over the untreated control cells, ± S.D. (n = 4). The data are from a single experiment typical of three.

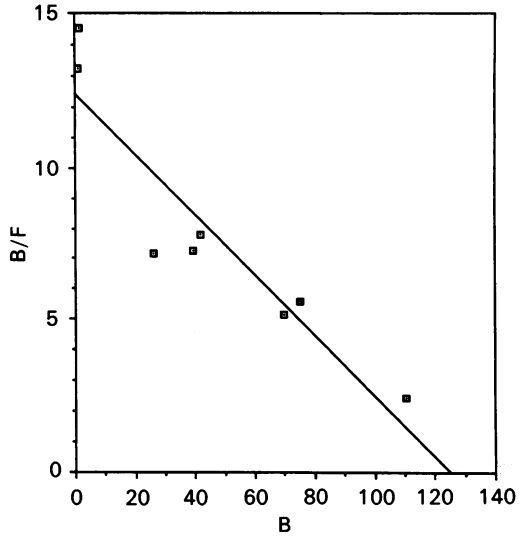

Fig. 6. Scatchard analysis of the binding of [^{3}H]$PGF_{2\alpha}$ to Am-NIH-3T3 cells

Binding was performed and assessed as described in the Materials and methods section. Bound $PGF_{2\alpha}$ (B) is expressed in fmol/10^6 cells; the $PGF_{2\alpha}$ concentration in the incubation medium (F) was in fmol. The data are from a single experiment typical of three.

Although stimulation of NIH-3T3 cells with $PGF_{2\alpha}$ induced PtdIns(4,5)P_2 breadown, it was without effect on the stimulation of DNA synthesis in this clone of NIH-3T3 cells (Tables 2 and 3; Fig. 3). This result was surprising, since it has been reported that the prostaglandin is a potent growth factor for both NIH-3T3 (Yu *et al.*, 1988) and Swiss 3T3 cells (MacPhee *et al.*, 1984). We have confirmed that $PGF_{2\alpha}$ is indeed a growth factor for Swiss 3T3 cells (Table 4) and for the clone of NIH-3T3 cells used by Yu *et al.* (1988) (Table 3; Fig. 4). It was therefore considered possible that some differences may exist in inositol phospholipid metabolism between these cells. No differences were observed in the stimulation of inositol phosphates generation by $PGF_{2\alpha}$ between the two clones of NIH-3T3 cells (Table 5; Fig. 2), nor were any differences detected in K_a, K_d or $PGF_{2\alpha}$-receptor number (Figs. 5 and 6). It is clear, however, in Table 4 that there are differences in responsiveness to other defined agonists, most noticably to bombesin; this may reflect a different receptor number, and requires investigation. Significantly, there is no difference in the response to calf serum (Table 5), which is equally effective in stimulating the proliferation of both cell types (Figs. 3 and 4). The stimulation of inositol phosphates accumulation by $PGF_{2\alpha}$ in Swiss 3T3 cells is less marked than in the two clones of NIH-3T3 cells, but it is clearly a growth factor in this cell type (Table 4). The apparent differences observed in the agonist-stimulated mitogenic and second-messenger responses (Table 5) observed between the two clones of NIH-3T3 cells may have a bearing upon the contradictory observations made on the effects of transfection of apparently the same cell type with the same *ras* gene on inositol phospholipid metabolism (Wakelam *et al.*, 1986; Seuwen *et al.*, 1988; Downward *et al.*, 1988).

Calf serum stimulates DNA synthesis in all three cell types (Tables 2, 3, 5; Figs. 3 and 4) and also induces inositol phosphates generation. This induction of DNA synthesis is dose-dependent (Table 2), but it is not potentiated in the NIH-3T3 cells by any concentration of $PGF_{2\alpha}$ (results not shown). This lack of potentiation is despite a synergistic effect on inositol phosphates generation (Table 1). The mechanism underlying the synergistic stimulation of inositol phosphates induced by $PGF_{2\alpha}$ and calf serum is unknown, and requires investigation.

The results in this paper therefore place a question on the role of inositol phospholipid metabolism in growth-factor-stimulated cell proliferation. Tones *et al.* (1988) have demonstrated no requirement for inositol phospholipid breakdown in serum-stimulated mitogenesis in CHO-K1 cells. Taylor *et al.* (1988) have found that the proliferation of Swiss 3T3 cells can be inhibited by the addition of pertussis toxin, with no inhibition of the stimulation of inositol phosphate generation. These studies and the experiments reported in the present paper suggest that, although mitogens can activate the breakdown of inositol phospholipids, it is either not obligatory for the onset of mitosis, or in itself not a complete signal. Consequently some additional signal must be being generated in the Am-NIH-3T3 and Swiss 3T3 cells, but not in the NIH-3T3 cells, in response to $PGF_{2\alpha}$, either in early G_1 phase or at a later stage in the cell cycle, which is required for the onset of DNA synthesis and mitosis. The identity of this signal is unclear; there is no difference in $PGF_{2\alpha}$-stimulated phosphatidylcholine breakdown between the two NIH-3T3 lines (F. M. Black & M. J. O. Wakelam, unpublished work), and therefore further signal(s) obligatory for growth-factor-stimulated cell proliferation remain to be identified.

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