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Early cell cycle diacylglycerol (DAG) content and protein kinase C (PKC) activity enhancement potentiates prostaglandin F2α (PGF2α) induced mitogenesis in Swiss 3T3 cells

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R59022, a diacylglycerol kinase inhibitor, enhances the prostaglandin F2α (PGF2α)-induced diacylglycerol (DAG) synthesis in Swiss 3T3 cells. It also potentiates the PGF2α-mediated protein kinase C (PKC)-dependent 80 kDa protein (80K) phosphorylation and initiation of DNA replication. R59022 enhances the PGF2α mitogenic response by increasing the rate of entry into the S phase. Insulin does not cause 80K phosphorylation, and does not enhance its induction but it potentiates the PGF2α mitogenic response. These results suggest that mitogenically triggered fluctuations in DAG content and PKC activity play a pivotal role in controlling the PGF2α-induced DNA synthesis while insulin acts via a different mechanism.

Prostaglandin F2α; R 59022; Diacylglycerol kinase; Protein kinase C; DNA synthesis

1. INTRODUCTION

Mammalian cell proliferation is a highly coordinated process [1–3]. Mitogens cause its induction by triggering a signalling network leading to the onset of DNA synthesis and division [1–3]. In Swiss 3T3 cells, prostaglandin F2α (PGF2α) rapidly increases the inositol 1,4,5 triphosphate (IP3) and diacylglycerol (DAG) content [4–7]. The latter causes protein kinase C (PKC) activation, a PGF2α requirement, to stimulate various events. They include, 80K phosphorylation, decreases in ^{125}I-EGF binding affinity properties and the mitogenic response [8–10].

Much evidence reveals that the diacylglycerol kinase (DGK) enzyme, which converts DAG into phosphatidic acid, tightly regulates the cellular DAG content, and thereby several PKC-dependent responses [11–14]. Whether changes in DGK activity and DAG levels can modulate PKC-dependent cell cycle events is of crucial importance to understand their role in mitogenesis.

Here we show that R59022 [15], a DGK inhibitor, potentiates the PGF2α induced-DAG increase as well as the 80K phosphorylation and proliferative response. R-59022 enhances the PGF2α induced entry into the S phase without altering the G1 phase length. Insulin, which does not cause PKC activation, potentiates PGF2α-induced mitogenesis. These results indicate that DAG content and PKC activity fluctuations triggered by PGF2α play a crucial role controlling the rate of initiation of DNA replication. We also conclude that insulin enhances this effect via a separate signal(s).

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Abbreviations: PGF2α, prostaglandin F2α; PKC, protein kinase C; DAG, diacylglycerol; DGK, diacylglycerol kinase; R59022, 6-[2-(4-[(4-fluoro phenyl) phenylmethylene]-1-piperdinyl]-]-methyl)5H-thiazolo[3,2-α]-pyrimidin-5-one.

2. MATERIALS AND METHODS

2.1. *Cell culture, DNA synthesis and 80K phosphorylation assay*

Swiss mouse 3T3 cells [16] culture, assay of DNA synthesis by autoradiography and calculation of the rate constant *K* value for entry into the S phase were as previously reported [1,10]. 80K phosphorylation culture conditions, labeling, determination procedures and densitometric analysis were as described [8,17].

2.2. *1,2 Diacylglycerol content*

Cells were plated on 100 mm Petri dishes similar to the DNA synthesis assay [4]. After four days, cells received 10 ml of fresh medium with 25 μCi [^{3}H]glycerol and 4 days later they become confluent and resting [4]. Upon isolation, 103 cpm [^{14}C]cholesteryl oleate was added to lysates for recovery estimation [18]. Chromatography of lipids and detection were as described before [4,18].

2.3. *Materials*

R59022 was a gift from Dr. D. Chaffay de Courcelles, Jansen Life Science. Chemicals were obtained from Sigma Chemical Company. [*methyl*-^{3}H]Thymidine (18 Ci/mmol), $^{32}P_i$ (8,500 Ci/mmol) and [^{14}C]cholesteryl oleate (59 Ci/mmol) were purchased from New England Nuclear. [^{3}H]Glycerol (15 Ci/mmol) was from American Radiolabeled Chemicals.

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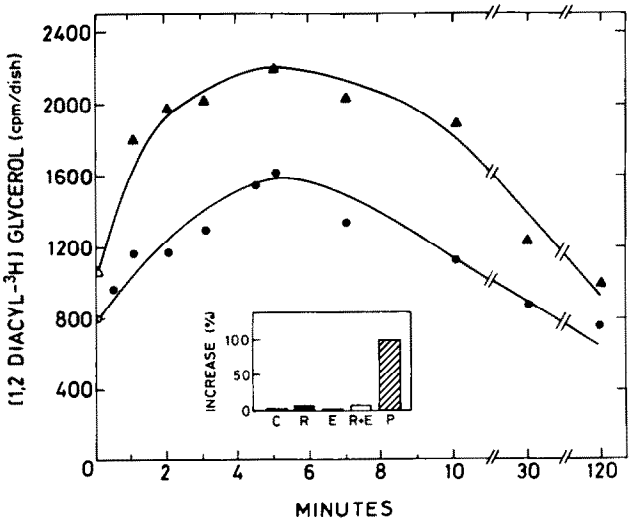

Fig. 1. Time-dependent DAG increase upon PGF2α or PGF2α–R59022 addition. R59022 (8 μM) was added 1 h prior to PGF2α. (○), control; (△), R59022 (8 μM); (●), PGF2α (300 ng/ml) and (▲), PGF2α plus R59022. 1,3-Diacylglycerol and triacylglycerol content did not vary under these conditions. The inset shows the effect of R59022 upon EGF (20 ng/ml) induction after 5 min. Additions were as follows: C, control; R, R59022; E, EGF; R+E; P, PGF2α. The assay was carried out as described in section 2.

3. RESULTS

3.1. *R59022 enhances PGF2α-induced DAG content*

The R59022 potentiation of the PGF2α-dependent DAG increase at various times upon mitogenic induction of confluent resting Swiss 3T3 cells is shown in Fig. 1. PGF2α added at 300 ng/ml caused a rapid maximal raise (two-fold) in DAG content within 5 min of stimulation. Thereafter DAG levels decline to or even below the unstimulated level (Fig. 1). R59022 by itself has little effect on DAG levels. However it enhances the rate at which PGF2α induces DAG content increase, reaching a value that is 1.4-fold higher than with PGF2α alone (Fig. 1). Neither EGF alone [4] nor EGF plus R59022 caused DAG increases (Fig. 1 inset).

3.2. *R59022 potentiates 80K phosphorylation*

PGF2α (30 or 300 ng/ml) stimulation for 10 min triggers a PKC-dependent 80K phosphorylation to a different extent (Fig. 2A). R59022 (8 μM) added by itself has no effect, but it increases the PGF2α-induced 80K phosphorylation almost in a similar fashion for both PGF2α concentrations (Fig. 2B). TPA, 12-*O*-tetradecanoylphorbol-13-acetate, a PKC activator, renders maximal effect (Fig. 2A,B). In contrast, insulin neither causes

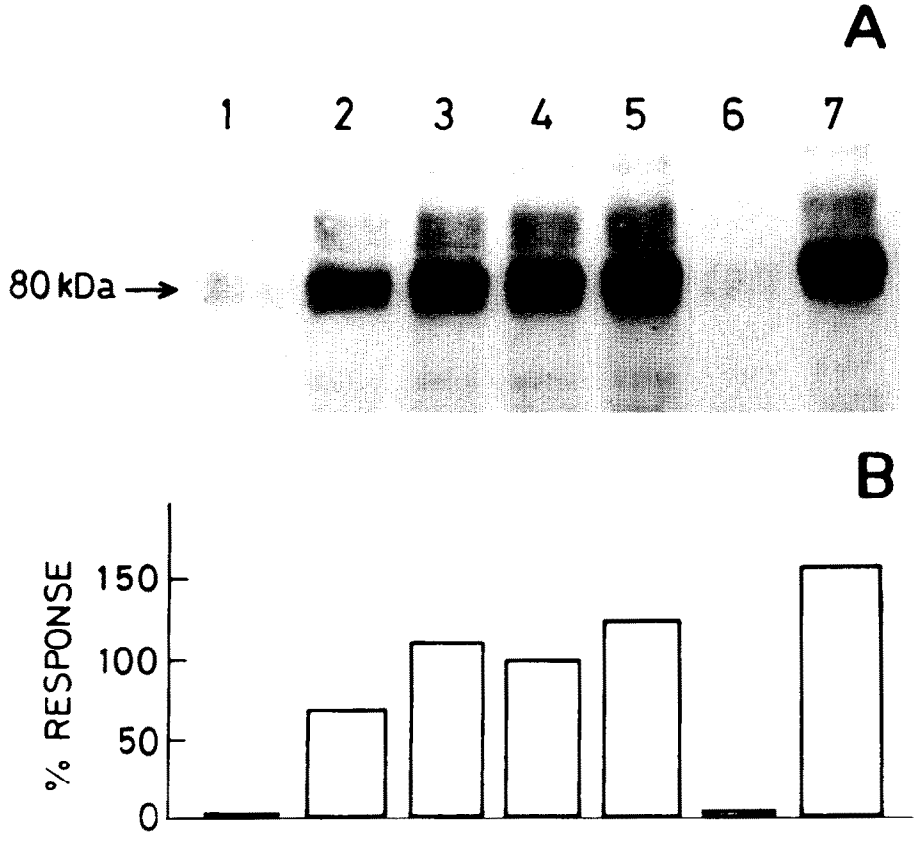

Fig. 2. (A) R59022 potentiation of 80K phosphorylation induced by two PGF2α concentrations. Additions were for 10 min as indicated: (1) R59022; (2) PGF2α 30 ng/ml; (3) PGF2α 30 ng/ml plus R59022; (4) PGF2α 300 ng/ml; (5) PGF2α 300 ng/ml plus R59022; (6) control; and (7) TPA. (B) Densitometric analysis. PGF2α 300 ng/ml represents the 100% value. R59022 was added as in Fig. 1.

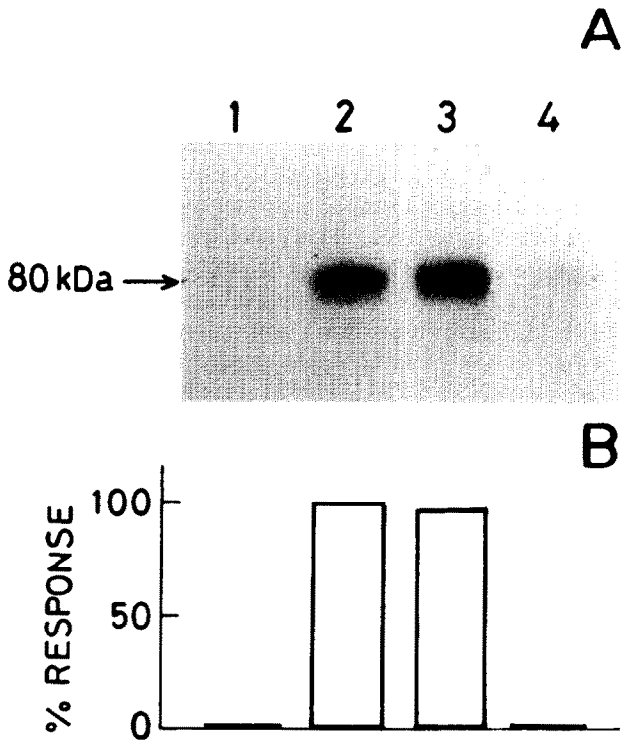

Fig. 3. (A) PGF2α (300 ng/ml) and insulin (50 ng/ml) differential action on 80K phosphorylation. Additions were as indicated: (1) control; (2) PGF2α; (3) PGF2α plus insulin; and (4) insulin. (B) Densitometric quantification. Assay conditions were as in Fig. 2.

80K phosphorylation nor potentiates this PGF2α-induced event (Fig. 3).

3.3. *R59022 enhances PGF2α mitogenic response*

The R59022 potentiation of PGF2α-induced mitogenesis is shown in Fig. 4A. R59022 (1–20 μM) by itself has no effect but with PGF2α at 30 or 300 ng/ml causes a dose-dependent labeling index increase reaching a maximum at 10 μM (Fig. 4A). R59022 also exerts synergistic action upon PGF2α induction by lowering the PGF2α dose requirement (Fig. 4B). Insulin (10^{-9} M), which in these cells is not mitogenic [1,10], further increases the PGF2α- or PGF2α–R59022-induced DNA synthesis (Fig. 4C. The R59022 action is exerted by enhancing the K value for entry into the S phase without altering the G1 length regardless of the PGF2α concentration (Fig. 5). In addition, R59022 does not change the PGF2α–insulin induced DNA synthesis initiation kinetics (Fig. 5).

4. DISCUSSION

Cumulative findings indicate that in mammalian cells the DGK enzyme is a key regulator of the DAG content [19,20]. Hence, DGK exerts control upon the PKC-activation threshold which is reflected on PKC-dependent events [21,22]. R59022-DGK inhibition-induced platelets or neutrophils cause potentiation of PKC-con-

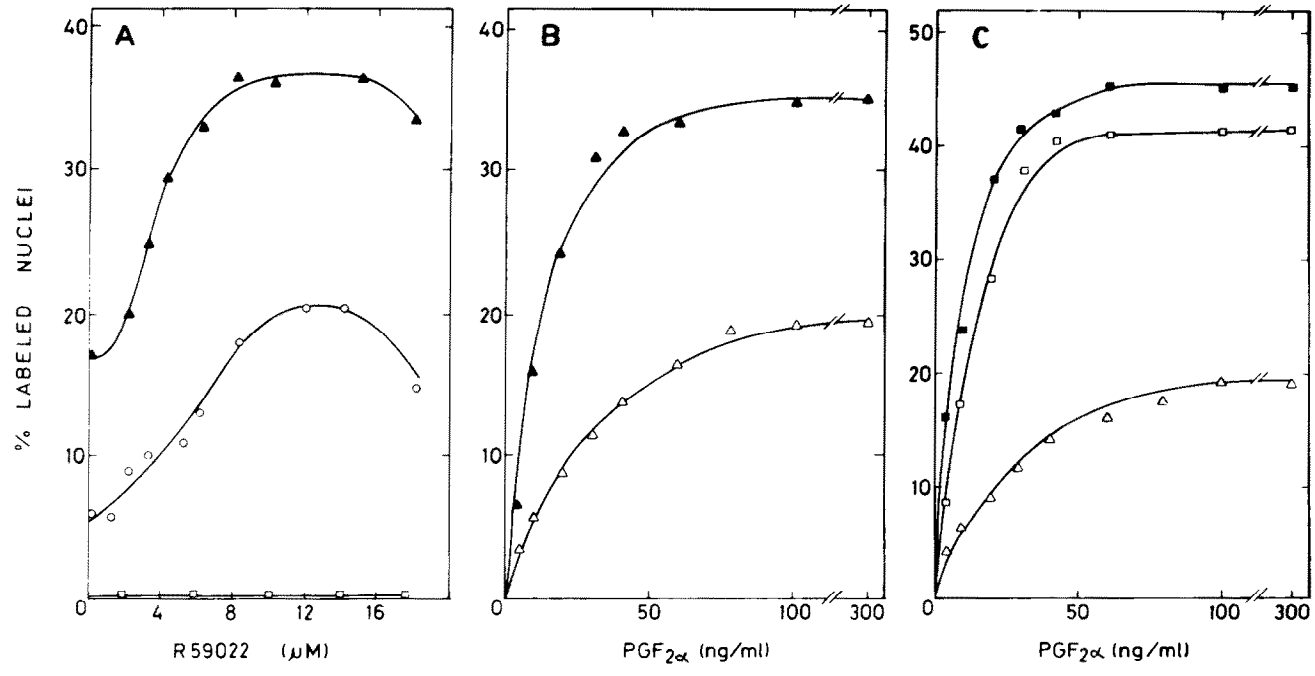

Fig. 4. (A) R59022 potentiation of PGF2α mitogenic response. (□), R59022; (○), R59022 plus PGF2α 30 ng/ml; (▲), R59022 plus PGF2α 300 ng/ml. (B) PGF2α dose-response minus (△) or (▲) plus R59022 at 8 μM. (C) Effect of insulin (50 ng/ml) on the PGF2α mitogenic response minus or plus 8 μM R59022. (△), PGF2α; (□), PGF2α plus insulin; and (■), PGF2α plus insulin and R59022. DNA synthesis was assayed as indicated in section 2.

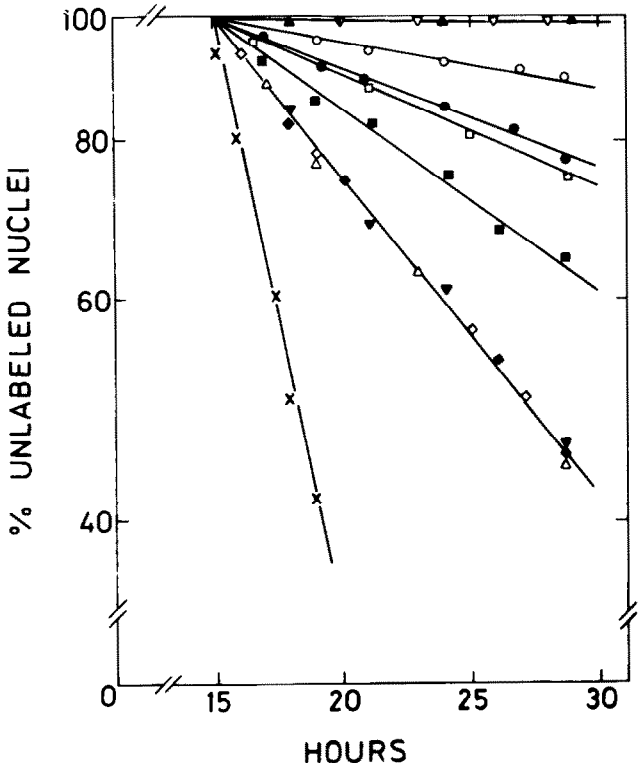

Fig. 5. Kinetics of entry into the S phase triggered by PGF2α minus or plus R59022 (8 μM) without or with insulin (50 ng/ml). Additions were as follows: (▽), Control (K = 0.05); (▲), insulin plus R59022 (K = 0.07); (○), PGF2α (30 ng/ml) (K = 0.80); (●), PGF2α (30 ng/ml) plus R59022 (K = 1.80); (▼), PGF2α (30 ng/ml) plus insulin (K = 5.67); (◆), PGF2α (30 ng/ml) plus R59022 and insulin (K = 5.69); (□), PGF2α (300 ng/ml) (K = 2.11); (■), PGF2α (300 ng/ml) plus R59022 (K = 3.21); (◇), PGF2α (300 ng/ml) plus insulin (K = 5.71); (△), PGF2α (300 ng/ml) plus R59022 and insulin (K = 5.70); (×), serum (K = 23.0). K values are given in 10^{-2} h. In all cases the duration of G1 was 14 h. Labeling procedures were as in Fig. 4.

trolled processes [14–18]. Also, in bombesin-stimulated Swiss 3T3 cells, R59022 enhances some early PKC-mediated events and mitogenesis [17]. Yet, it is not clear whether R59022 causes further bombesin-induced DAG-content increases [23] and its occurrence in the cell cycle.

Here we show that in Swiss 3T3 cells, R59022-DGK inhibition reduces the PGF2α-DAG generation time and transiently increases its content. R59022 also enhances the PGF2α-triggered PKC-dependent 80K phosphorylation and mitogenesis. PKC down-modulation prevents PGF2α or PGF2α–R59022 from causing the latter events, indicating their PKC requirement (not shown). Our results also suggest that PGF2α triggers both DAG-content increases and possibly DGK activity. Plateled-derived growth factor, in these cells, also stimulates both processes [24]. The cause of the inability of R59022 to maintain higher PGF2α-induced DAG levels is unknown, but likely to be a consequence of alternative DAG metabolic conversion that occurs in mitogenically induced Swiss 3T3 cells [25].

Of relevance is the fact that R59022 enhances the PGF2α-induced DNA synthesis by increasing the rate of entry into the S phase without altering the length of G1. This implies that either PGF2α- or PGF2α–R59022-triggered DAG content and PKC activity increases are crucial transient events to determine G1 progression and exert control upon processes regulating the onset of DNA replication [10]. In contrast, insulin, which in these cells neither raises DAG content [4] nor causes 80K phosphorylation or enhances the effect of PGF2α on both events, only increased the PGF2α-induced rate of entry into the S phase.

These findings also indicate that cell cycle control is exerted by two independent signalling mechanisms which, acting in concert, modulate the initiation of DNA replication. PGF2α triggers a DAG-PKC-dependent cascade of events which is causative of mitogenesis. Nevertheless we have shown that PKC activation cannot solely account for the PGF2α-triggered proliferative response but it requires complementary signal(s) [10]. In contrast, insulin synergistically enhances it by a separate amplifying mechanism. The latter is not exerted in early [8,9], but in late PKC-downstream-dependent events involving protein synthesis [10,26–28]. To unravel crucial time-dependent cell cycle events that occur upon transient DAG-content change and PKC activation is our present research endeavour.

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