Volume 297, number 1,2, 175–178 FEBS 10659 February 1992

Stimulation of protein kinase C (PKC) activity in resting Swiss 3T3 cells by prostaglandin $F_{2\alpha}$

Mercedes Goin and Luis Jimenez de Asua

Instituto de Investigaciones en Ingenieria Genética y Biologia Molecular (INGEBI), Obligado 2490, 1428 Buenos Aires, Argentina

Received 28 November 1991

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), a mitogen for resting Swiss 3T3 cells, rapidly stimulates phosphorylation of an 80 kDa protein (80 K). 1-Oleoyl-2-acetylglycerol (OAG) and 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) both protein kinase C (PKC) activators, also elicit 80 K phosphorylation. In contrast PGE_1, PGE_2 or $PGF_{2\beta}$, which are non-mitogenic in these cells, had little or no action on this event. However PGE_1 and PGE_2 potentiate the $PGF_{2\alpha}$ proliferative effect but do not enhance its action on 80 K phosphorylation. These results suggest that $PGF_{2\alpha}$ mitogenic induction involves PKC signalling pathway activation while its enhancement by PGE_1 or PGE_2 occurs through a different mechanism(s).

Swiss 3T3 cell; Prostaglandin $F_{2\alpha}$; Protein kinase C; 80 K phosphorylation; DNA synthesis

1. INTRODUCTION

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), a lipid molecule derived from arachidonic acid metabolism and released by a variety of tissues, has been shown to be a mitogen for different fibroblastic cell lines as well as for those of other origin [1–5].

In confluent resting Swiss mouse 3T3 cells $PGF_{2\alpha}$ rapidly increases the intracellular inositol 1,4,5-trisphosphate(1,4,5-IP_3) as well as diacylglycerol (DAG) content and induces the initiation of DNA replication and cell division [6,7]. In contrast, prostaglandin E_1 or E_2 (PGE_1, PGE_2) which at low concentrations (10^{-8} M) are non-mitogenic for these cells, fail to produce any significant changes in phospholipid metabolism, but increase the $PGF_{2\alpha}$ proliferative response [7–9]. Several findings reveal that stimulation with growth factors such as bombesin, vasopressin or platelet derived growth factor (PDGF), which in Swiss 3T3 cells raise cellular DAG content [10–12], leads to rapid protein kinase C (PKC) activation resulting in the phosphorylation of a cytosolic 80 kDa protein [13–15].

Here we show that in these cells $PGF_{2\alpha}$ selectively induces 80 K phosphorylation through the activation of the PKC signalling pathway. Both PGE_1 and $PGF_{2\beta}$ have no effect, whilst PGE_2, which stimulates phosphatidylinositol turnover and increases DAG content less than $PGF_{2\alpha}$ [7], has a marginal action in 80 K phosphorylation. In addition, PGE_1 or PGE_2 potentiate the $PGF_{2\alpha}$ mitogenic effect but without affecting the $PGF_{2\alpha}$-stimulated protein phosphorylation. These results also indicate that the enhancement of the $PGF_{2\alpha}$ proliferative response by PGE_1 or PGE_2 is exerted via a separate mechanism not involving PKC activation.

Abbreviations: $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; $PGF_{2\beta}$, prostaglandin $F_{2\beta}$; PGE_1, prostaglandin E_1; PGE_2, prostaglandin E_2; PKC, protein kinase C; OAG, 1-oleoyl-2-acetylglycerol; DAG, diacylglycerol; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PB_{12}, phorbol-12,13-dibutyrate.

Correspondence address: L. Jimenez de Asua, INGEBI, Obligado 2490, 1428 Buenos Aires, Argentina.

2. MATERIALS AND METHODS

2.1. *Cell culture, and mitogenic assay conditions*

Swiss mouse 3T3 cells [16] were maintained as previously described [17]. For experimental purposes, cells were plated into 35 mm Petri dishes in Dulbecco's modified Eagle's medium supplemented with low molecular weight nutrients and 6% fetal calf serum [17]. Three days later, the cultures received fresh medium containing only 100 μM phosphate and dialysed fetal calf serum. They then became confluent and quiescent in 3–4 days [17]. OAG and prostaglandins were dissolved in ethanol so that the final concentration added to the culture medium was less than 0.5%. Unstimulated cultures received similar amounts of solvent. Initiation of DNA synthesis upon mitogenic stimulation was measured by autoradiography as described before [17].

2.2. *Protein phosphorylation*

For 80 K phosphorylation, confluent quiescent cells received 200 μCi of carrier-free $^{32}P_i$ four hours prior to stimulation to allow steady-state labeling of the endogenous ATP pool [7]. The reaction was stopped by removal of medium and washing the cultures with Tris/saline solution (20 mM Tris-HCl/0.15 M NaCl). Heat-stable protein extracts were prepared according to a described procedure [18]. Thereafter, similar amounts of phosphoproteins were resolved in one-dimensional SDS-PAGE [19]. The resulting autoradiographs were scanned with a LKB laser densitometer.

2.3. *Materials*

PGE_1, PGE_2, $PGE_{2\alpha}$ and $PGF_{2\beta}$ were the generous gift of Dr. John Meringh, Upjohn Company, Kalamazoo. All remaining chemicals

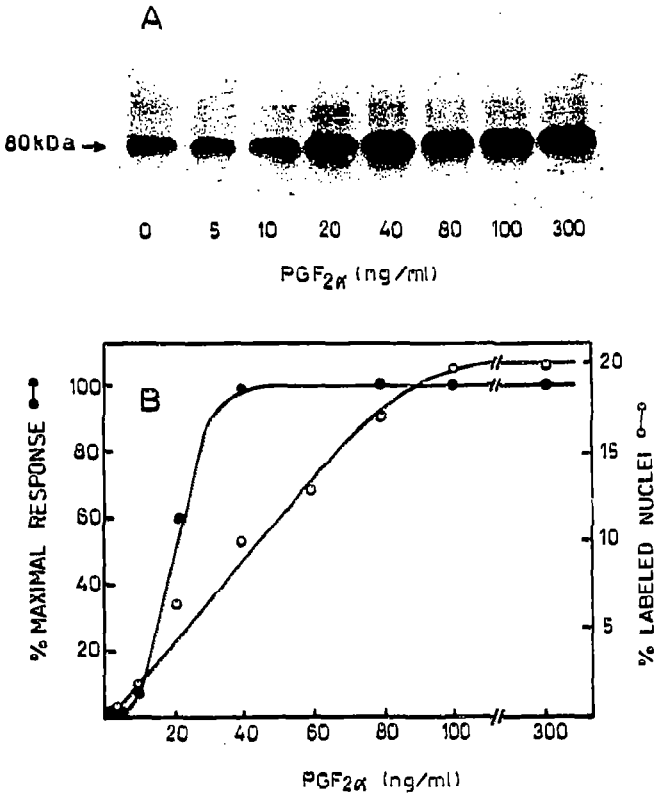

Fig. 1. (A) $PGF_{2\alpha}$ dose-dependent 80 K phosphorylation. (B) Scanning densitometric analysis of the 80 K phosphorylated band and $PGF_{2\alpha}$ dose-dependent initiation of DNA synthesis. Phosphorylation of 80 K to 100% is the maximal value obtained with $PGF_{2\alpha}$ (300 ng/ml). Cultures were labeled and stimulated for 80 K phosphorylation for 10 min and for the initiation of DNA synthesis as indicated in section 2.

were obtained from Sigma. Methyl[^{3}H]thymidine (18 Ci/mmol) and $^{32}P_i$ (8,500 Ci/mmol) were purchased from New England Nuclear.

3. RESULTS

3.1. *80 K phosphorylation is stimulated by $PGF_{2\alpha}$*

The $PGF_{2\alpha}$ dose-dependency for 80 kDa protein phosphorylation of confluent resting Swiss 3T3 cells after 10 min stimulation is shown in Fig. 1A. $PGF_{2\alpha}$ added from 5.0 to 300 ng/ml progressively increases the incorporation of $^{32}P_i$ into the 80 K reaching a plateau at 40 ng/ml. Densitometric analysis of the phosphorylated 80 K reveals an identical pattern taking the maximal value obtained with $PGF_{2\alpha}$ at 300 ng/ml as 100% (Fig. 1B). The $PGF_{2\alpha}$ dose-response for the initiation of DNA synthesis closely correlates with that observed for 80 K phosphorylation (Fig. 1C).

3.2. *Time-dependent 80 K phosphorylation in response to $PGF_{2\alpha}$*

The time course of 80 K phosphorylation in Swiss 3T3 cells upon $PGF_{2\alpha}$ stimulation is shown in Fig. 2A. $PGF_{2\alpha}$ (300 ng/ml) added to these cells from 0 to 10 min rendered an increase in the phosphorylation of the 80 kDa protein. A detectable $^{32}P_i$ incorporation into the 80 K was observed at 5 s, reaching to a plateau after 1 min of cell exposure to $PGF_{2\alpha}$. Plotting densitometric analysis values as a percentage of maximal phosphorylation obtained after 10 min gave identical results (Fig. 2B).

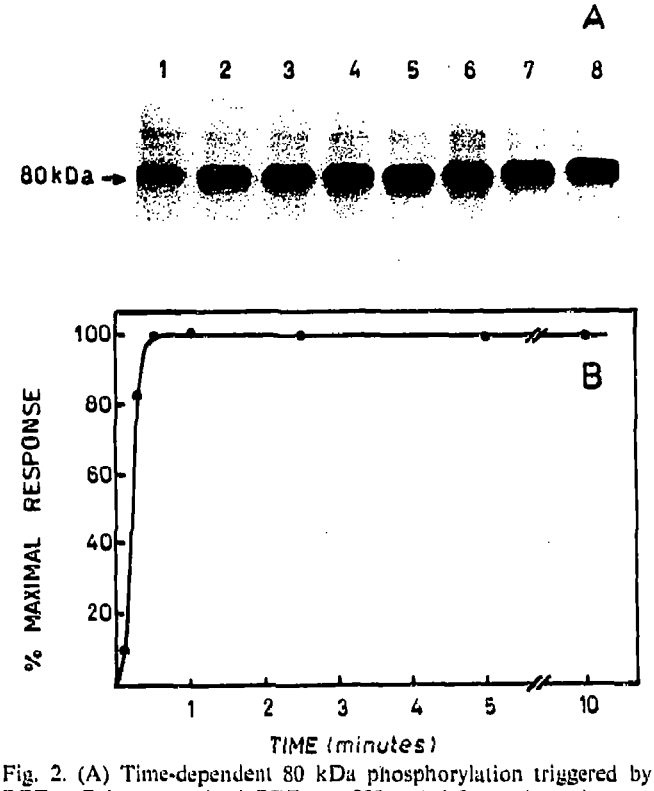

Fig. 2. (A) Time-dependent 80 kDa phosphorylation triggered by $PGF_{2\alpha}$. Cultures received $PGF_{2\alpha}$ at 300 ng/ml for various times as indicated: (1) Control; (2) 5 s; (3) 15 s; (4) 30 s; (5) 1 min; (6) 2.5 min; (7) 5 min, and (8) 10 min. (B) Densitometric analysis of the phosphorylated 80 K was expressed as 100% of the maximal value observed after 10 min of stimulation. Procedure and stimulation as in Fig. 1.

3.3 *$PGF_{2\alpha}$-stimulated 80 K phosphorylation reflects PKC activation*

Addition to these cells of 1-oleoyl-2-acetylglycerol (OAG) (100 μg/ml) or TPA (200 ng/ml), both PKC activators, produce similar results on 80 K phosphorylation

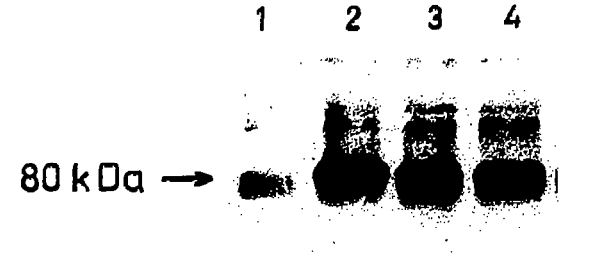

Fig. 3. Effect of TPA, OAG and $PGF_{2\alpha}$ on 80 K phosphorylation in Swiss 3T3 cells as indicated: (1) Control; (2) TPA (200 ng/ml); (3) OAG (100 μg/ml) and (4) $PGF_{2\alpha}$ at 300 ng/ml. Cells were labeled and stimulated as in Fig. 1.

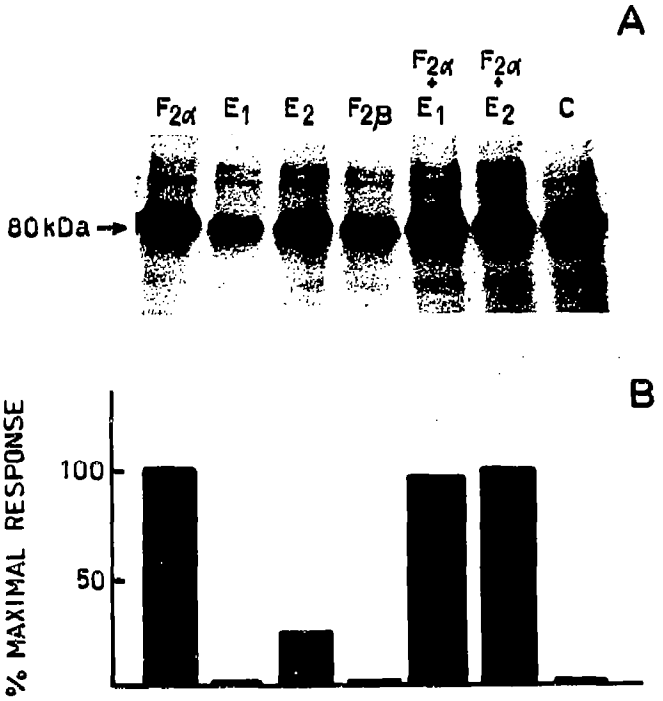

Fig. 4. (A) Differential stimulatory effect of PGE_1, PGE_2, $PGF_{2\beta}$ and $PGF_{2\alpha}$ added at 100 ng/ml on 80 K phosphorylation. (B) Densitometric quantification of response. The maximal value (100%) was obtained with $PGF_{2\alpha}$. Conditions for labeling and stimulation as in Fig. 1.

as with $PGF_{2\alpha}$ indicating that this response to $PGF_{2\alpha}$ involves PKC activation (Fig. 3).

3.4. *Specific 80 K phosphorylation by $PGF_{2\alpha}$ correlates with its mitogenic effect*

The $PGF_{2\alpha}$-specific action compared with those prostaglandins with a related chemical structure such as PGE_1, PGE_2, or $PGF_{2\beta}$ on 80 K phosphorylation is shown in Fig. 4A. PGE_1, PGE_2, $PGF_{2\alpha}$, or $PGF_{2\beta}$ added at 100 ng/ml to resting cells produced different effects. Only $PGF_{2\alpha}$ renders mitogenic induction and a marked increase in 80 K phosphorylation. In contrast, PGE_1 and $PGF_{2\beta}$ did not have any effect while PGE_2 produces less stimulation than $PGF_{2\alpha}$ (Fig. 4B). PGE_1 or PGE_2, which enhance the $PGF_{2\alpha}$ mitogenic effect (Table I), do not further increase the $PGF_{2\alpha}$-induced 80 K phosphorylation as judged by the radioautography densitometric analysis (Fig. 4B). Other results showed that EGF or insulin, which do not increase DAG intracellular content [7], also have no effect on 80 K phosphorylation [18] (data not shown).

4. DISCUSSION

Numerous reports have shown that mitogenic stimulation of Swiss mouse 3T3 cells by bombesin, vasopressin of PDGF elicits an early array of transducing signalling events [20]. They involve increases in

Table I

Effect of Prostaglandins E_1, E_2 and $F_{2\alpha}$ on the initiation of DNA synthesis and cell division

Additions	Labeled nuclei (%)	Cell number ($\times 10^4$)
None	0.5	8.0
$PGF_{2\alpha}$	20.0	9.3
PGE_1	0.8	7.8
PGE_2	0.6	8.1
$PGF_{2\alpha}$ + PGE_1	49.0	12.0
$PGF_{2\alpha}$ + PGE_2	51.0	11.7
Fetal calf serum	95.0	16.0

Additions were as follows: $PGF_{2\alpha}$ 100 ng/ml; PGE_1 100 ng/ml, and PGE_2 100 ng/ml. Prostaglandins were dissolved as in Fig. 4. For the assay of the initiation of DNA synthesis, cultures were labeled as indicated in section 2. Cell proliferation was measured in 60 mm Petri dishes after 60 h of stimulation [8].

phospholipase C activity and intracellular DAG content which leads to rapid PKC activation and phosphorylation of a cytosolic 80 kDa protein [13,15,21]. In addition, other results showed that in these cells either phorbol esters such as TPA and PB_{12}, or OAG, a synthetic permeable DAG analogue which causes PKC activation, elicit 80 K phosphorylation [13,14]. Furthermore, treatment of resting Swiss 3T3 cells with PB_{12}, to down-modulate the PKC activity, abolishes the bombesin- or vasopressin-stimulated 80 K phosphorylation. These facts indicate that this event involves PKC activation [13,14].

Previous findings have shown that $PGF_{2\alpha}$ rapidly stimulates phosphatidylinositol turnover, and increases the intracellular DAG content [7]. This suggests that the $PGF_{2\alpha}$ mitogenic effect might be mediated through phospholipid metabolism and possibly by the activation of the PKC signalling pathway. This is consistent with the fact that OAG causes mitogenesis only when added in combination with either insulin, PGE_1 or PGE_2 which alone are not PKC activators [20,22]. In addition it has been shown that PGE_1 or PGE_2, which are non-mitogenic for these cells, can enhance the $PGF_{2\alpha}$ proliferative response without further increases in $PGF_{2\alpha}$-raised DAG cellular content [7].

Here we show that $PGF_{2\alpha}$ rapidly stimulates 80 K phosphorylation, suggesting that this mitogen might induce Swiss mouse 3T3 cell proliferation through PKC activation. In addition, we provide evidence indicating that the synergistic effect of PGE_1 or PGE_2 with $PGF_{2\alpha}$ does not occur at the level of the 80 K phosphorylation. This strongly suggests that the PGE_1 or PGE_2 potentiation of the $PGF_{2\alpha}$ mitogenic effect occurs via an alternative mechanism(s), different from PKC activation, possibly involving another crucial PKC-dependent $PGF_{2\alpha}$ mitogenic event. The latter interpretation agrees with our previous results showing that the enhancement by PGE_1 or PGE_2 of the $PGF_{2\alpha}$ action occurs at the level

of protein synthesis-specific events such as the late-phase 2-deoxyglucose transport induction [8], which is related to increases in PKC activity [23]. In fact, other findings have also shown that in these cells $PGF_{2\alpha}$ stimulates ribosomal S6 protein phosphorylation, polysome formation and protein synthesis via PKC activation [24]. Our future research is directed to establish other PKC-dependent cell cycle events controlling the initiation of DNA replication.

Acknowledgements: We thank Gabriela Calamante for technical assistance and Mr. Joseph Welch, Becton Dickinson Labware, USA, for Falcon tissue culture material donation used in this study. This work was supported by grants from the Association for International Cancer Research (United Kingdom) and CONICET (Argentina) awarded to LJA. MG is a fellow and LJA principal investigator of CONICET.

REFERENCES

[1] Jimenez de Asua, L., Otto, A.M., Lindgrem, J.A. and Hammarstrom, S. (1983) J. Biol. Chem. 258, 8774–8780.
[2] Smith, C., Otto, A.M. and Jimenez de Asua, L. (1984) in: Growth and Maturation Factors Vol. 2 (Guroff, G. ed.) pp. 132–140, John Wiley and Sons, New York.
[3] Orlicky, D.J., Lieberman, R. and Gerschenson, L.E. (1986) J. Cell. Physiol. 127, 55–60.
[4] Hakeda, Y., Hotta, T., Kurihara, N., Ikeda, E., Maeda, N., Yaggu, Y. and Kunegawa, M. (1987) Endocrinology 121, 1966–1974.
[5] Goin, M., Calamante, G., Estevez, A.G. and Jimenez de Asua, L. (1991) in: Leukotrienes, Prostaglandins and Cancer (Hammarstron, S. ed.) (Honn, K.V. and Marnett, L.J., series eds.) Kluwer Academic Publishers, Norwell, Massachusetts (in press).
[6] Taylor, C.W., Blakeley, D.M., Corps, A.N., Berridge, M.J. and Brown, K.D. (1988) Biochem. J. 249, 917–920.
[7] Macphee, C.H., Drummond, A.H., Otto, A.M. and Jimenez de Asua, L. (1984) J. Cell. Physiol. 119, 35–49.
[8] Otto, A.M., Nilsen-Hamilton, M., Boss, B.D., Ulrich, M.D. and Jimenez de Asua, L. (1982) Proc. Natl. Acad. Sci. USA 79, 4992–4996.
[9] Jimenez de Asua, L., Macphee, C.H., Drummond, A.H. and Otto, A.M. (1984) in: Icosanoids and Cancer (Crastes de Paulet, A., Thaler-Dao, H. and Paoletti, R., eds.) pp. 139–153, Raven Press, New York.
[10] Takuwa, N., Takuwa, Y., Bollag, W.E. and Rasmussen, H. (1987) J. Biol. Chem. 262, 182–188.
[11] Cook, S.G. and Wakelam, M.G.O. (1991) Biochem. Biophys. Acta 1092, 265–272.
[12] Habenicht, A.J.R., Glomzel, J.A., King, W.C., Nist, C., Mitchel, C.D. and Ross, R. (1981) J. Biol. Chem. 256, 12329–12335.
[13] Zachary, I., Sinnet-Smith, J.W. and Rozengurt, E. (1986) J. Cell. Biol. 102, 2211–2222.
[14] Rodriguez-Pena, A. and Rozengurt, E. (1986) J. Cell. Physiol. 129, 127–130.
[15] Rozengurt, E., Rodriguez-Pena, M. and Smith, K.A. (1983) Proc. Natl. Acad. Sci. USA 80, 7244–7248.
[16] Todaro, G.J. and Green, H. (1963) J. Cell. Biol. 17, 299–313.
[17] Jimenez de Asua, L., O'Farrell, M.K., Clingan, D. and Rudland, P.S. (1977) Proc. Natl. Acad. Sci. USA 74, 3845–3849.
[18] Morris, C., Rice, P. and Rozengurt, E. (1988) Biochem. Biophys. Res. Commun. 155, 561–568.
[19] Laemmli, U.K. (1970) Nature 227, 680–685.
[20] Rozengurt, E., Erusalimsky, I., Mehnet, H., Morris, C., Nanberg, E. and Sinnett-Smith, J.W. (1987) Cold Spring Harbor Symposia on Quantitative Biology 53, 945–954.
[21] Isacke, C.M., Meisenhelder, J., Brown, K.D., Gould, K.L., Gould, S.J. and Hunter, T. (1986) EMBO J. 5, 2889–2898.
[22] Jimenez de Asua, L., Estevez, A.G. and Goin, M. (1991) in: Eicosanoids and other Bioactive Lipids in Cancer, Inflammation and Radiation Injury (Nigam, S., Honn, K.V., Marnett, L.J. and Walden, T. eds.) Kluwer Academic Publishers, Norwell, Massachusetts (in press).
[23] Takuwa, N., Takuwa, Y. and Rasmussen, H. (1988) J. Biol. Chem. 263, 9738–9745.
[24] Pelech, S.L. and Krebs, E. (1987) J. Biol. Chem. 262, 11598–11606.