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

Marc Issandou, Francis Bayard, and Jean-Marie Darbon

ABSTRACT

We have investigated the effects of phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) and permeant diacylglycerol 1,2-dioctanoyl-sn-glycerol (DiC8) on MCF-7 cell proliferation and protein kinase C activity. DiC8 mimics the effects of TPA on both cell morphology and proliferation, with an ED50 value of 11 µg/ml for cell growth inhibition. As with TPA and phorbol 12,13-dibutyrate, DiC8 enhances the degree of phosphorylation of an endogenous M, 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 ED50 observed are 5 ng/ml and 20 µg/ml, respectively, for phorbol esters and DiC8. The M, 28,000 protein is found in the cytosolic fraction and is phosphorylated on serine residues by both TPA and DiC8. Further characterization of the phosphorylated proteins using a highly resolving 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 DiC8 stimulation. Contrary to TPA, DiC8 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 DiC8, while only 10% of the initial protein kinase C level remains present in cells treated with TPA for 48 h. However, this remaining enzymatic activity is sufficient to induce the phosphorylation of the M, 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 DiC8 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 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++ and phospholipid-dependent protein kinase C (7–9), which most probably represents the high affinity phorbol ester receptor in target cells (8–10). 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-me-

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1 Supported by Institut National de la Santé et de la Recherche Médicale and U168, Department of Endocrinology, CHU Rangueil, Université Paul Sabatier, 31054 Toulouse Cedex, France.

2 To whom requests for reprints should be addressed.

3 The abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; PDBu, phorbol 12,13-dibutyrate; DAG, 1,2-diacylglycerol; DiC8, 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.
TPA exerts a slight cytotoxic effect on cells, leading to a small earlier than phorbol esters do, as a partial inhibition of MCF-7 cell growth. DiCg as well as TPA induce a dose-dependent decrease in the cell number after 4 days of treatment. Moreover, at the concentration of 5 ng/ml used in this study, inhibition. Paradoxically, DiCg reduces the rate of cell growth.

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 DiCg 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 DiCg is observed when this compound is added to the culture dishes into cold PBS and then sonicated. After incubation at 4°C for 45 min it was centrifuged for 1 h at 105,000 x g. The supernatant was considered as the cytosolic fraction. The cell lysate was centrifuged for 1 h at 105,000 x 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 x g. The supernatant recovered was used as the 0.5% NP-40 extract of the particulate fraction.

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 7.5, containing 10% glycerol, 2 mM EDTA, 6 mM ß-mercaptoethanol, and 100 mM sodium fluoride. Nuclear fraction was obtained by centrifugation of the cell lysate at 900 x g for 30 min and the pellet was solubilized in electrophoresis sample buffer. The nonnuclear fraction was centrifuged for 60 min at 105,000 x g. The resulting pellet was considered as the 105,000 x 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 mM sucrose, 2 mM EDTA, 2 mM EGTA, 100 μg/ml leupeptin, and 5 mM ß-mercaptoethanol (buffer A). After incubation at 4°C for 45 min it was centrifuged for 1 h at 105,000 x 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 x 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 x 10^6 cells was applied at 105,000 x g. The supernatant was used as the cytosolic fraction. Nuclear fraction was obtained by centrifugation of the cell lysate at 105,000 x g for 30 min and the pellet was solubilized in electrophoresis sample buffer. The nonnuclear fraction was centrifuged for 60 min at 105,000 x g. The supernatant was considered as the cytosolic fraction.

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

Fig. 2 illustrates the changes in MCF-7 cell morphology occurring upon treatment with phorbol esters and DAG. Control cultures show typical epithelial 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 DiCg 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_28,000 protein. We show treatment. The ED_{50} observed were 11 μg/ml (32 μM) and 0.05 ng/ml (0.08 nm), respectively, for DiCg and TPA.

Fig. 3 illustrates the changes in MCF-7 cell morphology occurring upon treatment with phorbol esters and DAG. Control cultures show typical epithelial 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 DiCg induces similar changes. Cells became rounded and spread out, and some giant cells are observable.
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Fig. 3. Effects of TPA and DiC₈ 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 DiC₈. Phase-contrast microscopy was performed at x100 magnification.

now that the permeant diacylglycerol DiC₈ mimics the effect of phorbol esters on this protein phosphorylation. On the contrary, the inactive tumor promoter 4α-phorbol is unable to induce the Mᵇ 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 Mᵇ 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 Mᵇ 28,000 protein demonstrates that only serine residues are phosphorylated under TPA as well as DiC₈ stimulation.

Time Course and Dose-Response Studies of the Mᵇ 28,000 Protein Phosphorylation. Fig. 7 illustrates the effects of varying incubation times on the phosphorylation of the Mᵇ 28,000 protein, when saturating concentrations of TPA, PDBu, or DiC₈ were used. Results indicate that the increase in ³²P 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 DiC₈ after 30-min incubation have also been explored. As shown on Fig. 8, the three activators induce a dose-dependent increase of the Mᵇ 28,000 protein phosphorylation with respective ED₅₀ of 5 ng/ml (8 nM), 5 ng/ml (10 nM), and 20 μg/ml (58 μM) for TPA, PDBu, and DiC₈.

Further Characterization of the Mᵇ 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 Mᵇ 28,000 protein exists as two isoforms (arrows a) with pi 6.1 and 5.9. Both forms are phosphorylated by TPA while DiC₈ enhances the labeling of the less acidic form more markedly. (b) TPA but not DiC₈ leads to the phosphorylation of an extra set of proteins (arrows b) with Mᵇ around 68,000 and pi ranging from 6.3 to 5.9. (c) TPA, but not DiC₈, induces a decrease of the ³²P incorporation into a Mᵇ 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 DiC₈ on protein kinase C activity were investigated by using the histone kinase activity assay. As shown on Fig. 10, 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, DiC₈ induces only a 25% drop in the cytosolic activity. We have recently shown that the DiC₈ effect was reversible, as the
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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 μCi [32P]phosphoric acid in the absence (C) or in the presence of 100 ng/ml TPA (T), 90 ng/ml 4α-phorbol (αP), 43 μg/ml DiC₈ (D), or 100 ng/ml PDBu (P). TCA-precipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The Mᵦ values were evaluated by the use of standard protein markers.

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 μg/ml DiC₈ (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 Mᵦ 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. 1(B), DiC₈ 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. 6. Phosphoamino acid analysis of the Mᵦ 28,000 phosphoprotein. Phosphoamino acid of the Mᵦ 28,000 protein from TPA- or DiC₈-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 DiC₈. Subconfluent cells were incubated in the presence of 100 ng/ml TPA (△), 100 ng/ml PDBu (○), or 43 μg/ml DiC₈ (●) for various lengths of time (0–60 min). 32P incorporation into the Mᵦ 28,000 protein was evaluated by densitometric scanning of the autoradiographs. a.u., arbitrary units.
from 3 separate experiments. The dose-response curves shown were obtained of the autoradiographs corresponding to the M, 28,000 protein are shown. The 3 incorporations of TPA, PDBu, and DiC8 on protein phosphorylation. Subconfluent cells were incubated for 30 min in the absence or in the presence of increasing concentrations of TPA (•), PDBu (○), and DiC8 (□). \( ^{32}P \) incorporation into the M, 28,000 protein was evaluated as indicated in "Materials and Methods." H.H., arbitrary units. Parts of the autoradiographs corresponding to the M, 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, 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, 28,000 endogenous substrate and what is the extent of this phosphorylation. To answer this question, cells were pretreated with TPA, PDBu, and DiC8 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, 28,000 protein is always maximally phosphorylated under TPA, PDBu, or DiC8 stimulation whatever the previous cell treatment.

DISCUSSION

We demonstrate here that permeant diacylglycerol DiC8 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 DiC8 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 DiC8 for both protein phosphorylation and cell proliferation show similar profiles with respective ED\(_{50}\) of 20 and 11 \( \mu \)g/ml. By contrast, the doses of TPA inhibiting cell growth appear much lower than those increasing the M, 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, 28,000 protein. In any case, although the ED\(_{50}\) values for both enzyme translocation (7.5 ng/ml, Reference 21) and M, 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, 28,000 substrate is apparently a cytosolic protein. However, the real subcellular localization of the M, 28,000 phosphopeptide in intact cells can be questioned as this protein could be artifactualy released from membranes or nuclei during cell homogenization. The fact that the protein kinase C redistribution induced by DiC8 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 DiC8 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 DiC8 effect was only transient while the one of TPA was irreversible. Furthermore, we clearly demonstrate that, contrary to TPA, DiC8 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 DiC8 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
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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 μCi [³²P]phosphoric acid in the absence (C) or in the presence of 100 ng/ml TPA (T) or 43 ng/ml DiC₈ (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 ³²P background of the gels, autoradiographs shown are representative of three similar experiments.

with membranes is quite fragile; (b) on the contrary, the lipophilic 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 DiC₈ 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 DiC₈ 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, 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, 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 DiC₈ 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.
was added three times a day. Protein kinase activity was measured in the 0.5% (D). The labeling of the M, 28,000 protein observed in TPA-pretreated cells (TPA) without (C) or with 100 ng/ml TPA (T), 100 ng/ml PDBu (P), or 43 ng/ml DiC8 exposure with 50 nCi ["Pjphosphoric acid, cells were incubated for 30 min days), or 43 Mg/ml DiC8 (3 days). DiCs was added three times a day. Cells were

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, 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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