Biochemical and Biophysical Research Communications **270,** 11–16 (2000)
doi:10.1006/bbrc.2000.2383, available online at http://www.idealibrary.com on IDEAL®

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) Induces Cyclin D1 Expression and DNA Synthesis via Early Signaling Mechanisms in Swiss Mouse 3T3 Cells

Moira Sauane,*,† Laura Correa,* Florencia Rogers,* Martín Krasnapolski,* Roger Barraclough,† Philip S. Rudland,† and Luis Jiménez de Asúa*,†,[1]

**Instituto de Investigaciones Bioquímicas "Luis F. Leloir" Fundación Campomar, Avenida Patricias Argentinas 435, 1405, Buenos Aires, Argentina; and †Molecular Medicine Group, School of Biological Sciences, University of Liverpool, Liverpool L69 3BX, United Kingdom*

Received February 9, 2000

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), a mitogen for Swiss 3T3 cells, triggers cyclin D1 mRNA/protein expression prior to cellular entry into the S phase, but fails to raise cdk4 or cyclin D3 levels, while 1-oleoyl-2-diacylglycerol (OAG), a protein kinase C (PKC) and tyrosine kinase (TK) activator, induces only cyclin D1 expression with no mitogenic response. In contrast, in PKC-depleted or -inhibited cells, $PGF_{2\alpha}$, but not OAG, increases cyclin D1 expression with no mitogenic response. Finally, OAG, in the presence of orthovanadate (Na_3VO_4) or $TGF_{\beta1}$, induces DNA synthesis. Thus, it appears that $PGF_{2\alpha}$ triggers cyclin D1 expression via two independent signaling events that complement with $TGF_{\beta1}$-triggered events to induce DNA synthesis.

Key Words: **protein kinase C; tyrosine phosphorylation; cyclin D1 mRNA; DNA replication.**

Mammalian cell multiplication is a highly coordinated phenomenon (1–3). Most mitogens are known to act by eliciting a set of specific signals, which, in turn, concertedly modulate the initiation of DNA replication and cell division (4). Much evidence has revealed that different mitogen-induced signals control the levels of cyclins, key molecules that regulate crucial cell cycle transitions (5, 6). Consequently, the identification of these signals is important for understanding the regulatory events controlling the normal cell cycle commitment (6). Cyclins are a conserved family of proteins that play a pivotal role in cyclin-dependent kinase (CDK) activation, which in turn, are required to complete cell cycle transitions (6, 7). Cyclin Ds are the first cyclins to be induced when mammalian cells leave the G_0 resting state and progress into the G_1-phase (7). Cyclin D1 is known to play a regulatory role in cellular commitment to initiate DNA replication (7–9), by interacting with its partner protein kinases cdk4 and cdk6 (7, 10). Its expression is controlled by various mitogen- and non-mitogen-dependent signaling processes (9, 11–13). Thus, mitogen deprivation of normal cells in early G_1-phase causes a rapid decline in cyclin D1 mRNA and protein levels, rendering such cells unable to enter the S-phase (8, 14). In addition, microinjection of antibodies against cyclin D1 during the early, but not the late, G_1-phase, impairs initiation of DNA replication (14).

In Swiss mouse 3T3 cells, prostaglandin $F_{2\alpha}$ mitogenic action involves signaling events, including increases in diacylglycerol (DAG), inositol 1,4,5-trisphosphate (IP3), intracellular Ca^{2+} ion mobilization, and protein kinase C (PKC) activation (15, 16). These events are known to be required to induce cellular entry into the S phase. In contrast, when these, or NIH-3T3 cells, are treated with 13-tetradecanoyl-12-phorbolacetate (TPA) to cause PKC down modulation, $PGF_{2\alpha}$ can still stimulate intracellular Ca^{2+} mobilization as well as tyrosine phosphorylation of proteins such as the focal adhesion protein $p125^{FAK}$, but without eliciting mitogenic response (17, 18). Both PKC-dependent and independent $PGF_{2\alpha}$-triggered events appear to be concertedly required for cells to initiate DNA synthesis. Nevertheless, transforming growth factor $\beta1$ ($TGF_{\beta1}$), which has no mitogenic action on these cells, potentiates the $PGF_{2\alpha}$ proliferative re-

Abbreviations used: A23187, 4-bromo-calcium ionophore A23187; OV^- or Na_3VO_4, sodium orthovanadate; OAG, 1-oleoyl-2-acetyl glycerol; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PKC, protein kinase C; TPA, 13-tetradecanoyl-12-phorbolacetate; $TGF_{\beta1}$, transforming growth factor $\beta1$; GF, GF109203X, 3[1(dimethylaminopropyl) (indol-3yl)-4(indol-3-il) maleimide.

[1] To whom correspondence should be addressed at Instituto de Investigaciones Bioquímicas "Luis F. Leloir" Fundación Campomar, Avenida Patricias Argentinas 435, 1405, Buenos Aires, Argentina. Fax: +54-11-4865-2246. E-mail: ljasua.fc@iib.uba.ar.

0006-291X/00 $35.00

sponse via signals that neither involves PKC activation nor intracellular Ca^{2+} ion mobilization (19, 20). However, $TGF_{\beta 1}$-triggered events can complement with those processes involving PKC activity, and hence confer Swiss 3T3 cells with the ability to initiate DNA replication (20)

A basic question regarding $PGF_{2\alpha}$ signaling mechanisms is whether, and how, each personalized $PGF_{2\alpha}$ signal regulates cyclin D1 expression, and how such events can ultimately control cellular commitment to enter the S-phase. The present study shows that in Swiss 3T3 cells, $PGF_{2\alpha}$-triggered events involved in cyclin D1 expression include either TK and/or PKC activation. However, $PGF_{2\alpha}$ appears to induce DNA synthesis via the combined actions of the induction of cyclin D1 gene expression and other PKC-, TK-, or $TGF_{\beta 1}$-triggered events. These results should help to clarify the mechanism(s) by which mitogenic signals separately control both cyclin D1 expression and normal cell multiplication.

MATERIALS AND METHODS

Cell culture and DNA synthesis assay. The maintenance of cultured Swiss mouse 3T3 cells (21) and the DNA synthesis assay have been previously described (1). In experiments involving different times of $PGF_{2\alpha}$ exposure, culture medium was removed by aspiration, after which cultures were washed twice with serum-free medium and the cells exposed to conditioned medium obtained from parallel cultures. Then, were radioactively labeled for 28 h by continuous exposure to 3 μCi/ml, 1 μM [*methyl*-^{3}H] thymidine (1).

Northern blotting analysis for cyclins D1, D2, D3, and cdk4. Cells (3×10^5) were plated in 100 mm dishes, similar as for DNA synthesis assay (15–19). Under such conditions, cultures became confluent and quiescent at 3×10^6 cells/plate. Total RNA was prepared using the TRIzol reagent, and samples (15 μg) were subjected to 1% Mops/formaldehyde agarose gel electrophoresis, and blotted onto nylon membranes (22). cDNA probes were ^{32}P-labeled using a Promega kit, and used to hybridize to the membranes, following which the membranes were washed and exposed to X-ray film (22). For cyclins D1, D2, and D3, the 1.3-kbp *Eco*RI fragment of pcBZ04.1, the 1.2-kbp *Eco*RI fragment of pcN9, and the 1.7-kbp *Eco*RI fragment of pcn2, were used, respectively (8); cyclin Ds, ckd4, and the 18S RNA cDNA probes were kindly provided by Drs. C. D. Sherr (St. Jude's Hospital, Memphis, TN) and A. R. Kornblihtt (Physiology and Molecular Biology Laboratory, School of Sciences, University of Buenos Aires, Argentina).

Western blotting. Cells were plated as described for RNA isolation. Following the different treatments, cells were lysed in RIPA buffer (25 mM Tris–HCl [pH 8.0], 150 mM NaCl, 1% NP-40, 2 mM EDTA–Na_2, 0.1% sodium deoxycholate) (22), supplemented with 2.5 mg/ml leupeptin and 2.5 mg/ml aprotinin, and the extracted proteins quantified (17). A total of 200 ng extracted protein were separated on SDS–polyacrylamide gels, and transferred to nitrocellulose membranes. Membranes were incubated for 1 h at room temperature with a solution containing 140 mM NaCl, 8 mM Na_2HPO_4, 3 mM NaH_2PO_4, 1.5 mM KCl, 3% low-fat powdered milk, and 0.1% Tween 20 (22). For Western blotting, mouse monoclonal antibody (mAB) against cyclin D1, rabbit polyclonal antibody against cyclin D2, mouse mAB against cyclin D3, and rabbit polyclonal antibody against CDK4 (all from Santa Cruz Biotechnology), were used. Antigens were detected by incubating membranes for 16 h at 4°C with the specific antibodies (23). After washing, membranes were incubated with horseradish peroxidase-conjugated second antibody (DAKO), and visualized with the ECL enhanced chemiluminescence kit.

Chemicals. Prostaglandin $F_{2\alpha}$ and GF109203X were kindly provided by Dr. Mary Tokleston (Upjohn Co., Kalamazoo, MI), and by Simon Lister (Glaxo Wellcome, UK); A23187 and $TGF_{\beta 1}$ were obtained from Calbiochem (La Jolla, CA) and R & D (Minneapolis, MN), respectively, TRIzol and was from purchased Gibco BRL and Promega Kit was from Promega. Other high purity compounds were purchased from Sigma Chemical Co. [*methyl*-^{3}H]Thymidine (18 Ci/mmol) was from New England Nuclear and ECL Western blotting detection reagent was from Amersham Life Sciences.

RESULTS AND DISCUSSION

The $PGF_{2\alpha}$ induction of cyclin D1 mRNA in Swiss mouse 3T3 cells was measured at different times (Fig. 1). Densitometric analysis of Northern blots of cyclin D1 mRNA standardized to 18S RNA, revealed that $PGF_{2\alpha}$ (300 ng/ml) increased cyclin D1 mRNA levels after 6 h, attaining a maximum at 10–12 h, followed by a rapid decline at 14 h (Figs. 1A and 1C). Western blotting analysis indicated that $PGF_{2\alpha}$ raised cyclin D1 protein levels at 6–9 h, reaching a plateau after 12–15 h, and that protein levels continued to remain high up to 21 h. $PGF_{2\alpha}$ also substantially raised cyclin D2 levels, but only at later times, when cells had undergone 12 to 21 h of G_1-phase progression. In contrast, $PGF_{2\alpha}$ failed to increase significantly cyclin D3 or CDK4 protein levels (Fig. 1B). Both the time required for $PGF_{2\alpha}$ to increase intracellular levels of cyclin D1 mRNA/protein, and that for $PGF_{2\alpha}$ to commit cells to enter S-phase, exhibited similar patterns (Fig. 1C). Cells continuously exposed to $PGF_{2\alpha}$ for 4–6 h failed to promote entry into the S-phase (Fig. 1C). In contrast, longer $PGF_{2\alpha}$ stimulus (9 to 15 h) conferred an increased ability of cells to enter into S-phase (Fig. 1C). Finally, continuous exposure of cells to $PGF_{2\alpha}$ for 28 h resulted in 20% of them initiating DNA synthesis (15). Thus, it appears that $PGF_{2\alpha}$-dependent cyclin D1 mRNA/protein increases exhibit a timing similar to that observed for cells to acquire the ability to enter the S-phase (Fig. 1C).

Mitogenic activity in Swiss, as well as NIH, 3T3 cells, involves both PKC- and TK-dependent events. PKC-triggered events can be dissected by stimulating, inhibiting, or depleting PKC activity (16–18). Exposure of Swiss 3T3 cells to 100 μg/ml 1-oleoyl-2-acetylglycerol (OAG, a DAG analogue and PKC activator) or 300 ng/ml $PGF_{2\alpha}$ for 10 h, caused a 15-fold increase in cyclin D1 mRNA levels (Figs. 2A and 2C). In cells pretreated with 10 μM GF109203X (GF) (24), a PKC inhibitor, 2 h prior addition of $PGF_{2\alpha}$, a 12-fold increase in cyclin D1 mRNA levels was observed, while no effect was detected when OAG was used (Figs. 2A and 2C). The addition of GF at various concentrations also impaired $PGF_{2\alpha}$'s mitogenic response (Fig. 2E). Furthermore, in TPA-mediated, PKC downmodulated

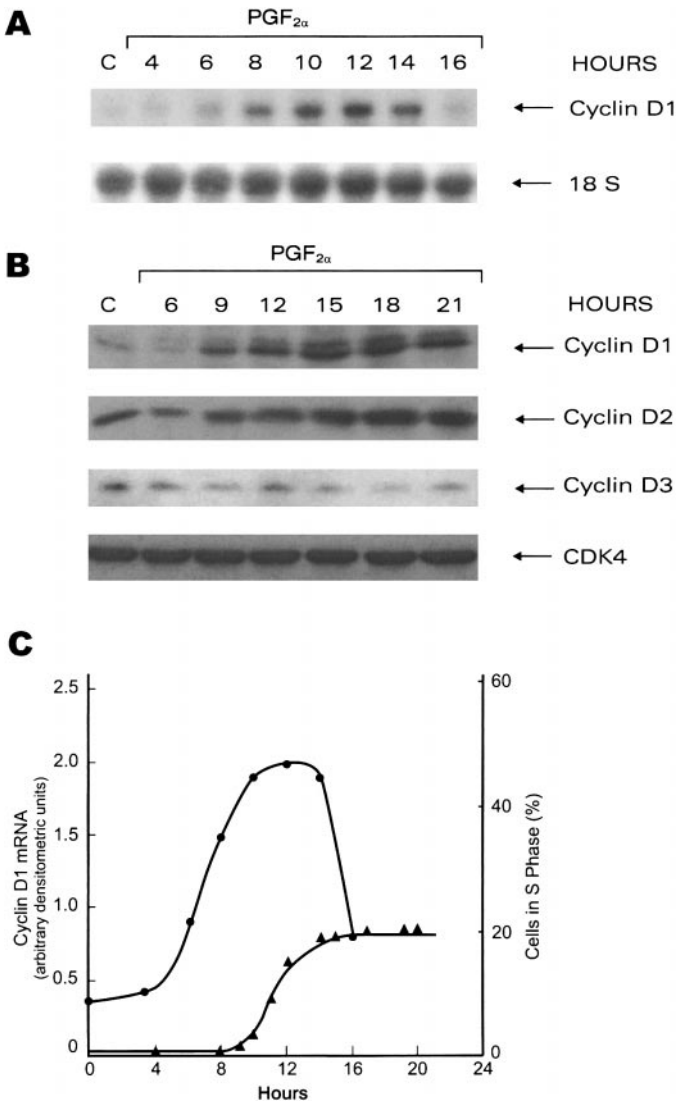

FIG. 1. $PGF_{2\alpha}$ mitogenic response and cyclins D1, D2, D3, and CDK4 expression in confluent resting Swiss 3T3 cells. $PGF_{2\alpha}$ (300 ng/ml) was added at zero time, and cells were prepared at various times for Northern and Western blot assays, and for assays for DNA synthesis, as described under Materials and Methods. (A) Northern blot assays of cyclin D1 mRNA and 18S RNA. Autoradiographic exposures were 24 h and 4 h, respectively. (B) Western blot analysis of cyclin D1, D2, D3, and CDK4. (C) (●) Northern blot quantitation of cyclin D1 mRNA from A (above) (standardized to 18S RNA) and (▲) effects of $PGF_{2\alpha}$ exposure for different times on the percentage of cells in S-phase measured after 28 h. Procedures are described under Materials and Methods.

cells, only $PGF_{2\alpha}$ raised cyclin D1 mRNA levels (Figs. 2B and 2D), but it still failed to induce entry into S-phase (17, 20).

Exposure of Swiss 3T3 cells to either $PGF_{2\alpha}$ (300 ng/ml), OAG (100 μg/ml), or to the tyrosine phosphatase inhibitor, Na_3VO_4 (20 nM), for 10 h, increased cyclin D1 protein levels, but $TGF_{\beta 1}$ (1.5 ng/ml) and Ca^{2+} ionophore A23187 (500 nM), which in these cells causes Ca^{2+} mobilization (24), failed to do so (Fig. 3). In addition, the combination of OAG and $TGF_{\beta 1}$ or Na_3VO_4, caused further increases in cyclin D1 protein levels, while OAG combined with Ca^{2+} ionophore had no additional effect (Figs. 3A and 3B). Moreover, 28 h of exposure to $PGF_{2\alpha}$ alone, or to the combination of OAG and $TGF_{\beta 1}$ or Na_3VO_4, enabled cells to initiate DNA synthesis (Figs. 3C and 3D). In contrast, A23187 and OAG, or A23187 and Na_3VO_4, which partially mimic some of the $PGF_{2\alpha}$-dependent signals (16), failed to do so (Fig. 3C and not shown). Nevertheless, Ca^{2+} ionophore enhanced the combined action of OAG and Na_3VO_4 (Fig. 3D), while A23187 and Na_3VO_4, Na_3VO_4 and $TGF_{\beta 1}$, or A23187 and $TGF_{\beta 1}$, failed to induce both cyclin D1 expression and a mitogenic response (not shown).

A large body of evidence supports the fact that cyclin Ds' expression controls mammalian cell commitment to enter the S-phase (8–14). Mitogens control this process via specific signals, ultimately causing selective cyclin Ds' expression (4, 13). Mann *et al.* have shown that in Swiss 3T3 cells, both bombesin- as well as TPA-triggered PKC activation induces cyclin D1 but not cyclin D3 expression (13), In contrast, agents activating the cAMP-dependent protein kinase (PKA) stimulate only cyclin D3 expression (13). Other findings show that, in Balb/c 3T3 cells, platelet-derived growth factor (PDGF) increases cyclin D1 mRNA transcripts via PKC activation, but when such cells are PKC-depleted, PDGF exhibits some ability to induce cyclin D1 gene expression (12), suggesting that PDGF can also stimulate cyclin D1 expression via PKC-independent events (12).

In conclusion, $PGF_{2\alpha}$ increases cyclin D1 mRNA/protein levels in Swiss mouse 3T3 cells, but fails to induce either cyclin D3 or cdk4 expression. Nevertheless, it is likely that the effects of $PGF_{2\alpha}$ may differ from that of bombesin in signaling cyclin Ds expression, since $PGF_{2\alpha}$, unlike bombesin, caused increases in cyclin D2 protein in late G_1 (13). Furthermore, $PGF_{2\alpha}$ induction of cyclin D1 expression appears to play a pivotal role in controlling cellular commitment to initiate DNA replication, since the timing required for commitment (25) is closely correlated with the maximum increases in cyclin D1 mRNA/protein levels (Fig. 1). In addition, early $PGF_{2\alpha}$ deprivation causes rapid cyclin D1 degradation, impairing cellular commitment ability (not shown). It is unknown whether or not, the $PGF_{2\alpha}$-induced cyclin D2 expression may also have a role in executing commitment for DNA replication. It appears that $PGF_{2\alpha}$-induced cyclin D1 expression involves at least two separate $PGF_{2\alpha}$-signaled processes. While one process is dependent upon PKC activity, the other is not, since $PGF_{2\alpha}$, unlike OAG, triggered cyclin D1 mRNA/protein increases in PKC-depleted cells. Such a PKC-independent process may correspond to other early $PGF_{2\alpha}$-triggered events, such as TK activation (16, 17).

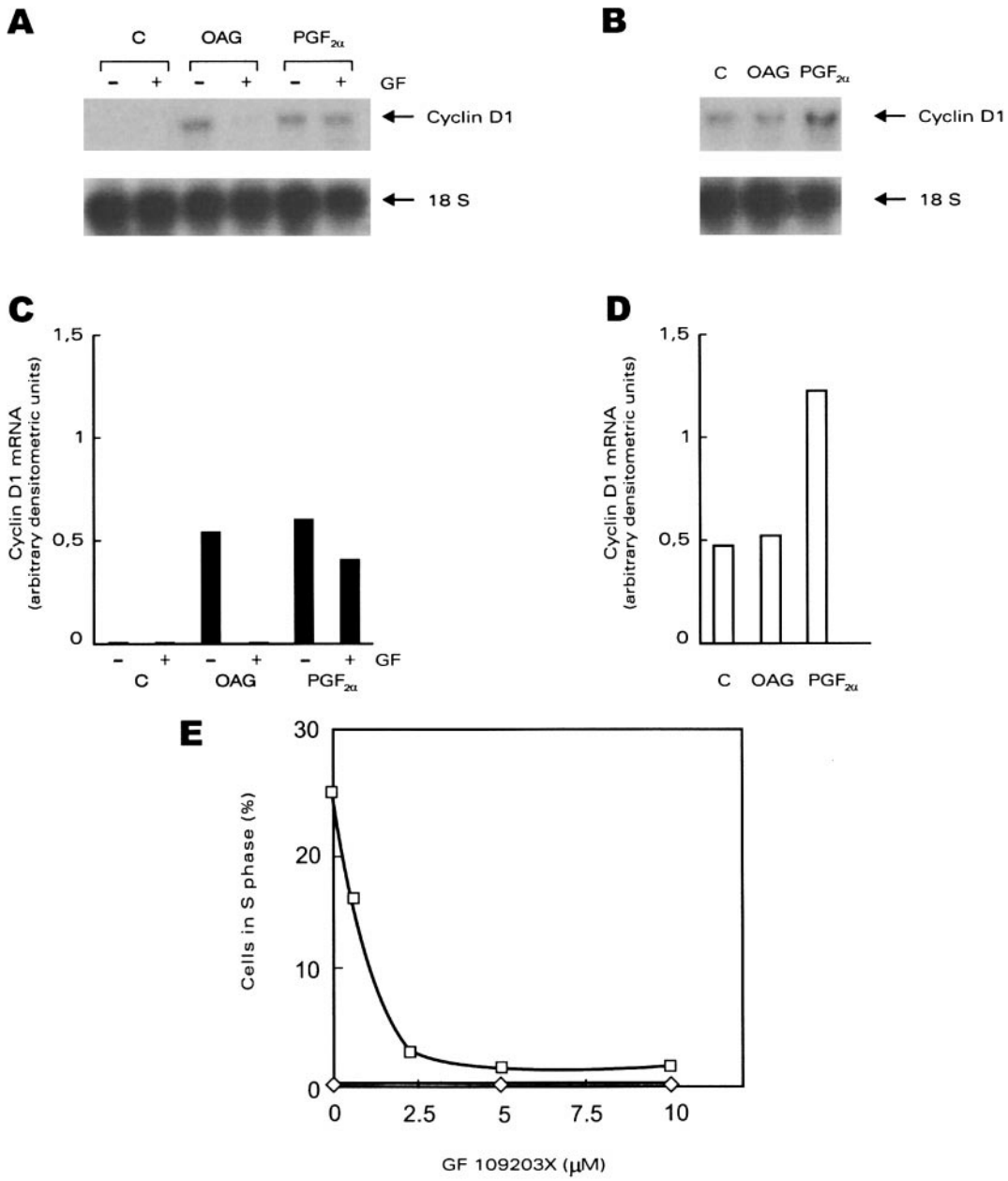

FIG. 2. Northern blot analysis of the effects of PKC inhibitors upon the $PGF_{2\alpha}$ (300 mg/ml) or OAG (100 μg/ml) stimulation of cyclin D1 mRNA expression. (A) Untreated cells (-GF) and cells treated with GF (10 μM) (+GF) 2 h prior to exposure to $PGF_{2\alpha}$ or OAG for 10 h. (B) Effects of PKC depletion on cyclin D1 mRNA. Cells were PKC depleted by adding TPA (800 nM) 72 h prior to stimulation with $PGF_{2\alpha}$ or OAG for 10 h. (C, D) Northern blot densitometric analysis (standardized to 18S RNA). The films in upper and lower panels were exposed for different times (as in Fig. 1). (E) Effect of various concentrations of GF alone ($\diamond$) or GF and 300 ng/ml $PGF_{2\alpha}$ ($\square$) upon induction of DNA synthesis for 28 h. Cultures were labeled and processed as indicated under Materials and Methods.

The Ca^{2+} ionophore by itself fails to increase cyclin D1 mRNA/protein levels, indicating that $PGF_{2\alpha}$-triggered Ca^{2+} ion mobilization does not cause cyclin D1 expression. However, Na_3VO_4, which increases tyrosine phosphorylation independently of PKC activation (17, 26), raises cyclin D1 expression, suggesting that $PGF_{2\alpha}$-increased TK activity may also induce cyclin D1 gene expression. This is supported by the fact that, regardless of PKC activity, both $PGF_{2\alpha}$ and bombesin stimulate tyrosine phosphorylation of various proteins (17, 18). Candidates for the role of a $PGF_{2\alpha}$-activated TK-dependent process controlling cyclin D1 gene expression include those causing either $p125^{FAK}$ or $pp60^{SRC}$ phosphorylation (18, 26–28). Both $PGF_{2\alpha}$-triggered PKC- and TK-dependent events can separately trigger cyclin D1 expression, but only their concerted action, together with other events, lead to DNA synthesis (15–17). However, independently of cyclin

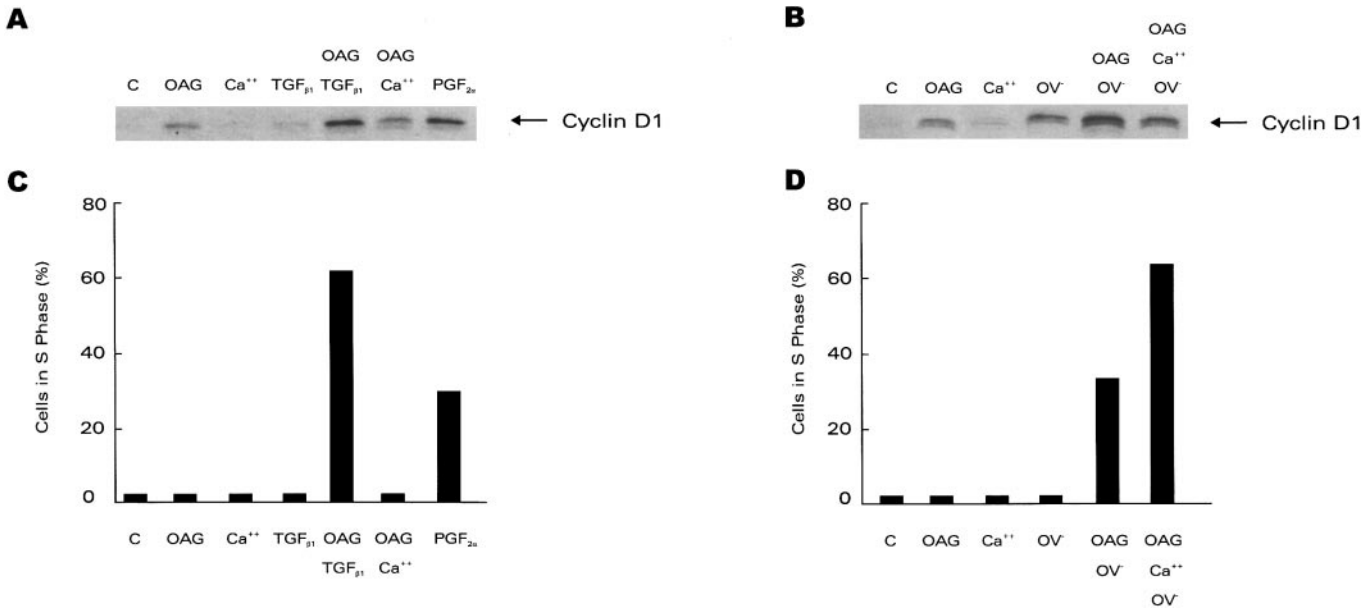

FIG. 3. Effects of Na_3VO_4, $TGF_{\beta 1}$, Ca^{2+} ionophore, and OAG, on cyclin D_1 protein levels and DNA synthesis. (A) Western blot analysis of cyclin D_1 in cells incubated 10 h without or with OAG (100 μg/ml), $TGF_{\beta 1}$ (1.5 ng/ml), Ca^{2+} ionophore (500 nM), or $PGF_{2\alpha}$ (300 ng/ml), as indicated. (B) Western blot analysis of cyclin D1 in cells stimulated for 10 h with Na_3VO_4, OAG or Ca^{2+} ionophore. (C, D) Effects of the additions in A and B, on the percentage of cells on S-phase after 28 h, respectively.

D1 expression, Ca^{2+} mobilization potentiates the concerted action of the later events (Fig. 4). It is important to establish whether these PKC- and/or TK-dependent processes exert differential control over cyclin D1 mRNA/protein turnover. Of significance is the fact that $TGF_{\beta 1}$ acts via events separate from those of $PGF_{2\alpha}$ (19), and could potentiate and/or complement OAG-activated PKC-dependent processes. Indeed, $TGF_{\beta 1}$ increases OAG-triggered, PKC-mediated cyclin D1 protein levels, and, acting together with other PKC-dependent events, confers on a cell the ability to respond to mitogens (19). Moreover, It is unlikely that $PGF_{2\alpha}$ might act solely via the PKC-mediated increases in the RAF/MEK/MAPK-signaled events that appear to induce cyclin D1 expression (13, 29, 30). Nevertheless, the complete identity of all the $PGF_{2\alpha}$-triggered events underlying cyclin D1 expression remains to be elucidated.

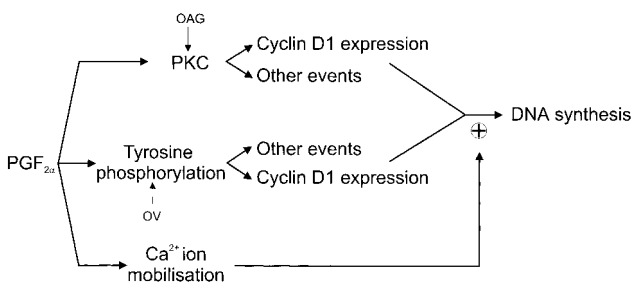

FIG. 4. Summary of $PGF_{2\alpha}$-triggered personalized signals. $PGF_{2\alpha}$ induces PKC activation, tyrosine phosphorylation and Ca^{2+} ion mobilization. The first two events can, independently, trigger cyclin D1 mRNA/protein expression, as well as other processes, but only their concerted action can trigger DNA synthesis. Ca^{2+} ion mobilization, in combination with the other two signaling pathways, only potentiates the entry into the S-phase.

These signaling events triggered by $PGF_{2\alpha}$ as well as by $TGF_{\beta 1}$ may bear some relevance to the control of other processes underlying regulation of the commitment for cellular entry into S-phase. Indeed, in Balb/c 3T3 cells, both PKC-dependent and $TGF_{\beta 1}$-mediated, events, are known to confer stability to ribonucleotide reductase mRNA, via transactivation of specific elements located at its 3′-untranslated region (31, 32). Those elements resemble those located in the 3′untranslated region of cyclin D1 mRNA (33). Thus, our future endeavor will be targeted to elucidating whether and how these $PGF_{2\alpha}$-triggered signals may also control cyclin D1 mRNA stability and commitment in normal cells, and to establishing whether such events can account for the cyclin D1 mRNA overproduction observed in various cancerous cells (34).

ACKNOWLEDGMENTS

L. J. de Asua has been a Royal Society Visiting Professor (London, UK). He is deeply indebted to Susanne Cividini for her encouragement. We are grateful to Ms. Liz Shannon, Becton–Dickinson (NJ), for her generous donation of sterile plastic materials and to Ms. Daniela Conesa for her helpful corrections to the manuscript. This study was supported by Association for International Cancer Research Grant 099-018; the Scotland Cancer and Polio Research Fund (Liverpool, UK), and the Agencia de Ciencia y Tecnologia, Argentina. L.C. and F.R. are recipients of Glaxo-Wellcome Argentina studentships. M.S. is a Fellow of the National Research Council of Argentina (CONICET).

REFERENCES

1. Jimenez de Asua, L., Richmond, K. M. C., and Otto, A. M. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 1004–1008.
2. Rozengurt, E. (1986) *Science* **234,** 161–166.
3. Pardee, A. B. (1989) *Science* **246,** 603–608.
4. Berridge, M. J. (1993) *Nature* **361,** 315–325.
5. Peter, M., and Herskowitz, I. (1994) *Cell* **79,** 573–582.
6. Hunter, T., and Pines, J. (1994) *Cell* **79,** 573–582.
7. Pines, J., and Hunter, T. (1995) *in* Cell Cycle Control (Hutchinson, C., and Glover, D. M., Eds.), pp. 228–263, IRL Publications, Oxford Univ. Press.
8. Matsushime, H., Roussel, M. F., Ashmun, R. A., and Sherr, C. J. (1991) *Cell* **65,** 701–713.
9. Matsushime, H., Even, M. E., Strom, D. K., Kato, J. Y., Hanks, S. K., Rousse, M. F., and Sherr, C. J. (1992) *Cell* **71,** 323–334.
10. Bates, S., Bonetta, L., MacAllan, D., Parry, D., Holder, A., Dickson, C., and Peters, G. (1994) *Oncogene* **9,** 71–79.
11. Won, K. A., Xiong, Y., Beach, D., and Gilman, M. Z. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 9910–9914.
12. Winston, J. T., and Pledger, W. J. (1993) *Mol. Biol. Cell* **4,** 1133–1144.
13. Mann, D. J., Higgins, T., Jones, N. C., and Rozengurt, E. (1997) *Oncogene* **14,** 1759–1766.
14. Baldin, V., Lukas, J., Mascote, M. J., Pagano, M., and Draetta, G. (1993) *Genes Dev.* **7,** 812–821.
15. Goin, M., Pignataro, O., and Jimenez de Asua, L. (1993) *FEBS Lett.* **316,** 68–72.
16. Corps, A. N., Cheek, T. R., Moreton, R. B., Berridge, M. J., and Brown, K. D. (1989) *Cell Reg.* **1,** 75–85.
17. Jimenez de Asua, L., and Goin, M. (1997) *Adv. Exp. Med. Biol.* **400,** 531–538.
18. Watanabe, T., Nakao, A., Emerling, D., Hashimoto, Y., Tsukamoto, K., Horie, Y., Kinshita, M., and Kurokawa, K. (1994) *J. Biol. Chem.* **269,** 17619–17625.
19. Gomez de Alzaga, M., Goin, M., Ortiz, M., and Jimenez de Asua, L. (1994) *FEBS Lett.* **356,** 21–24.
20. Schere-Levy, C., Sauane, M., Rudland, P. S., and Jimenez de Asua, L. (1997) *Biochem. Biophys. Res. Commun.* **236,** 814–818.
21. Todaro, G. J., and Green, H. (1963) *J. Cell Biol.* **17,** 299–313.
22. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning. A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
23. Towbin, J., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.
24. Sinnett-Smith, J., Zachary, I., Valverde, A., and Rozengurt, E. (1993) *J. Biol. Chem.* **268,** 14261–14268.
25. Jimenez de Asua, L., Goin, M., Ortiz, M. B., and Gomez de Alzaga, M. (1997) *Adv. Exp. Med. Biol.* **400,** 449–454.
26. Gould, K. L., and Hunter, T. (1988) *Mol. Cell. Biol.* **8,** 3345–3356.
27. Zhao, J. H., Reiske, H., and Guan, J. L. (1998) *J. Cell Biol.* **143,** 1997–2008.
28. Lee, R. J., Albanese, C., Stenger, R. J., Watanabe, G., Inghirami, G., Haines, G. K., III, Webster, M., Muller, W. J., Brugge, J. S., Davis, R. J., and Pestell, R. G. (1999) *J. Biol. Chem.* **274,** 7341–7350.
29. Albanese, C., Johnson, J., Watanabe, G., Eklund, N., Vu, D., Arnold, A., and Pestell, R. G. (1995) *J. Biol. Chem.* **270,** 23589–23597.
30. Lavole, J. N., L'allemain, G., Brunet, A., Muller, R., and Pouyssegur, J. (1996) *J. Biol. Chem.* **271,** 20608–20616.
31. Chen, F. Y., Amara, F. M., and Wright, J. A. (1993) *EMBO J.* **12,** 2977–3986.
32. Amara, F. M., Chen, F. Y., and Wright, J. A. (1995) *Nucleic Acids Res.* **23,** 1461–1467.
33. Smith, R., Peters, G., and Dickson, C. (1995) *Genomics* **25,** 85–92.
34. Lees, E. M., and Harlow, E. (1995) *in* Cell Cycle Control (Hutchinson, C., and Glover, D. M., Eds.), IRL Publications, Oxford Univ. Press.