Antagonistic Actions of Phorbol Ester in Mammalian G₀→G₁→S Cell Cycle Transition

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Abstract
We have developed a protocol that reveals two antagonistic effects of phorbol-12-myristate-12-acetate (PMA) on the G₀→G₁→S transition of mammalian cell cycle. Balb-3T3 (Clone A31) cells arrested in G₀ by serum starvation can be stimulated to traverse the G₁ phase and initiate DNA synthesis 12 h later by a 2-h pulse with PMA. In contrast with this early stimulatory effect, PMA has an inhibitory effect when presented to the cells during the last 6 h of G₁. PMA is able to inhibit DNA synthesis initiation irrespective of the triggering agent, i.e., serum, fibroblast growth factor, epidermal growth factor, platelet-derived growth factor, or PMA itself (presented as an early pulse). We have established that the critical period for the PMA inhibitory effect is between 6 and 8 h after cell stimulation. This dual effect of PMA is not a peculiarity of Balb-3T3 (clone A31) cells because it is also observed with other fibroblastic cell lines, namely, SWISS 3T3, NIL 8, and RAT 1, and also with the epithelial Y-1 adenocortical cell line.

Treatment with PMA for 0.5 or 2 h activates protein kinase C (PKC) in Balb-3T3-A31 cells, but is not sufficient to down-regulate the enzyme because a second 30-min PMA pulse applied between 6 and 6.5 h activates PKC again. On the other hand, a continuous 6.5-h PMA treatment causes PKC down-regulation; therefore, the inhibitory effect of PMA could be mediated by PKC.

Growth factor early response proto-oncogenes c-myc, c-fos, and c-jun are induced transiently by both early and late PMA pulses, suggesting that these genes are not involved in the PMA inhibitory effect.

Introduction
Mammalian cell cycle control is exerted mainly at the G₀→G₁→S transition (1, 2). Balb-3T3 cells subjected to G₀ arrest by serum deprivation can be stimulated to traverse G₁ and enter the S phase by peptide growth factors like PDGF³ and FGF (3). These growth factors are known to induce a large set of early response genes among which are the c-myc, c-fos and c-jun proto-oncogenes (4–8).

The phorbol ester PMA, a well-known PKC activator (9), mimics the mitogenic actions of growth factors, inducing the same early response genes and stimulating S phase entry (8). These results have prompted the notion that PKC is an essential component in the regulatory routes activated by PDGF and/or FGF receptors.

In this paper we show that PMA has a dual effect in the G₀→G₁→S transition: it is stimulatory in early G₁ (G₀→G₁ transition) and inhibitory in late G₁ (G₁→S transition). This novel PMA inhibitory effect was observed in several fibroblastic cell lines, suggesting that it is a general phenomenon in the cell cycle of mammalian cells. PKC isoforms are likely to be mediators of this PMA inhibitory effect, suggesting a link between this enzyme and the pathway that regulates G₁→S traversing.

Results
The protocols used are shown in Fig. 1. Fig. 1A shows the protocols designed for the DNA synthesis stimulation experiments of Figs. 2–4; Fig. 1B refers to PKC activity assays (Fig. 5) and the induction of early genes (Fig. 6).

G₀-arrested Balb-3T3 cells initiate DNA synthesis on treatment with a 2-h pulse of FGF or PMA (Fig. 2). However, a second PMA pulse, between 6 and 12 h, blocks G₁ traversing, abolishing DNA synthesis initiation (Fig. 2). The stimulatory effects of long (12 h) treatment with FGF, EGF, or PDGF are also blocked by a late PMA pulse (Fig. 2). In a 12-h PMA treatment, the inhibitory effect prevails (Fig. 2).

The time of exposure to PMA is critical for its mitogenic effect. Thus, a 1-h pulse is not sufficient but a maximal effect is observed on a 2-h treatment, decreasing upon a 4-h treatment (Fig. 3). The mitogenic effects of both 2- and 4-h PMA pulses are equally abolished on a second PMA treatment applied between 6 and 12 h after the initiation of the first pulse (Fig. 3).

This DNA synthesis inhibitory effect of PMA seems to be restricted to a narrow period of the G₁ phase between 6 and 8 h: PMA pulses between 6 and 8, 6 and 9, and 6 and 10 h, respectively, abolish the mitogenic effect of FGF (Fig. 4), whereas later pulses, between 9 and 12 or 10 and 12 h, are not effective (Fig. 4).

³ The abbreviations used are: PDGF, platelet-derived growth factor; PMA, phorbol-12-myristate-12-acetate; PPP, platelet poor plasma; FGF, fibroblast growth factor; EGF, epidermal growth factor; CDK, cyclin-dependent kinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

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The early response genes are transiently induced during the G0→G1→S transition; therefore, we monitored c-fos mRNA levels after 0.5 h and c-myc and c-jun after 3 h stimulation with PMA and/or FGF by Northern hybridizations (Fig. 5). PMA and FGF separately or combined cause identical c-fos, c-myc, and c-jun induction (Fig. 5). It is important to note that a 2-h FGF pulse triggers DNA synthesis stimulation to levels that are higher than that of a 2-h PMA pulse (Fig. 2). However, the DNA synthesis stimulation due to the combination of FGF plus PMA, in a 2 h pulse, even at non-saturating concentrations, does not overcome the effect of FGF alone (data not shown), indicating that there is no additive effect between FGF and PMA in this response.

Balb-3T3 cells treated with an early 2-h PMA pulse do not display detectable levels of c-fos mRNA at 6.5 h or of c-myc and c-jun mRNA at 9 h (Fig. 5). However, a second PMA
treatment initiated at 6 h triggers c-fos mRNA induction again at 6.5 h and c-myc and c-jun induction at 9 h (Fig. 5), therefore, following the same induction pattern observed with the first PMA pulse. The results obtained with the second PMA treatment are the same irrespective of the first mitogenic factor used in the early treatment, i.e., FGF or PMA (Fig. 5). In conclusion, a second PMA treatment in the middle of the G0→G1→S transition, i.e., at 6 h, causes the same induction pattern of c-fos, c-myc, and c-jun observed in the first few h, at least at the mRNA level, even though PMA is blocking G1 transverse and abolishing DNA synthesis initiation.

We also measured modulation of PKC activity by PMA by assaying the activity of all kinases dependent on diol and phosphatidyserine, in both membrane and soluble fractions. PKC activation is clearly evidenced by a 5-fold increase in activity in the membrane fraction of PMA-treated (0.5 h) cells (Fig. 6A). A 2-h PMA pulse leads to similar levels of activation, whereas a 6.5-h PMA treatment is sufficient to cause a pronounced PKC down-regulation, indicated by a significant reduction in activity in both membrane and soluble fractions (Fig. 6A). However, a 2-h PMA pulse followed by PMA withdrawal does not cause down-regulation because within 6 h the cells resemble control cells by two criteria: (a) low, rela-
mammalian cell cycle. Results equivalent to those presented here for the Balb-3T3 cells were found for three fibroblastic lines, namely RAT 1 (rat cells), NIL 8 (hamster cells), and Swiss-3T3 (mouse cells) and, also, for the Y-1 adrenocortical cells, a tumorigenic mouse line of functional cells.

Discussion
The $G_0\rightarrow G_1\rightarrow S$ transition of the cell cycle of Balb-3T3 cells is a well-known cell response that has been studied extensively for the last 30 years. PMA is known as a mitogenic agent that mimics growth factors in promoting the $G_0\rightarrow G_1\rightarrow S$ transition. Here we show that this statement must be reevaluated because PMA in fact has a dual effect in the $G_0\rightarrow G_1\rightarrow S$ transition: it is stimulatory at the early stages of the $G_0\rightarrow G_1\rightarrow S$ transition and inhibitory when applied later in $G_1$. The late inhibitory effect of PMA seems to prevail over its early stimulatory effect.

This phenomenon was underlined by a protocol (Fig. 1A) designed to explore the $G_0\rightarrow G_1\rightarrow S$ transition in $G_0$-arrested Balb-3T3 cells stimulated by serum, growth factors (FGF, PDGF, and EGF), and PMA (Fig. 2). Our protocol has provided evidence that PMA blocks $G_1$ cell traversing between the 6 and 8 h of stimulation (Fig. 4). However, this inhibitory effect is not restricted to $G_0$-arrested cells or to the particular experimental conditions used here. In fact, exponentially growing cells are also inhibited by a PMA pulse (Fig. 7), indicating that these cells are susceptible to the PMA inhibitory effect between 2 and 4 h before entering S phase. Moreover, this PMA inhibitory effect was also observed with other mammalian cell lines (data not shown). Therefore, the $G_1$-blocking action of PMA described here is likely to be a common feature of the mammalian cell cycle. Measurements of PKC activity during PMA treatments of Balb-3T3 cells (Fig. 6) suggest that both stimulatory and inhibitory PMA effects are mediated by PKC activation.

Induction of the early response genes, particularly of the proto-oncogenes c-myc, c-fos, and c-jun, by PMA, closely resembles the activity of growth factors like PDGF and FGF. Results from several laboratories indicate that the induction of these proto-oncogenes is necessary for $G_0$-arrested cells to traverse $G_1$ and initiate DNA synthesis (10). However, the induction of these proto-oncogenes is not cell cycle specific as shown by us (11) and others (10). Thus, it is not surprising that the pattern of c-myc, c-fos, and c-jun induction by PMA (Fig. 5) is the same irrespective of the period of PMA treatment, i.e., between 0 and 3 h or between 6 and 9 h of stimulation. These results suggest that the PMA inhibitory effect on $G_1\rightarrow S$ traversing is unrelated to its ability to induce the expression of these genes.

Data from a number of laboratories during the last ten years have led to the notion that the products of proto-oncogenes and of tumor suppressor genes (anti-oncogenes) are organized into two separated circuits that regulate the controls on the $G_0\rightarrow G_1\rightarrow S$ transition (2, 8). Proto-oncogene products are concentrated in the regulatory pathways that are activated by growth factor receptors and stimulate $G_0$ exit toward $G_1$. PMA, through PKC activation, induces the early response genes by the pathway of this growth factor. This mechanism can explain the mitogenic action of PMA.
On the other hand, the anti-oncogene products (retinoblastoma protein, p53, p21, and p16) seem to play a role in the CDKs circuit that regulates the G1→S transition. CDKs activation, in contrast with the early response genes induction, seems to be a cell cycle-specific event (2, 12). We propose that the PMA inhibitory effect of G1→S transition may be due to activation of a PKC isoform that has a connection with the circuit of CDK and anti-oncogenes products, thus, blocking Rb phosphorylation that is required for the G1→S transition to occur.

Materials and Methods

Cells and Cell Culture Conditions

Frozen Stocks of Balb-3T3 Cells. Clone A31 (13) was kept in liquid nitrogen. Cultures were grown in DMEM with 1.2 g/liter sodium bicarbonate plus 10% FCS under a 5% CO2/air atmosphere. Cultures were grown continuously to near confluence and were subcultured every 3–4 days.

Mitogenic Activity Assays

DNA Synthesis Stimulation—Synchronous Populations. Confluent cultures were serum starved for 24 h in medium containing 3% PPP. DNA synthesis of these G0→G1-arrested cells was stimulated by serum, growth factors, or PMA. [3H]thymidine (0.25 μCi/ml; 10−7 M) was incorporated between 12 and 24 h after triggering DNA synthesis stimulation and after incorporation into DNA was measured by scintillation counting as described previously (14).

Labeling Index—Asynchronous Populations. Exponentially growing coverslip cultures were treated with PMA (10 ng/ml) for 2 h and then pulse-labeled with [3H] thymidine (5 μCi/ml; 10−7 M) for 15 min every 2 h after the PMA pulse. Coverslips were extracted twice with cold 10% trichloroacetic acid, washed with 95% ethanol, air dried, and covered with Kodak AR-10 stripping film. After 10 days of exposure, the film was developed and cells were stained with Giemsa. Labeling index was estimated by scoring the percentage of labeled nuclei under light microscope by counting at least 300 cells/cover slip.

The treatment referred to as early PMA pulse consists of: (a) PMA addition at time zero; (b) incubation for the desired period of time; (c) medium removal; (d) washing of cell monolayers twice with PBS; and (e) addition of fresh medium. Control dishes were subjected to the same
Fig. 6. PKC activation by PMA treatments. After a 24 h starvation in medium containing 3% PPP, Balb-3T3 cells were stimulated with 10 ng/ml PMA (A) and 20 ng/ml FGF (B) for the indicated times. PKC activity was measured as described in "Materials and Methods" for the membrane (■) and soluble (□) fractions. Columns, mean of three independent experiments, each with duplicates. *, statistically significant differences (student t test; P < 0.001) between control and experimental conditions, comparing either membrane activities or membrane:soluble activities ratios.

Fig. 7. PMA inhibitory action is exhibited by exponentially growing cultures. Exponentially growing Balb-3T3 cells were treated for 2 h with PMA (■) and then washed twice with PBS. Every 2 h, duplicate coverslips were pulse labeled for 15 min with [3H]thymidine and extracted as described in the "Materials and Methods." The same procedure was adopted for the control population growing in 10% FCS (□) for a 10-h period.

RNA Extraction and Northern Hybridization Analysis
Confluent cultures in 150-mm diameter dishes were serum starved for 24 h in medium containing 3% PPP. Treatment with FGF (20 ng/ml), PMA (10 ng/ml), or a combination of both was performed for different periods of time as indicated in "Results." Cell monolayers were lysed in 4 % guanidine isothiocyanate (15), layered on top of a 5.7 M CsCl-25 mM sodium acetate (pH 5.0) solution, and centrifuged to equilibrium in a SW50.1 rotor. Total RNA recovered from the CsCl pellet was dissolved in water, quantified by UV absorption, and used for Northern hybridization analysis as follows: 10 ng total RNA in 4-morpholinepropanesulfonic acid-formaldehyde was heated for 15 min at 65°C, fractionated in 1% agarose gels, and transferred to nitrocellulose filters in 15x SSC. The DNA probes used for hybridization were: pFBJ-2fos for c-fos (16), LK58B-c-junR for c-jun, pBIC01myc for c-myc (17), and pGAPDH1 for GAPDH (18). All of the plasmids were labeled with [α-32p]dCTP by random primer extension (19).

Protein Kinase C Activity Determination
Confluent cultures in 150-mm diameter dishes were serum starved for 24 h in medium containing 3% PPP. PMA, FGF, or a combination of both washing procedures. PMA removal by PBS washing was monitored by using a radioactively labeled PMA analogue, i.e., [20 nCi]phorbol-12:13-dibutyrate. Results shown in Figs. 1–3, and 6 are representative of at least three independent experiments in each case.

R. Bravo, unpublished data.
were washed twice with a physiological saline buffer without calcium; cells were collected in 1.5 ml buffer A [20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 0.5 mM EGTA, 2 mM phenylmethylsulfonyl fluoride, 25 ng/ml leupeptin, and 0.33 mM Sucrose] and homogenized with 20 strokes of a Teflon glass homogenizer. The homogenates were centrifuged at 100,000 × g for 45 min at 4°C, and the supernatants were collected and kept at 4°C to assay for PKC activity associated with the soluble fraction. The pellets were washed in 1.5 ml buffer B (buffer A without the sucrose) and homogenized with 20 strokes, with NP40 added to a 1% final concentration. The pellet homogenates were mixed for 40 min at 4°C and centrifuged at 100,000 × g for 45 min at 4°C. The supernatants were collected to assay for PKC activity associated with the membrane fraction. Soluble and membrane samples were loaded onto a DEAE-cellulose column (300-μl bed volume), equilibrated in 20 mM Tris-HCl (pH 7.5), and kept under agitation for 40 min at 4°C. Columns were washed with 2 ml buffer B for 40 min and eluted with 0.5 ml buffer B containing 0.15 mM NaCl. PKC activity was assayed in 50-μl aliquots containing 20 mM Tris-HCl, 25 ng histone H1, 0.75 mM CaCl2, 10 mM magnesium acetate, 50 mg/ml leupeptin, 100 μM [γ-32P]ATP (120 cpm/pmol), and with or without lipids as described (20).

**Growth Factors, Drugs, and Isotopes**

FGF and EGF from Boehringer Mannheim; PMA from Sigma; and [3H]-methylthymidine (83 Ci/mmol), [γ-32P]ATP (3000 Ci/mmol), and [α-32P]dCTP (3000 Ci/mmol) were from Amersham.

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**References**