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# Inhibition of MCF-7 Cell Growth by 12-*O*-Tetradecanoylphorbol-13-acetate and 1,2-Dioctanoyl-*sn*-glycerol: Distinct Effects on Protein Kinase C Activity[1]

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## ABSTRACT

**We have investigated the effects of phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and permeant diacylglycerol 1,2-dioctanoyl-*sn*-glycerol ($DiC_8$) on MCF-7 cell proliferation and protein kinase C activity. $DiC_8$ mimics the effects of TPA on both cell morphology and proliferation, with an $ED_{50}$ value of 11 μg/ml for cell growth inhibition. As with TPA and phorbol 12,13-dibutyrate, $DiC_8$ enhances the degree of phosphorylation of an endogenous $M_r$ 28,000 protein in a time- and dose-dependent manner. The effect is measurable upon 5 min of cell treatment with each protein kinase C activator and reaches a maximum at 30 min. The $ED_{50}$s observed are 5 ng/ml and 20 μg/ml, respectively, for phorbol esters and $DiC_8$. The $M_r$ 28,000 protein is found in the cytosolic fraction and is phosphorylated on serine residues by both TPA and $DiC_8$. Further characterization of the phosphorylated proteins using a highly resolutive two-dimensional electrophoresis demonstrates that the two-protein kinase C activators lead to slightly distinct protein phosphorylation patterns with an extra set of proteins phosphorylated under TPA but not $DiC_8$ stimulation. Contrary to TPA, $DiC_8$ induces only a partial and transient translocation of protein kinase C activity from the cytosolic to the particulate compartment. Moreover, no down-regulation of protein kinase C is observed after prolonged treatment of MCF-7 cells with $DiC_8$, while only 10% of the initial protein kinase C level remains present in cells treated with TPA for 48 h. However, this remainder enzymatic activity is sufficient to induce the phosphorylation of the $M_r$ 28,000 protein at its maximal level.**

**In conclusion, our results reinforce the hypothesis of a negative modulatory role of protein kinase C in MCF-7 cell proliferation but suggest that the two activators TPA and $DiC_8$ could induce distinct molecular events with regard to the enzyme recruitment and activity as well as to its further processing.**

## INTRODUCTION

Tumor promoter phorbol esters such as TPA[3] induce various biochemical and biological effects in cultured cells, including striking stimulatory or inhibitory effects on cell proliferation and differentiation (1, 2). In MCF-7 human breast cancer cells, TPA and other active phorbol esters cause growth arrest (3–5) and changes in cell morphology (6). The only currently recognized mediator of the TPA action is the $Ca^{2+}$- and phospholipid-dependent protein kinase C (7–9), which most probably represents the high affinity phorbol ester receptor in target cells (8–12). There is increasing evidence that protein kinase C plays a pivotal role in the transmembrane signaling of a wide variety of extracellular stimuli including growth factors, hormones and other biologically active substances (for reviews, see References 7, 13, 14).

The physiological activator of protein kinase C is DAG which accumulates transiently as a consequence of the receptor-mediated inositol phospholipid breakdown (7, 14, 15). TPA can substitute for intracellular messenger DAG to activate protein kinase C (16, 17). As in many other cell types (17, 18) TPA induces a rapid subcellular redistribution of protein kinase C in MCF-7 cells, followed by a progressive disappearance of the enzyme upon prolonged cell treatment (19–22).

In order to further define the role of protein kinase C in the inhibition of MCF-7 cell proliferation, we have investigated whether the permeant synthetic $DiC_8$ could mimic the effects of TPA on protein kinase C activity and cell growth. We report that the two activators induce similar changes in cell morphology and inhibit cell proliferation identically. $DiC_8$ and TPA similarly increase the degree of phosphorylation of a cytosolic protein with 28,000 molecular weight in intact MCF-7 cells but slightly distinct protein phosphorylation patterns can be demonstrated by using two-dimensional electrophoresis. Moreover, contrary to TPA and PDBu, $DiC_8$ causes only a partial and transient translocation of protein kinase C from the cytosolic to the particulate compartment. Finally, $DiC_8$ is unable to trigger the down-regulation of protein kinase C which occurs at the membrane level after TPA or PDBu stimulation.

## MATERIALS AND METHODS

**Chemicals.** Histone $H_1$, TPA, phosphatidyl-serine, 1,2-dioleoyl-glycerol and $DiC_8$ were obtained from Sigma. [γ-$^{32}$P]ATP (0.5–3 Ci/mmol) and [$^{32}$P]phosphoric acid were purchased from Amersham. Acrylamide and bisacrylamide were obtained from Biorad. All other chemicals were from Merck.

**Cell Culture.** MCF-7 cells were grown at 37°C in RPMI 1640 (GIBCO), pH 7.3, supplemented with 2 g/liter of sodium bicarbonate, 2 mM L-glutamine, 1 μM insulin, and 5% fetal calf serum (FCS, Seromed). Culture media were changed every 2 days. For cell growth measurement, MCF-7 cells were plated at an initial density of 1–1.5 × $10^4$ cells per 35-mm dish. After 48 h (Day 0) the medium was replaced by fresh RPMI-5% FCS and phorbol esters or $DiC_8$ were added at various concentrations. Addition of the permeant diacylglycerol was repeated three times a day whereas phorbol esters were added every 2 or 3 days. Control dishes received the same volume of the solvent acetone (final concentration of 0.1%). Cells were harvested with 0.05% trypsin-0.02% EDTA and cell number was determined by using a Coulter Counter (Coultronics).

**Protein Phosphorylation.** Subconfluent cultures (1 × $10^6$ cells/dish) were washed twice in a phosphate-free Krebs-Ringer buffer, pH 7.2, containing 20 mM HEPES, 0.1% BSA, and 0.2% glucose, and incubated for 2 h at 37°C in 1 ml of the same buffer containing 50 μCi [$^{32}$P]-phosphoric acid. Stimuli were then added for a further 30-min period. Cells were washed twice with cold PBS and 10% TCA was added. TCA-precipitated proteins were dissolved in 150 μl of electrophoresis sample buffer containing 0.06 M Tris-HCl, pH 6.7, 2% SDS, 8% glycerol, 2% β-mercaptoethanol and 0.005% bromophenol blue. Samples were then boiled for 5 min at 90°C. Proteins were fractionated by electrophoresis on 4.5 and 12% (w/v) discontinuous SDS-polyacrylamide slab gel as described by Laemmli (23). After protein fixation by cold trichloroacetic acid and Coomassie blue staining, the gels were dried, then exposed to Hyperfilms-MP for 48–72 h. The autoradiographs were scanned by densitometry and the $^{32}$P incorporated into the proteins was evaluated by measuring the respective peak areas as previously indicated (24).

Alternatively, TCA-precipitated proteins were subjected to a two-

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[3] The abbreviations used are: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PDBu, phorbol 12,13-dibutyrate; DAG, 1,2-*sn*-diacylglycerol; $DiC_8$, 1,2-dioctanoyl-*sn*-glycerol; EGTA, ethylene glycol bis (β-aminoethylether)-*N,N,N',N'*-tetraacetic acid; SDS, sodium dodecyl sulfate; NP-40, nonidet P-40; PBS, phosphate buffered saline; TCA, trichloroacetic acid.

dimensional gel electrophoresis according to O'Farrell (25). Proteins were dissolved in lysis buffer containing 9.5 M urea, 2% NP-40, 5% β-mercaptoethanol, and 2% ampholine (pH 3.5–9.5, Biorad). After sonication the lysate was centrifuged at 10,000 × *g* for 30 min. Proteins were subjected to isoelectric focusing using 10.5-cm tube gels of 1.5-mm diameter, followed by electrophoresis on SDS-polyacrylamide (12%) slab gel and autoradiography. The amount of $^{32}P$ incorporated into proteins was evaluated by comparison with a standard $^{32}P$ label scale autoradiograph.

**Phosphoamino Acid Analysis.** Phosphoproteins were recovered from the dried polyacrylamide gel and then acid hydrolyzed by 6 N HCl at 110°C for 2 h. Phosphoserine, phosphothreonine, and phosphotyrosine standards were added to the radioactive samples. Phosphoamino acids were separated according to Hunter and Sefton (26) by electrophoresis at 1000 V for 1 h in pyridine/acetic acid/$H_2O$ (1:10:189, V/V), pH 3.5, on thin-layer cellulose plates.

**Subcellular Localization of the Phosphorylated Proteins.** After incubation in the presence of stimuli for 30 min, cells were scraped from the culture dishes into cold PBS and then sonicated in 10 mM Tris-HCl, pH 7.3, containing 10% glycerol, 2 mM EDTA, 6 mM β-mercaptoethanol, and 100 mM sodium fluoride. Nuclear fraction was obtained by centrifugation of the cell lysate at 900 × *g* for 30 min and the pellet was solubilized in electrophoresis sample buffer. The nonnuclear fraction was centrifuged for 60 min at 105,000 × *g*. The resulting pellet was considered as the 105,000 × *g* membrane fraction and the supernatant was considered as the cytosolic fraction.

**Protein Kinase C Assay.** Subconfluent cells were rapidly harvested in cold PBS, and homogenized in 20 mM Tris-HCl, pH 7.5, containing 0.25 M sucrose, 2 mM EDTA, 2 mM EGTA, 100 μg/ml leupeptin, and 5 mM β-mercaptoethanol (buffer A).

The cell lysate was centrifuged for 1 h at 105,000 × *g*. The supernatant was used as the cytosolic fraction. The corresponding pellet was resuspended in buffer A, containing 0.5% NP-40 and briefly sonicated. After incubation at 4°C for 45 min it was centrifuged for 1 h at 105,000 × *g*. The supernatant recovered was used as the 0.5% NP-40 extract of the particulate fraction.

When indicated, the cell lysate was directly treated with buffer A containing 0.5% NP-40 for 45 min at 4°C and then centrifuged for 1 h at 105,000 × *g*. The supernatant was used as the 0.5% NP-40 extract of the cell homogenate. Cytosol, 0.5% NP-40 extract of particulate fraction or cell homogenate obtained from 40 × $10^6$ cells was applied to a DEAE-cellulose column (DE52, 0.8 × 3 cm) equilibrated with 20 mM Tris-HCl, pH 7.5, containing 2 mM EDTA, 1 mM EGTA, 50 μg/ml phenylmethylsulfonyl fluoride, and 5 mM β-mercaptoethanol (buffer B). Columns were washed with 10 ml of buffer B, and protein kinase C was eluted with 5 ml of buffer B containing 0.13 M NaCl.

Protein kinase C was immediately assayed as previously indicated (19).

## RESULTS

**Effects of Phorbol Esters and Permeant Diacylglycerol on Cell Growth and Morphology.** Fig. 1 shows the time course study of the effects of TPA, PDBu, and $DiC_8$ on MCF-7 cell proliferation. Maximal growth inhibition was reached after 2 and 4 days of cell treatment, respectively, with phorbol esters and DAG. The inhibitory effect of $DiC_8$ is observed when this compound was added three times a day while one addition of phorbol esters TPA and PDBu was sufficient to produce maximal inhibition. Paradoxically, $DiC_8$ reduces the rate of cell growth earlier than phorbol esters do, as a partial inhibition of MCF-7 proliferation was observed after 1 day of treatment with DAG. Moreover, at the concentration of 5 ng/ml used in this study, TPA exerts a slight cytotoxic effect on cells, leading to a small decrease in the cell number after 4 days of treatment.

Fig. 2 shows the effects of increasing concentrations of TPA and $DiC_8$ on cell growth. $DiC_8$ as well as TPA induce a dose-dependent reduction in cell number measured after 5 days of

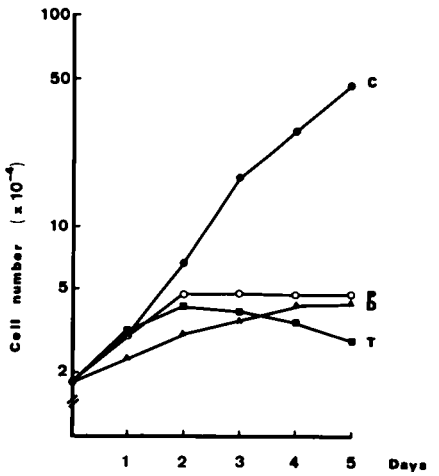


Fig. 1. Effects of TPA, PDBu, and $DiC_8$ on the growth of MCF-7 cells. Cells were cultured as indicated in "Materials and Methods." in the absence (*C*) or in the presence of 5 ng/ml TPA (*T*), 10 ng/ml PDBu (*P*), or 21.5 μg/ml $DiC_8$ (*D*). $DiC_8$ was added 3 times a day. TPA was added every 3 days and PDBu every 2 days. Cell counting was performed on 3 separate dishes. The maximum variation of cell number did not exceed 15%.

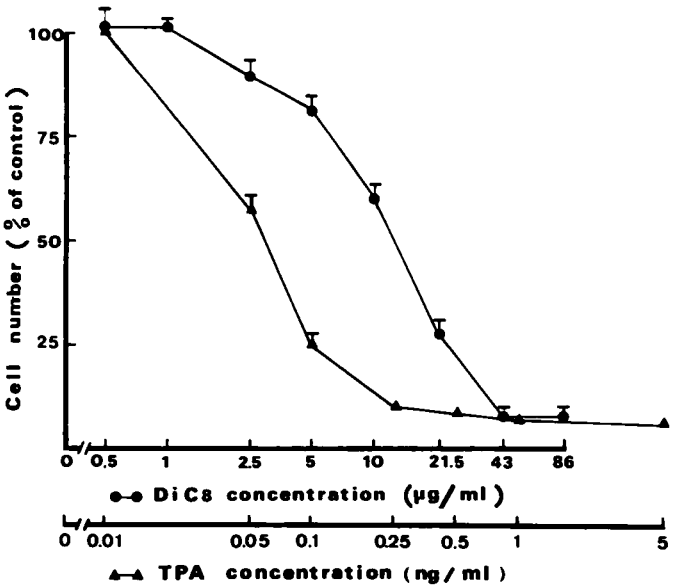


Fig. 2. TPA and $DiC_8$ dose-dependent inhibition of MCF-7 cell proliferation. Cells were cultured in the absence (*control*) or in the presence of increasing concentrations of $DiC_8$ (●) or TPA (▲). $DiC_8$ was added three times a day whereas TPA was added every 3 days. Cells were counted after 5 days of treatment. The number of control cells increased from 4 × $10^4$ cells at Day 0 to 1 × $10^6$ cells at Day 5. Data are the means ± SD of five measurements from one representative experiment. Results are expressed as percent of the control cell number.

treatment. The $ED_{50}$ observed were 11 μg/ml (32 μM) and 0.05 ng/ml (0.08 nM), respectively, for $DiC_8$ and TPA.

Fig. 3 illustrates the changes in MCF-7 cell morphology occurring upon treatment with phorbol esters and DAG. Control cultures show typical epithelioid characteristics with several clusters of small and polygonal cells. As previously reported (6), TPA produces striking modifications in MCF-7 cell morphology. We show now that $DiC_8$ induces similar changes. Cells became rounded and spread out, and some giant cells are observable.

**Effects of Protein Kinase C Activators on Protein Phosphorylation Pattern in Intact Cells.** Fig. 4 shows the protein phosphorylation pattern obtained when MCF-7 cells were stimulated for 30 min by different activators of protein kinase C. As previously reported (19), TPA, and PDBu markedly enhance the degree of phosphorylation of a $M_r$ 28,000 protein. We show

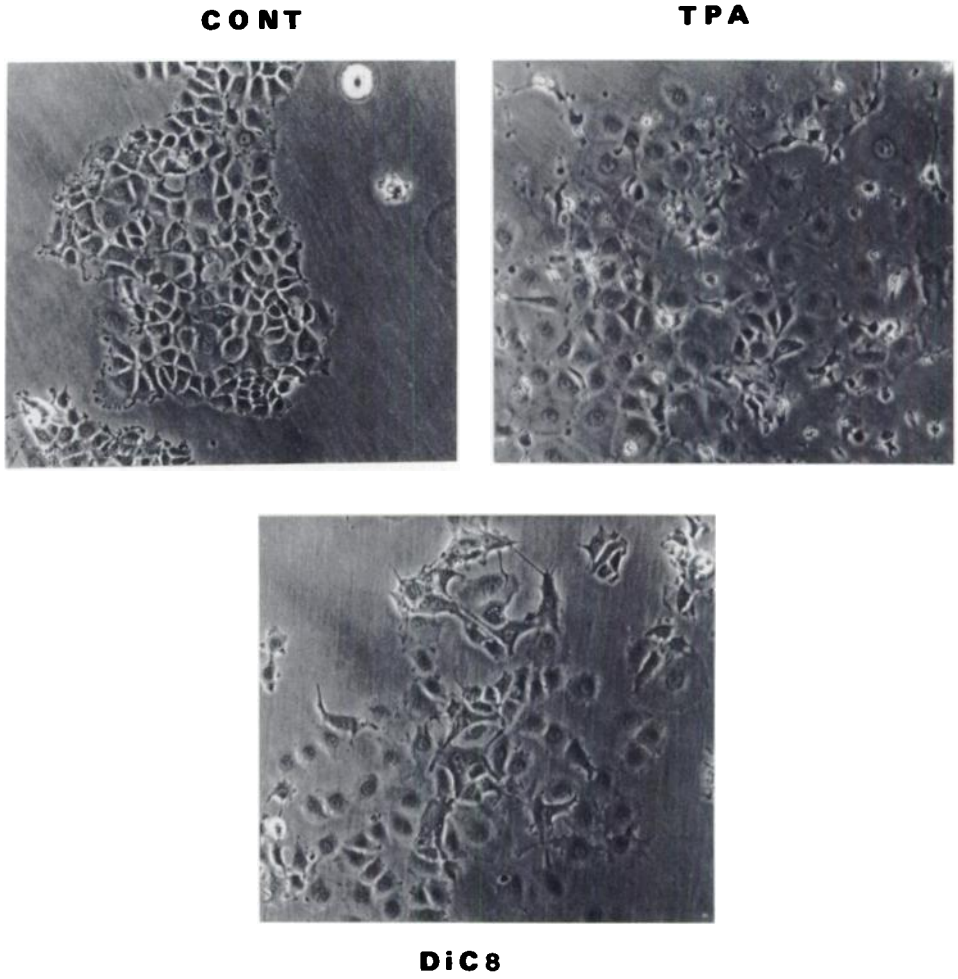


Fig. 3. Effects of TPA and $DiC_8$ on MCF-7 cell morphology. Cells were cultured for 3 days in the absence (*Cont*) or in the presence of 5 ng/ml TPA or 21.5 μg/ml $DiC_8$. Phase-contrast microscopy was performed at ×100 magnification.

now that the permeant diacylglycerol $DiC_8$ mimics the effect of phorbol esters on this protein phosphorylation. On the contrary, the inactive tumor promoter 4α-phorbol is unable to induce the $M_r$ 28,000 protein phosphorylation. We have previously reported that this compound was also ineffective in inhibiting MCF-7 cell growth (5).

We have investigated the subcellular localization of the $M_r$ 28,000 protein kinase C substrate. Fig. 5 clearly demonstrates that this protein is mainly recovered in the cytosolic fraction of MCF-7 cells. No significant labeling was observed in the nuclear and membrane fractions. As shown in Fig. 6, phosphoamino acid analysis of the $M_r$ 28,000 protein demonstrates that only serine residues are phosphorylated under TPA as well as $DiC_8$ stimulation.

**Time Course and Dose-Response Studies of the $M_r$ 28,000 Protein Phosphorylation.** Fig. 7 illustrates the effects of varying incubation times on the phosphorylation of the $M_r$ 28,000 protein, when saturating concentrations of TPA, PDBu, or $DiC_8$ were used. Results indicate that the increase in $^{32}P$ labeling was already observable after 5 min of incubation with each protein kinase C activator and reached a maximum after 30 min of cell treatment.

Effects of increasing concentrations of TPA, PDBu, and $DiC_8$ after 30-min incubation have also been explored. As shown on Fig. 8, the three activators induce a dose-dependent increase of the $M_r$ 28,000 protein phosphorylation with respective $ED_{50}$ of 5 ng/ml (8 nM), 5 ng/ml (10 nM), and 20 μg/ml (58 μM) for TPA, PDBu, and $DiC_8$.

**Further Characterization of the $M_r$ 28,000 Protein by Two-Dimensional Gel Analysis.** As it was surprising that the protein kinase C activators induced an increase in the phosphorylation of only one protein, we anticipated that a more complex protein phosphorylation pattern could be observed by using a higher resolutive fractionation technique. Comparison of the autoradiographs obtained after two-dimensional electrophoresis (Fig. 9) revealed that: (*a*) the $M_r$ 28,000 protein exists as two isoforms (*arrows a*) with pi 6.1 and 5.9. Both forms are phosphorylated by TPA while $DiC_8$ enhances the labeling of the less acidic form more markedly. (*b*) TPA but not $DiC_8$ leads to the phosphorylation of an extra set of proteins (*arrows b*) with $M_r$ around 68,000 and pi ranging from 6.3 to 5.9. (*c*) TPA, but not $DiC_8$, induces a decrease of the $^{32}P$ incorporation into a $M_r$ 54,000 protein with pi 4.5 (*arrow c*).

**Effects of Phorbol Esters and Diacylglycerol on Protein (Histone) Kinase C Activity.** The short term effects of TPA, PDBu, and $DiC_8$ on protein kinase C activity were investigated by using the histone kinase activity assay. As shown on Fig. 10*A*, the three activators clearly cause the subcellular translocation of the enzyme, probed as the drop in the cytosolic protein kinase C activity. However, the amplitude of this phenomenon appears quite different from one to another compound. TPA causes a nearly 85% decrease of the cytosolic activity upon 10 min of cell treatment while PDBu induces an approximate 45% translocation of protein kinase C. In similar conditions, $DiC_8$ induces only a 25% drop in the cytosolic activity. We have recently shown that the $DiC_8$ effect was reversible, as the

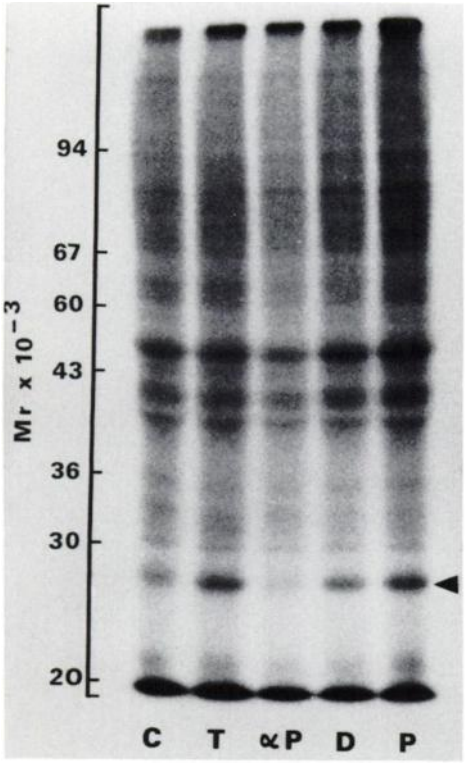


Fig. 4. Effects of protein kinase C activators on the protein phosphorylation pattern in intact MCF-7 cells. Subconfluent cells were incubated for 30 min in phosphate-free Krebs-Ringer buffer containing 50 $\mu$Ci [$^{32}$P]phosphoric acid in the absence (*C*) or in the presence of 100 ng/ml TPA (*T*), 90 ng/ml 4$\alpha$-phorbol ($\alpha P$), 43 $\mu$g/ml $DiC_8$ (*D*), or 100 ng/ml PDBu (*P*). TCA-precipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The $M_r$ values were evaluated by the use of standard protein markers.

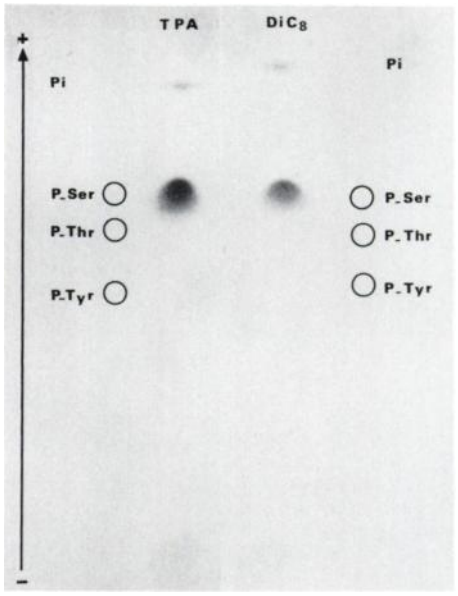


Fig. 6. Phosphoamino acid analysis of the *Mr* 28,000 phosphoprotein. Phosphoamino acid of the $M_r$ 28,000 protein from TPA- or $DiC_8$-treated cells were analyzed as indicated in "Materials and Methods." The position of standards was revealed by ninhydrin staining.

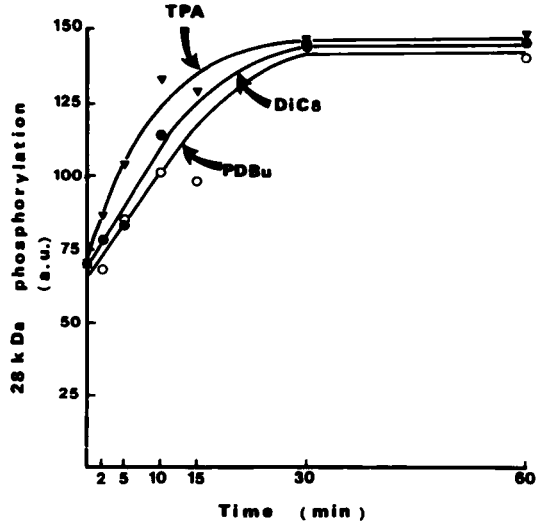


Fig. 7. Time course of protein phosphorylation induced by a saturating dose of TPA, PDBu, and $DiC_8$. Subconfluent cells were incubated in the presence of 100 ng/ml TPA (▼), 100 ng/ml PDBu (○), or 43 $\mu$g/ml $DiC_8$ (●) for various lengths of time (0–60 min). $^{32}$P incorporation into the $M_r$ 28,000 protein was evaluated by densitometric scanning of the autoradiographs. a.u., arbitrary units.

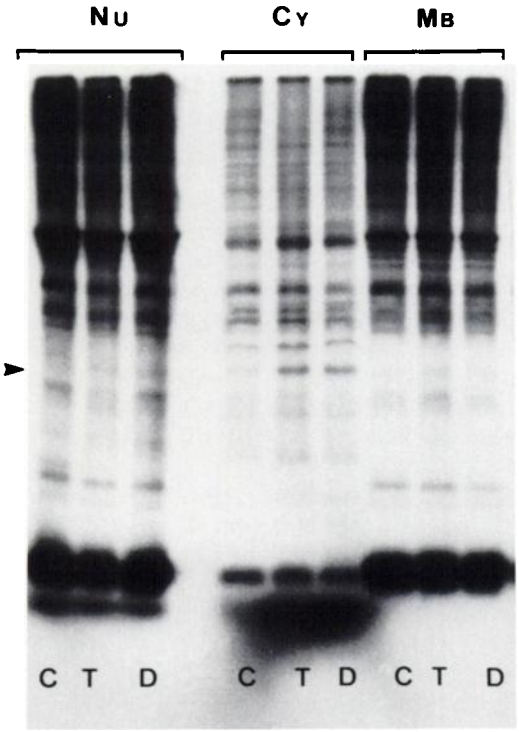


Fig. 5. Subcellular localization of the phosphoproteins. After incubation for 30 min in the absence (*C*) or in the presence of 100 ng/ml TPA (*T*) or 43 $\mu$g/ml $DiC_8$ (*D*), cells were fractionated as indicated under "Materials and Methods." The different subcellular fractions were analyzed by electrophoresis on SDS-polyacrylamide gel and autoradiography. *NU*, nuclei; *CY*, cytosol; *MB*, 105,000 g membrane fraction. *Arrow*, position of the $M_r$ 28,000 protein.

cytosolic protein kinase C activity returned to the control value after 60 min, while the phorbol ester effect appears, on the contrary, irreversible (27).

We have also investigated the effects of phorbol esters and DAG on protein kinase C when cells are treated with these compounds for much longer periods of time. As shown on Fig. 10*B*, $DiC_8$ is unable to significantly modify the cellular enzyme level upon a 72-h cell treatment, although the DAG was effective to inhibit cell proliferation after such a culture period (Fig. 1). Nearly identical results were obtained when cells were treated with phospholipase C, an enzyme known to induce the formation of diacylglycerol at the cell membrane level (data not shown). By contrast, TPA and, at a lower extent, PDBu induce the down-regulation of protein kinase C with only 10 and 55% of the initial enzyme level remaining present in cells treated with respectively TPA and PDBu.

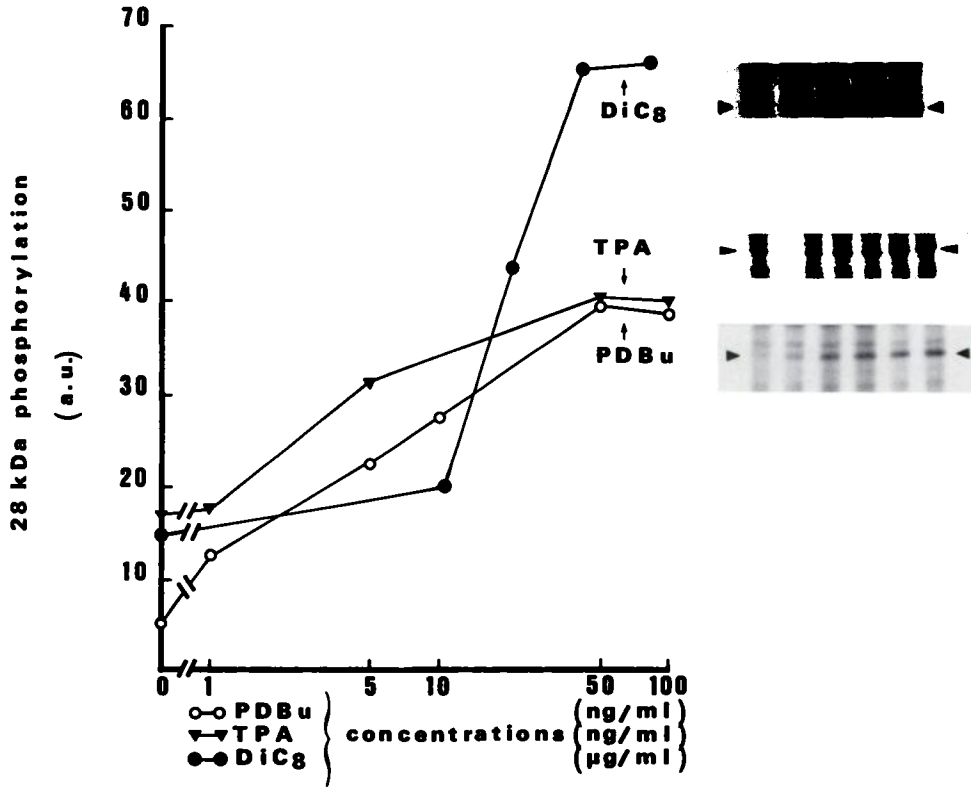


Fig. 8. Effects of increasing concentrations of TPA, PDBu, and $DiC_8$ on protein phosphorylation. Subconfluent cells were incubated for 30 min in the absence or in the presence of increasing concentrations of TPA (▼), PDBu (○), and $DiC_8$ (●). $^{32}P$ incorporation into the $M_r$ 28,000 protein was evaluated as indicated in "Materials and Methods." a.u., arbitrary units. Parts of the autoradiographs corresponding to the $M_r$ 28,000 protein are shown. The 3 dose-response curves shown were obtained from 3 separate experiments.

**Effect of Prolonged Cell Treatment with Phorbol Esters on the $M_r$ 28,000 Protein Phosphorylation.** As long-term exposure of cells to phorbol esters leads to a profound decline of the cellular protein kinase C, the question arises whether the remainder enzyme is always able to phosphorylate the $M_r$ 28,000 endogenous substrate and what is the extent of this phosphorylation. To answer this question, cells were pretreated with TPA, PDBu, and $DiC_8$ for respectively 48, 72, and 72 h. Then, cells were extensively washed and incubated for 30 min with the different protein kinase C activators. Fig. 11 clearly shows that the $M_r$ 28,000 protein is always maximally phosphorylated under TPA, PDBu, or $DiC_8$ stimulation whatever the previous cell treatment.

## DISCUSSION

We demonstrate here that permeant diacylglycerol $DiC_8$ mimics the effects of phorbol ester TPA on MCF-7 cell morphology and growth arrest. These data reinforce the hypothesis of an implication of protein kinase C in these biological cell responses and strongly suggest that this enzyme plays a negative modulatory role in the MCF-7 cell proliferation. The fact that repeated addition of $DiC_8$ was necessary to get the maximal response is very likely the consequence of the well-known rapid metabolization of DAG (28) rather than less efficiency of the physiological compound which finally produces more than 90% inhibition of cell proliferation.

Analysis of the dose-response curves obtained with $DiC_8$ for both protein phosphorylation and cell proliferation show similar profiles with respective $ED_{50}$ of 20 and 11 μg/ml. By contrast, the doses of TPA inhibiting cell growth appear much lower than those increasing the $M_r$ 28,000 protein phosphorylation (respective $ED_{50}$ values of 0.05 and 5 ng/ml). Such a discrepancy could be explained by the fact that cell growth was estimated after 5 days of TPA treatment. Because of its high lipophilicity and its inability to be metabolized, TPA could accumulate in some membrane compartments during the course of the culture period, leading to an under-estimation of the final concentrations. Alternatively, the analysis procedure that we used along the dose-response studies to assess the endogenous protein phosphorylation may not be sensitive enough to observe minor changes in $^{32}P$ labeling of the $M_r$ 28,000 protein. In any case, although the $ED_{50}$ values for both enzyme translocation (7.5 ng/ml, Reference 21) and $M_r$ 28,000 protein phosphorylation (5 ng/ml, Fig. 8) were found very close, the real implication of this protein in the biological cell response remains to be defined. In particular the link between this phosphorylation event and the protein kinase C translocation is not presently very clear as the $M_r$ 28,000 substrate is apparently a cytosolic protein. However, the real subcellular localization of the $M_r$ 28,000 phosphopeptide in intact cells can be questioned as this protein could be artifactually released from membranes or nuclei during cell homogenization. The fact that the protein kinase C redistribution induced by $DiC_8$ occurs in the range of DAG concentrations which are effective in inhibiting cell growth (27) suggests that the translocation process is very likely an absolute prerequisite for the activation of protein kinase C and its subsequent action. Nevertheless, the $DiC_8$ effect is only partial when compared to the one of TPA. That may be due to the methodology we use to assess the phenomenon. However, we have shown that the $DiC_8$ effect was only transient while the one of TPA was irreversible. Furthermore, we clearly demonstrate that, contrary to TPA, $DiC_8$ is totally unable to induce the cellular down-regulation of protein kinase C during long term treatment. On the contrary, PDBu mimics the effect of TPA although the extent of the enzyme disappearance appears lower (55% for PDBu *versus* 90% for TPA after 48–72 h treatment). Taking together these results suggests that TPA and $DiC_8$ may activate protein kinase C in a somewhat distinct manner and lead us to postulate that: (*a*) following DAG-induced translocation, protein kinase C is very rapidly released from the membrane compartment as the enzyme interaction

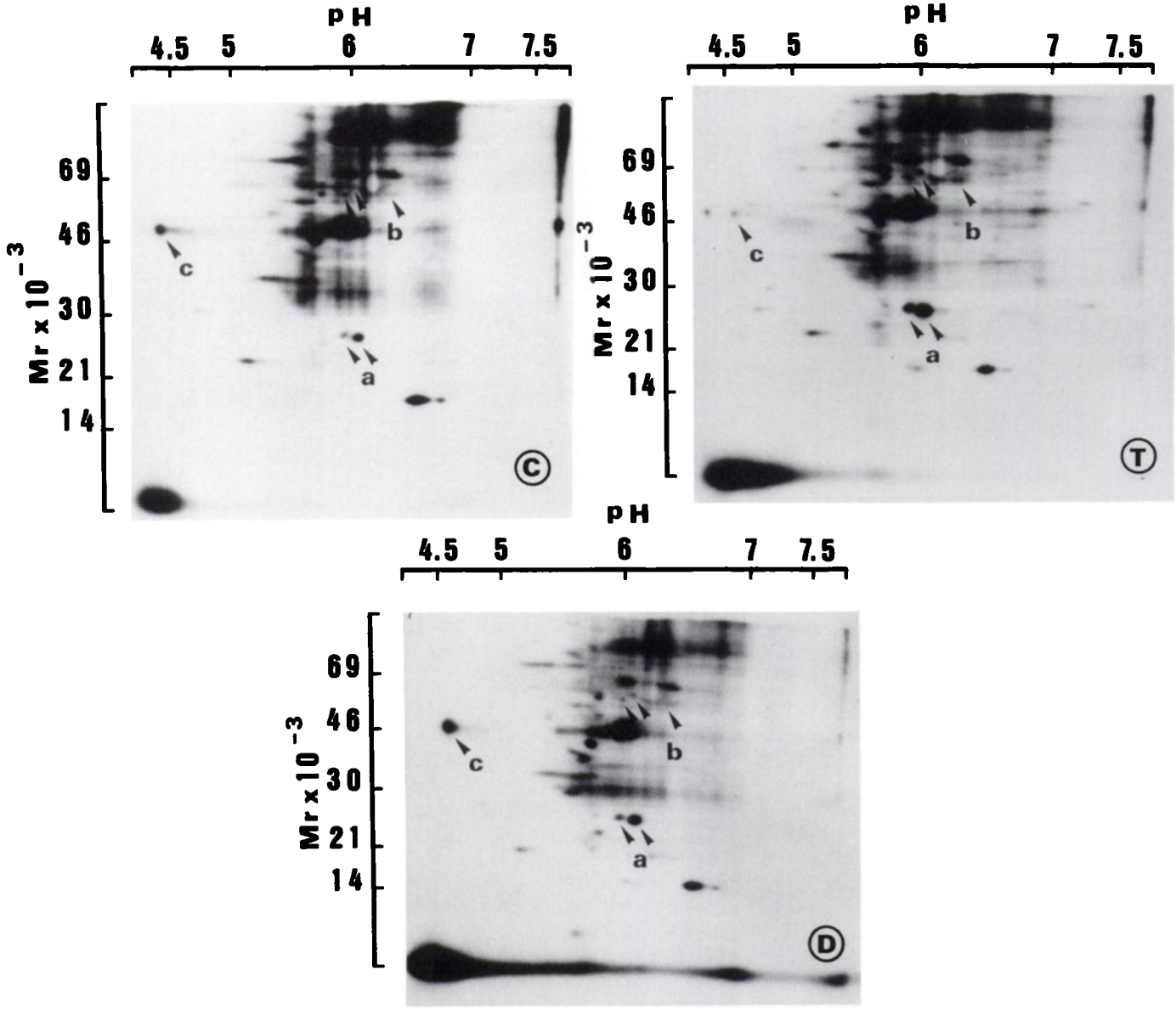


Fig. 9. Further analysis of phosphoproteins by two-dimensional electrophoresis. MCF-7 cells were incubated for 30 min in phosphate-free Krebs-Ringer buffer containing 50 $\mu$Ci [$^{32}$P]phosphoric acid in the absence (*C*) or in the presence of 100 ng/ml TPA (*T*) or 43 $\mu$g/ml $DiC_8$ (*D*). Phosphorylated proteins were extracted as described in "Materials and Methods" and were subjected to isoelectric focusing followed by SDS-polyacrylamide (12%) gel electrophoresis. *Arrows*, those proteins whose phosphorylation was affected by stimuli. Despite some variations in the nonspecific $^{32}$P background of the gels, autoradiographs shown are representative of three similar experiments.

with membranes is quite fragile; (*b*) on the contrary, the liphophilic and non-metabolized phorbol ester TPA binds the enzyme to membranes in a tight and irreversible manner; (*c*) as a consequence, DAG is unable to trigger the enzyme processing that occurs at the membrane level, during TPA treatment.

Alternatively, the differential effects of TPA and $DiC_8$ on protein kinase C activity could be due to the ability of the two activators to bind and activate different isozymic forms of the enzyme (29–31). Such a hypothesis is further sustained by our finding that distinct protein phosphorylation patterns can be observed under TPA and $DiC_8$ stimulation when using a highly resolutive two-dimensional electrophoresis. However, these latter data could also be due to differences in the content and localization of protein kinase C after stimulation by the two enzyme activators. Nevertheless such discrepancies in the protein phosphorylations induced by TPA and DAG have been already reported in other cell types (28, 32) and could explain that in some systems, TPA and DAG may cause various biological responses (28, 32–34).

Furthermore, the acute differences that we show between the effects of TPA and DAG on protein kinase C processing may be of interest to explain the fact that contrary to DAG, TPA has been reported as a potent tumor promoter (1, 2).

Finally, our data also demonstrate that the low percentage of protein kinase C activity remaining after long exposure to TPA are able to produce a maximal phosphorylation of the $M_r$ 28,000 protein. That could explain the continuous effect of the phorbol ester on cell growth arrest when the enzyme level is progressively going down. Further studies are needed to determine whether the $M_r$ 28,000 protein is a substrate for a sub-class of protein kinase C not affected by the TPA treatment or if MCF-7 cells contain a large excess of protein kinase C activity not involved in cellular response. In any case, the cell growth arrest caused by TPA and $DiC_8$ does not seem linked to disappearance of protein kinase C as the permeant diacylglycerol is effective in inhibiting cell proliferation without affecting the cellular content of the enzyme.

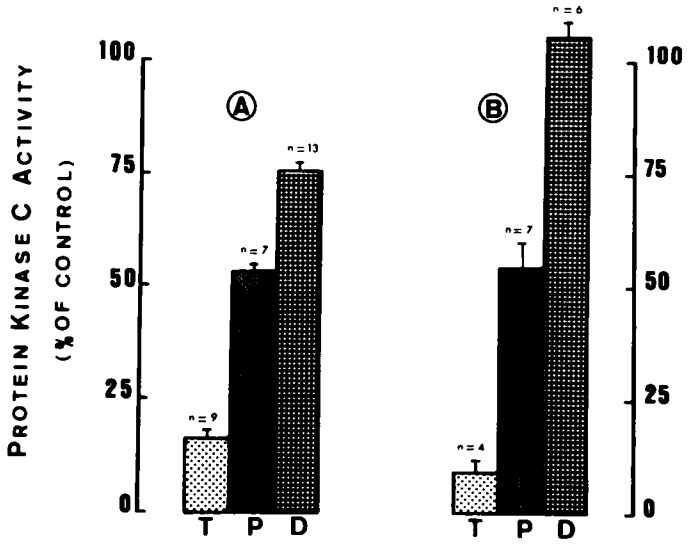


Fig. 10. Effects of TPA, PDBu, and $DiC_8$ on protein kinase C activity. *A*, MCF-7 cells were treated for 10 min with 100 ng/ml TPA (*T*), 100 ng/ml PDBu (*P*), or 43 μg/ml $DiC_8$ (*D*). Protein kinase activity was measured in cytosolic fractions following DEAE-cellulose chromatography. Data are the means ± SE of 7 to 13 different experiments. *B*, MCF-7 cells were treated for 2 days with 100 ng/ml TPA (*T*), or 3 days with 100 ng/ml PDBu (*P*), or 43 μg/ml $DiC_8$ (*D*). $DiC_8$ was added three times a day. Protein kinase activity was measured in the 0.5% NP-40 extract of the cell homogenate following DEAE-cellulose chromatography. Data are the means ± SE of 4 to 7 different experiments.

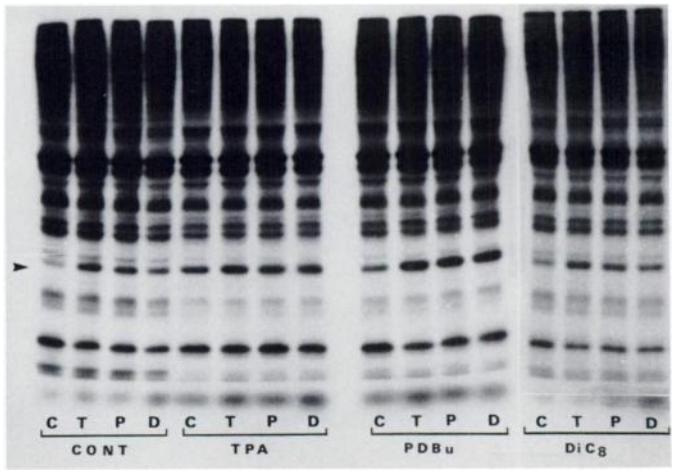


Fig. 11. Phosphorylation of the $M_r$ 28,000 protein after long exposure of the cells with TPA, PDBu, and $DiC_8$. Subconfluent cells were cultured in the absence (*Cont*) or in the presence of 100 ng/ml TPA (for 2 days), 100 ng/ml PDBu (3 days), or 43 μg/ml $DiC_8$ (3 days). $DiC_8$ was added three times a day. Cells were then repeatedly washed in 10% fetal calf serum to eliminate stimuli. After 2-h exposure with 50 μCi [$^{32}$P]phosphoric acid, cells were incubated for 30 min without (*C*) or with 100 ng/ml TPA (*T*), 100 ng/ml PDBu (*P*), or 43 μg/ml $DiC_8$ (*D*). The labeling of the $M_r$ 28,000 protein observed in TPA-pretreated cells (*TPA*) incubated without stimuli (*C*) was very likely due to small amounts of the highly lipophilic phorbol ester remaining after cell washing.

In conclusion our results suggest that protein kinase C could play a negative modulatory role in MCF-7 cell proliferation. While the protein kinase C translocation appears as a possible prerequisite in the biological cell response, *i.e.*, cell growth inhibition, the enzyme down-regulation observed after TPA action does not. Our study demonstrates that the $M_r$ 28,000 protein is a specific marker of protein kinase C activation in MCF-7 cells. The nature and function of this endogenous substrate remain to be defined.

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