Involvement of Protein Kinase C in Phorbol Ester-induced Sensitization of HeLa Cells to cis-Diamminedichloroplatinum(II)*

(Received for publication, September 5, 1989)

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We have investigated the effect of tumor promoting phorbol esters on the antiproliferative actions of several antitumor agents. Pretreatment of HeLa cells with 12-O-tetradecanoylphorbol 13-acetate (TPA) or phorbol 12,13-dibutyrate (PDBu) caused a significant (9-fold) increase in cellular sensitivity to cis-diamminedichloroplatinum(II) (CP). TPA also sensitized HeLa cells to melphanal (2.5-fold) but had no effect on the antiproliferative activity of bleomycin, doxorubicin, vincristine, or mitomycin C. The sensitization of HeLa cells by TPA was concentration-dependent up to 1 μM and paralleled the activation of protein kinase C by TPA measured in vitro. The maximum stimulation of protein kinase C (6-fold) was observed with 10 nM TPA. 4α-Phorbol 12,13-didecanoate neither activated protein kinase C nor sensitized HeLa cells to CP. 4-O-Methyl-TPA, which does not affect cell cycle distribution of HeLa cells, also sensitized these cells to CP by 6-fold and activated protein kinase C by 3-fold. Inhibitors of protein kinase C, such as palmitoylcarnitine and sphingosine, antagonized PDBu-induced sensitization of HeLa cells to CP. The maximum sensitization of HeLa cells to CP required prolonged pretreatment (≥24 h) with phorbol esters but could not be explained by down-regulation of protein kinase C. For example, 4-O-methyl-TPA caused no down-regulation of protein kinase C. Moreover, TPA caused substantial down-regulation of protein kinase C (1% of control) in A-253 cells but failed to sensitize A-253 cells to CP. TPA (100 nM), however, activated protein kinase C in A-253 cells by 5.5-fold. Therefore, activation of protein kinase C by TPA appears to be necessary but not sufficient for cellular sensitization to CP. The sensitization of HeLa cells by TPA was associated with a concentration- and time-dependent increase in cellular platinum content. The protein synthesis inhibitor cycloheximide (10 μg/ml) blocked sensitization of HeLa cells to CP as well as the increase in platinum content caused by a 24-h pretreatment with PDBu.

The calcium- and phospholipid-dependent protein kinase (protein kinase C) plays a pivotal role in transmembrane signaling (1). It mediates a wide variety of responses produced by growth factors, hormones, and mitogens and regulates many cellular processes including cell growth, differentiation, secretin, receptor interaction, and gene expression (1). Diacylglycerol (DAG), transiently generated by the hydrolysis of inositol phospholipids, activates protein kinase C physiologically by decreasing its cofactor requirements (2). The enzyme consists of multiple isoforms including α, β, γ, and δ (1, 3, 4). Protein kinase C is considered to be the primary receptor for tumor promoting phorbol esters (5, 6), which stimulate the enzyme by a mechanism similar to DAG (7) and induce a rapid translocation of the cytosolic enzyme to the plasma membrane (8). Extended exposure to these phorbol esters leads to proteolytic degradation or down-regulation of protein kinase C (9).

Phorbol esters, such as 12-O-tetradecanoylphorbol 13-acetate (TPA), elicit pleiotropic responses in different cell types. They both inhibit and stimulate cell proliferation and differentiation (10-13), alter gene expression (14, 15), and affect protein synthesis (16). They have also been reported to reduce cellular sensitivity to some anticancer agents (17-19). The increase in protein kinase C activity by phorbol esters has been reported to induce the “multiple drug-resistant” phenotype in human breast cancer cells (19). Although it is generally believed that the effects of phorbol esters are mediated by protein kinase C (14), the mechanism responsible for the reduced sensitivity to these anticancer drugs is not clear. It has been proposed that the transient protection of human KB carcinoma cells afforded by TPA against the toxic effects of etoposide, vincristine, methotrexate, and mitoxantrone was due to decreased drug accumulation (17). This implies protein kinase C level may regulate the cellular accumulation of some anticancer drugs. Others (18) have suggested that phorbol esters can affect drug sensitivity by a mechanism independent of protein kinase C. For example, the increased survival of murine cells to methotrexate caused by TPA has been linked to the effects of TPA on cell cycle progression (18).

cis-Diamminedichloroplatinum(II) (CP) is one of the most widely used antineoplastic agents, often in combination with other anticancer drugs. Its cytotoxicity is thought to be caused by covalent interactions with chromosomal DNA (20). How the drug is taken up by cells and delivered to the nucleus is not understood. Nevertheless, penuclear events, such as passage of CP through plasma membrane, its transport to the nucleus, and effects of membrane and cytosolic components on CP, are likely to influence its ultimate biologic action (21--

* This work was supported in part by National Institutes of Health Grant CA-43917 and Grants 100-R061 and 100-R031 from the Bristol-Meyers Company. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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§ The abbreviations used are: DAG, diacylglycerol; TPA, 12-O-tetradecanoylphorbol 13-acetate; PDBu, phorbol 12,13-dibutyrate; CP, cis-diamminedichloroplatinum(II); DACH, 1,2-diaminocyclohexane platinum sulfate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; HEPES, N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Me2SO, dimethyl sulfoxide.
Because tumor-promoting phorbol esters are key signal transducers, act at plasma membrane, and have been shown to reduce the cellular accumulation of some anticancer drugs, we began examining their effects on cellular sensitivity to CP.

In the course of our investigation, Hofmann et al. (24, 25) reported that inhibitors of protein kinase C, such as quercetin or either lipid analogue BM 43440, either independently or in combination with TPA, augmented the sensitivity of Walker carcinoma cells to CP. These authors postulated that depletion of protein kinase C either by the inhibitors or due to extended exposure to TPA was responsible for enhanced antiproliferative activity of CP. In contrast, Nobata et al. (26) failed to demonstrate any synergism between CP and other lipid analogues in human ovarian adenocarcinoma cells. Since these inhibitors of protein kinase C also affect other enzymes (24, 25), the mechanism underlying the sensitization to CP remains unidentified.

In the present report we have shown that phorbol esters cause significant sensitization of HeLa cells to CP and we have explored the possible mechanisms responsible for the changes in cellular sensitivity to CP.

**EXPERIMENTAL PROCEDURES**

**Materials**—Calf thymus histone (III-S), cycloheximide, TPA, PDBu, 4-O-methyl-TPA, diolein, d-sphingosine, palmitoylcarnitine, MITT, and melanophan were purchased from Sigma. Bleomycin and CP were obtained from Bristol-Myers Co. Doxorubicin was from Adria Laboratories Inc., Columbus, OH, and vincristine was obtained from Eli Lilly and Co. 1,2-Diaminocyclohexane platinum sulfate (DACH) was generously provided by Dr. Miles P. Hacker of the Department of Pharmacology, University of Vermont, Burlington, VT. Phosphatidylserine was from Supelco, Bellefonte, PA. [γ-32P]ATP was purchased from Amersham Corp., and DES2 was obtained from Whatman Lab Sales Inc., Hillisboro, OR. [S-methyl-3H]Bleomycin A2 was obtained from Du Pont-New England Nuclear.

**Cell Culture**—Human cervical carcinoma (HeLa) cells and human head and neck carcinoma (A-253) cells were maintained as monolayer cultures in Dulbecco’s modified minimal essential medium (GIBCO) and McCoy’s 5a medium (GIBCO), respectively, supplemented with 2 mM l-glutamine, 10% fetal bovine serum (HyClone, Logan, UT), 25 mM HEPES buffer (pH 7.4), penicillin (100 units/ml), and streptomycin (100 μg/ml) and kept in a humidified incubator at 37 °C with 95% air and 5% CO2.

**Cell Survival Assay**—Exponentially growing cells were plated in microtiter plates (Costar, Cambridge, MA) and incubated at 37 °C in 5% CO2. Phorbol esters dissolved in MeSO were diluted in the growth medium and were added to the plate as described in the text. Control cells received an equivalent amount of the solvent (<0.1%). Unless otherwise mentioned, cells were preincubated with the compounds for 24 h and then incubated with various concentrations of anticancer drugs. Following a 48–72 h exposure to the anticancer drug with or without phorbol esters, the number of viable cells was determined using MTT as described by Carmichael et al. (27). Control values were always calculated for each individual experiment based upon the results obtained in the absence of CP but in the presence of solvent (MeSO4 or phorbol ester).

**Isolation of Protein Kinase C**—Protein kinase C was partially purified from HeLa cells or A-253 cells using DES2-cellulose chromatography as described previously (28). Briefly, cells were harvested and cell pellets were washed with ice-cold phosphate-buffered saline without any divalent cations. All subsequent procedures were carried out at 0–4 °C. Cells were homogenized in ice-cold Tris-HCl buffer, 0.25 M sucrose, 2 mM EDTA, 5 mM EGTA, 10 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 0.01% leupeptin (buffer A). The homogenate was centrifuged at 100,000 × g for 1 h. The cytosolic fraction was subjected to DE52-cellulose chromatography and eluted with 0.1 M NaCl in 20 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 0.5 mM EDTA, 0.5 mM EGTA, and 10 mM β-mercaptoethanol. The pellet was resuspended in buffer A and membrane-bound protein kinase C was extracted from the pellet by stirring for 30 min with 0.5% Triton X-100 and centrifuged at 100,000 × g for 1 h. The supernatant was subjected to DE52 chromatography as mentioned above.

**Assay for Enzyme Activity**—The enzyme activity was determined by measuring the incorporation of 32P from [γ-32P]ATP into histone III-S (28). The reaction mixture (200 μl) contained the enzyme preparation (1–3 μg of protein), 20 mM Tris-HCl (pH 7.5), 400 μg of histone, 15 μM [γ-32P]ATP, 10 mM magnesium acetate, and 0.5 mM CaCl2, with or without 20 μg/ml phosphatidylserine, and 2 μg/ml DAG. Free Ca2+ concentration was calculated to be 0.4 μM by the method of Fabiato and Fabiato (29). Calcium-dependent protein kinase C activity was determined by subtracting the activity determined in the absence of phosphatidylserine and DAG from that in the presence of phosphatidylserine and DAG. In the presence of either Ca2+ or phospholipid alone the enzyme activity was less than 5% of the activity when both were present. To determine the activation of protein kinase C by phorbol esters in the in vitro assay, we used 100 nM phorbol esters (or as indicated in the text) and 0.5 mM EGTA instead of DAG and CaCl2. All reactions were incubated at 30 °C for 10 min and terminated with 1 ml of ice-cold 25% trichloroacetic acid. The assay was linear with regard to both time and amount of protein. The precipitated protein was washed 4 times with 25% trichloroacetic acid and counted in a liquid scintillation counter. Protein was determined by the method of Bradford (30) using bovine serum albumin as a standard.

One unit of protein kinase C activity is defined as that amount of enzyme which catalyzes the transfer of 1 pmol of phosphate from ATP to histone per min at 37 °C.

**Assessment of Cellular Platinum Content**—Cells in logarithmic phase of growth were pretreated for 24 h with varying concentrations of TPA. CP (0.75 μM) was then added and cells were incubated for an additional 24 h. In other studies, cells were pretreated with 100 nM PDBu or MeSO (0.05%) for 1–24 h in the presence or absence of 10 μg/ml cycloheximide. Cells were then exposed to 10 μM CP for 2 h. In all experiments, cells were washed twice with ice-cold phosphate-buffered saline, harvested using a solution of 0.05% trypsin and 0.5 mM EDTA and centrifuged; the cell pellets were washed 6 times in ice-cold phosphate-buffered saline and frozen at –70 °C until analyzed. Cell-associated platinum level was measured by flameless atomic absorption spectroscopy as described previously (31). Briefly, platinum from a 15-μl sample injection volume was atomized from the walls of pyrolytically coated graphite tubes. A Perkin-Elmer model 2380 atomic absorption spectrophotometer was used in conjunction with a Perkin-Elmer model 400 graphite furnace to measure the absolute mass of platinum in the cell samples. Each measurement was made in triplicate in three independent experiments.

**Assessment of Cellular Bleomycin A2 Content**—Exponentially growing HeLa cells were pretreated with 16 nM TPA for 24 h and then incubated with 1.5 μM [3H]bleomycin A2 for an additional 3 h. Cellular bleomycin A2 content was measured as described previously (32).

**RESULTS**

The Effect of TPA on the Antiproliferative Activity of Anticancer Agents—We investigated the effect of TPA on the antiproliferative activity of cis-diaminedichloroplatinum(II) (CP) in human cervical carcinoma (HeLa) and human head and neck carcinoma (A-253) cells. In the absence of TPA (100 nM), HeLa and A-253 cells exhibited similar concentration response profiles to CP (Fig. 1). The survival curve of HeLa cells seen with CP was shifted to the left by a 24-h pretreatment with TPA (100 nM), lowering the concentrations of CP required to inhibit cellular proliferation by 50% (IC50) and 90% (IC90) approximately 9- and 6-fold, respectively (Fig. 1A). In contrast, TPA pretreatment (0.1 μM) had no effect on the sensitivity of A-253 cells to CP (Fig. 1B).

Fig. 2 shows the effect of varying concentrations of TPA on the antiproliferative activity of CP. As little as 0.1 nM TPA, which alone did not affect HeLa cell proliferation, reduced the IC50 of HeLa cells for CP from 1.27 to 0.4 μM. The maximum sensitization was achieved with 1 nM TPA; this concentration of TPA alone inhibited cellular proliferation by 32% (data not shown).

We next examined the importance of duration of exposure to TPA on the sensitization of HeLa cells to CP. We used PDBu in these studies because it has better solubility properties than TPA in growth medium and was less likely to be
Cellular Sensitization to cis-Platinum by Phorbol Esters

The effect of duration of exposure to PDBu on the sensitivity of HeLa cells to CP

HeLa cells were pretreated with either PDBu (100 nM) or Me$_2$SO (0.05%) for various periods of time at 37 °C. Cells were then exposed to various concentrations of CP for either 72 or 2 h. Time of CP addition was considered as 0 h. The antiproliferative activity of CP was determined as described under “Experimental Procedures.” Wash indicates PDBu was removed at the time CP was added (0 h).

<table>
<thead>
<tr>
<th>Addition of PDBu</th>
<th>Wash</th>
<th>Length of exposure to CP</th>
<th>Sensitization$^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>h</td>
<td>h</td>
<td></td>
<td>-fold</td>
</tr>
<tr>
<td>-0.25</td>
<td>+</td>
<td>72</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>-2.0</td>
<td>+</td>
<td>72</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>-6.0</td>
<td>+</td>
<td>72</td>
<td>1.9 ± 0.04</td>
</tr>
<tr>
<td>-24.0</td>
<td>+</td>
<td>72</td>
<td>3.9 ± 0.3</td>
</tr>
<tr>
<td>-24.0</td>
<td>-</td>
<td>72</td>
<td>8.9 ± 0.4</td>
</tr>
<tr>
<td>-1.0</td>
<td>-</td>
<td>2</td>
<td>1.5 ± 0.4</td>
</tr>
<tr>
<td>-24.0</td>
<td>-</td>
<td>2</td>
<td>4.9 ± 1.2</td>
</tr>
</tbody>
</table>

$^*$The -fold sensitization was calculated as the ratio of IC$_{50}$ of HeLa cells for CP in the presence of solvent Me$_2$SO to that in the presence of PDBu (100 nM).

Fig. 1. Effect of TPA on the antiproliferative activity of CP. HeLa cells (A) or A-253 cells (B) were pretreated with either the solvent Me$_2$SO (0) or 100 nM TPA (●) for 24 h and the antiproliferative activity of CP was determined as described under “Experimental Procedures.” Control values (100%) were based upon the results obtained in the absence of any drug but in the presence of Me$_2$SO (○) or TPA (●). Each symbol represents the mean value of four determinations from a single microtiter plate. The S.D. was ±15%. These results are representative of 10 (A) or 3 (B) separate experiments.

Fig. 2. Effect of varying concentrations of phorbol esters on the antiproliferative activity of CP. The concentration of CP required to inhibit cellular proliferation by 50% (IC$_{50}$) was determined by MTT assay after pretreatment of HeLa cells with indicated concentrations of TPA (cross-hatched bars), 4-O-methyl-TPA (solid bars), or 4α-phorbol 12,13-didecanoate (hatched bars) for 24 h. The results are representative of two or more experiments.

Fig. 3. Effect of TPA on the antiproliferative activity of anticancer agents. HeLa cells were pretreated with either the solvent Me$_2$SO (○) or 16 nM TPA (●) for 24 h. We then added various concentrations of DACH (A), melphalan (B), mitomycin C (C), doxorubicin, bleomycin, and vincristine for 72 h later the cell number was determined by MTT assay as described under “Experimental Procedures.” Each symbol represents the mean value of four or more determinations, and the S.D. was ±15% of the mean.

The effect of 4-O-methyl-TPA on CP sensitivity. This non-tumor-promoting but hyperplasiogenic phorbol ester, which has previously been shown to lack any effect on HeLa cell cycle parameters (33), caused a concentration-dependent enhancement of CP sensitivity (Fig. 2). The concentration of 4-O-methyl-TPA required to induce maximum sensitization was...
Phorbol esters were 35 ± 1.7 and 25.2 ± 0.2 pmol/min/mg, respectively. The presence of varying concentrations of TPA (Fig. 4). The activity of 4α-phorbol 12,13-didecanoate was incapable of stimulating protein kinase C as well as sensitizing HeLa cells to CP. Thus, our results suggest that sensitization to CP is not linked to perturbation of cell cycle parameters because even 1 μM 4-0-methyl-TPA does not induce any alteration in cell cycle distribution of HeLa cells (33). The biologically inactive phorbol ester 4α-phorbol 12,13-didecanoate, was ineffective in sensitizing HeLa cells to CP even at 500 nM (Fig. 2).

Relationship between Protein Kinase C Activation and CP Sensitization—To establish whether protein kinase C activation is linked to the enhancement of antiproliferative activity of CP, we measured protein kinase C activity in vitro in the presence of varying concentrations of TPA (Fig. 4). The maximum stimulation of protein kinase C was achieved with 10 nM TPA. Thus, the concentration dependences for the increase in protein kinase C activity and the decrease in IC50 by TPA were similar (Fig. 2 versus Fig. 4).

We also compared the ability of TPA analogues to activate protein kinase C and sensitize HeLa cells to CP. As shown in Table II, PDBu (100 nM) was as effective as TPA (100 nM) in stimulating protein kinase C (6-fold) and sensitizing HeLa cells to CP (9-fold). 4-0-Methyl-TPA (100 nM) caused a 6-fold sensitization to CP and also enhanced protein kinase C activity 3-fold. 4α-Phorbol 12,13-didecanoate was incapable of stimulating protein kinase C as well as sensitizing HeLa cells to CP. Thus, our results suggest that sensitization to CP is related to stimulation of protein kinase C activity. Protein kinase C from A-253 cells, however, was also stimulated significantly by 100 nM TPA (5.5-fold) in the in vitro assay, although the sensitivity of these cells to CP was unaffected by TPA (Table II; Fig. 1B).

Effect of Protein Kinase C Inhibitors on Cellular Sensitivity to CP—We examined the ability of protein kinase C inhibitors such as sphingosine (34), palmitoylcarnitine (35), and staurosporine (36) to reverse TPA-induced sensitization of HeLa cells to CP. Both palmitoylcarnitine (10 μM) (Fig. 5A) and sphingosine (10 μM) (Fig. 5D) antagonized TPA-induced sensitization of HeLa cells to CP. Staurosporine (100 nM) had little effect in phorbol ester-induced sensitization of HeLa cells to CP (data not shown). At the concentrations used in our study all the inhibitors by themselves reduced cellular proliferation and caused a modest increase in cellular sensitivity to CP (Fig. 5).

Relationship between Protein Kinase C Down-regulation and CP Sensitization—Since long-term exposure of cells to TPA is known to down-regulate protein kinase C, we examined whether depletion of protein kinase C was related to CP sensitization. As shown in Table III, exposure of HeLa cells to TPA (100 nM) for 24 h caused significant down regulation of protein kinase C (5% of control). In contrast, PDBu (100 nM) led to only a 25% down-regulation of protein kinase C. 4-0-Methyl-TPA (100 nM) caused no down-regulation of protein kinase C. Therefore, there was no correlation between protein kinase C down-regulation and the ability of a phorbol ester to sensitize cells to CP.

![Graph showing the effect of varying concentrations of TPA on the activation of protein kinase C in HeLa cells.](http://example.com/graph1.png)

**FIG. 4.** Effect of varying concentrations of TPA on the activation of protein kinase C in HeLa cells. Protein kinase C activity was determined in vitro in the presence of various concentrations of TPA as described under "Experimental Procedures." The results are representative of two or more experiments.

**TABLE II**

<table>
<thead>
<tr>
<th>Cells</th>
<th>Additions</th>
<th>Sensitization of CP</th>
<th>Stimulation of protein kinase C activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>TPA</td>
<td>9.14 ± 1.20</td>
<td>6.78 ± 0.55</td>
</tr>
<tr>
<td></td>
<td>PDBu</td>
<td>9.33 ± 0.10</td>
<td>6.07 ± 0.85</td>
</tr>
<tr>
<td></td>
<td>4-0-Methyl-TPA</td>
<td>5.58 ± 0.11</td>
<td>2.70 ± 0.32</td>
</tr>
<tr>
<td></td>
<td>4α-PDD</td>
<td>1.12 ± 0.001</td>
<td>1.02 ± 0.01</td>
</tr>
<tr>
<td>A-253</td>
<td>TPA</td>
<td>0.95 ± 0.05</td>
<td>5.46 ± 0.16</td>
</tr>
</tbody>
</table>

*The -fold sensitization was calculated as the ratio of IC50 of CP in the presence of solvent MeSO to that in the presence of various phorbol esters (100 nM) as indicated in the table. The IC50 values (mean ± S.E.) of HeLa and A-253 cells in the presence of MeSO were 1.27 ± 0.10 and 1.00 ± 0.06, respectively.

4-0-Methyl-TPA and 4α-PDD, 4α-phorbol 12,13-didecanoate.

**FIG. 5.** Effect of protein kinase C inhibitors on the antiproliferative activity of CP. HeLa cells were preincubated with 10 μM palmitoylcarnitine (triangles) or 10 μM sphingosine (triangles) for 2 h prior to addition of MeSO (open symbols) or 10 nM TPA (closed symbols). Following 24 h, cells were exposed to various concentrations of CP and cell survival was determined by the MTT assay as described under "Experimental Procedures." Each symbol represents the mean value of four determinations and the S.D. was ±12%.

**TABLE III**

<table>
<thead>
<tr>
<th>Cell</th>
<th>Additions</th>
<th>Protein kinase C activity (pmol/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>MeSO</td>
<td>17.9 ± 0.30 15.4 ± 0.23 2.42 ± 0.20</td>
</tr>
<tr>
<td></td>
<td>TPA</td>
<td>0.95 ± 0.46 0.34 ± 0.13 0.61 ± 0.34</td>
</tr>
<tr>
<td>A-253</td>
<td>MeSO</td>
<td>13.9 ± 0.54 12.6 ± 0.25 1.34 ± 0.29</td>
</tr>
<tr>
<td></td>
<td>TPA</td>
<td>0.11 ± 0.03 0.04 ± 0.02 0.07 ± 0.04</td>
</tr>
</tbody>
</table>

Effect of phorbol esters on protein kinase C down-regulation

HeLa cells or A-253 cells were treated with either MeSO (0.05%) or phorbol esters (100 nM) for 24 h. Protein kinase C was isolated and the enzyme activity was determined as described under "Experimental Procedures."
ester to sensitize HeLa cells to CP. In addition, TPA (100 nM) caused substantial down regulation of protein kinase C (less than 1% of control) in A-253 cells but did not change cellular sensitivity to CP (Fig. 1B; Table II).

Effect of Protein Synthesis Inhibitor on CP Sensitization by Phorbol Ester—To examine if new protein synthesis is required for the phorbol ester-induced sensitization of HeLa cells to CP, we tested the effect of protein synthesis inhibitor cycloheximide on CP sensitivity. Cells were treated with a concentration of cycloheximide that inhibited protein synthesis in HeLa cells by 95% (37). Cycloheximide (10 μg/ml) by itself had no effect on the antiproliferative activity of CP in HeLa cells but it completely blocked the sensitization of HeLa cells to CP caused by PDBu (Fig. 6).

Effect of Phorbol Esters on Cellular Drug Content—There are various mechanisms by which TPA might influence the cytotoxic action of CP. We examined whether TPA altered cellular platinum levels. Cells were exposed for 24 h to a 24-h pretreatment with varying concentrations of TPA. As shown in Fig. 7, the increase in cell-associated platinum was concentration-dependent up to 16 nM TPA, raising the platinum level more than 2-fold. We also examined the effect of 16 nM TPA on the cellular content of bleomycin A₅, an agent to which no sensitization was seen (Fig. 3). In the presence of vehicle and 16 nM TPA, bleomycin A₅ content was 8.42 ± 0.30 and 9.20 ± 0.30 pmol/mg protein, respectively.

![Figure 6](http://example.com/fig6.jpg)

**Fig. 6.** Effect of cycloheximide on the antiproliferative activity of CP. HeLa cells were incubated with MeSO (open symbols) or 100 nM PDBu (closed symbols) for 24 h, in the presence (triangles) or absence (circles) of 10 μg/ml cycloheximide. Cells were then exposed to various concentrations of CP for additional 2 h, washed twice with medium, and cell survival was determined by the MTT assay as described under "Experimental Procedures." This result is representative of three individual experiments. Each symbol represents the mean value of four determinations in a single experiment and the S.D. was ±10% of the mean.

![Figure 7](http://example.com/fig7.jpg)

**Fig. 7.** Effect of TPA on cellular platinum content. HeLa cells were preincubated with various concentrations of TPA for 24 h and then exposed to 0.75 μM CP for an additional 24 h. Cell-associated platinum content was measured by flameless atomic absorption spectrophotometry. Each symbol represents the mean value of three to six individual experiments and the S.D. was ±15% of the mean.

**TABLE IV**

<table>
<thead>
<tr>
<th>PDBu pretreatment</th>
<th>ng platinum/10⁶ cells*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-Cycloheximide</td>
</tr>
<tr>
<td>1</td>
<td>7.81 ± 0.55</td>
</tr>
<tr>
<td>24</td>
<td>12.09 ± 0.62</td>
</tr>
<tr>
<td></td>
<td>12.11 ± 0.56</td>
</tr>
<tr>
<td></td>
<td>16.0 ± 0.84</td>
</tr>
<tr>
<td></td>
<td>9.68 ± 0.81</td>
</tr>
</tbody>
</table>

* Each value is the mean ± S.E. of three individual experiments performed in triplicate.

**DISCUSSION**

We have demonstrated that phorbol esters that activate protein kinase C cause a significant enhancement of the antiproliferative activity of CP in HeLa cells. This is a cell type-specific effect, being seen in HeLa but not in A-253 cells. The sensitization phenomenon was also specific for the anticancer drug tested. Pretreatment with TPA sensitized HeLa cells to both CP and melphalan, which covalently react with DNA, but not to another DNA alkylating agent, mitomycin C. Phorbol ester-pretreated HeLa cells were slightly more sensitive to DACH but not to doxorubicin, bleomycin, or vincristine.

Hofmann et al. (24) also have observed sensitization of Walker rat carcinoma cells to CP by long-term exposure to TPA, although it was rather limited (<2-fold). The authors postulated that the depletion of protein kinase C by TPA caused the sensitization of Walker cells to CP, because the observed enhancement occurred at a time when protein kinase C was down-regulated. Our data do not support this hypothesis. First, there was no correlation between the ability of a phorbol ester to down-regulate protein kinase C and to sensitize HeLa cells to CP (Table III). Second, 4-O-methyl-TPA at a concentration that does not cause any down-regulation of protein kinase C was almost as effective as TPA in inducing sensitivity to CP in HeLa cells. Finally, TPA caused substantial down-regulation of protein kinase C in A-253 cells but failed to sensitize these cells to CP.

Based upon the results of our study, there appears to be a good correlation between the activation of protein kinase C as measured in vitro and the sensitization of HeLa cells to CP by phorbol esters. Unlike doxorubicin which was shown to inhibit protein kinase C activity (38), CP by itself has no effect on protein kinase C activity (25). The enhancement in antiproliferative activity of CP by various concentrations of...
TPA paralleled the increase in protein kinase C activity. In addition, the ability of different phorbol esters to activate protein kinase C correlated with their ability to decrease the IC\textsubscript{50} for CP. Furthermore, inhibitors of protein kinase C, such as palmitylcarnitine and sphingosine, inhibited the TPA-mediated sensitization. We and others (24–26) have, however, observed sensitization of cells to CP by inhibitors themselves. Unfortunately, all known inhibitors of protein kinase C lack specificity and are known to have other pharmacological activities (24, 25, 36, 39, 40). Therefore, it is difficult to ascertain whether the mechanism of cellular sensitization to CP by these inhibitors involves protein kinase C or not.

Activation of protein kinase C appears to be necessary but not sufficient to explain the sensitization phenomenon. TPA caused significant stimulation of protein kinase C from A-253 cells but did not sensitize these cells to CP. The failure to sensitize A-253 cells may be due to lack of some important cellular component distal to the stimulation of protein kinase C.

The ability of cycloheximide to block the sensitization seen with a 24-h pretreatment is consistent with the hypothesis that the synthesis of additional protein(s) is required for maximal stimulation. At least seven different isoforms of protein kinase C have been isolated (1, 3, 4). Although the function of each isoform is not known, there appears to be tissue-specific expression of these isoforms (1). Recent results indicate that TPA can stimulate the synthesis of the γ form of protein kinase C and that this requires at least 6–24 h (15). We have found using Western blot analyses and a isoform specific monoclonal antibody to protein kinase C-γ that HeLa cells express very low amounts of protein kinase C-γ and these levels are unaffected by TPA treatment. It is conceivable that the sensitization of HeLa cells after a 24-h pretreatment may be mediated by the synthesis of some protein other than a subspecies of protein kinase C.

The sensitization of HeLa cells to CP by phorbol esters may be due to the increase in platinum content. Results from a number of laboratories indicate that resistance to CP is marked by a lower cellular content of CP (41-43). The magnitude of the increase in cellular platinum appears to depend on the concentration of TPA as well as the length of exposure to phorbol esters. The level of increase in platinum content (2-fold) is sufficient to explain the elevated sensitivity of HeLa cells. Teicher \textit{et al.} (41), for example, found that human cells with a 30-fold resistance to CP had a 3-fold decrease in platinum content. This is in agreement with the results of other laboratories (42, 43). It is interesting, however, that cycloheximide could block the increase seen with a 24-h PDBu pretreatment but not a 1-h pretreatment. This is consistent with the hypothesis that new protein synthesis is essential for maximum sensitization but may indicate a difference in the mechanism responsible for the modest sensitization seen with a 1-h pretreatment and the more substantive enhancement seen after 24 h.

The mode of entry and removal of CP in malignant and normal cells is not well defined. There is indirect evidence that some alkylating agents, such as CP and melphanal, are transported into the cells via a membrane carrier whereas the cellular entry of alkylating agents, such as busulfan or mitomycin C, does not involve any carrier (21). While protein kinase C has been implicated in regulating the “multiple drug resistance” phenotype (19), there is no evidence that PGlycoprotein, which is a drug efflux pump, recognizes CP or melphanal. It is noteworthy that phorbol esters increase the sensitivity of HeLa cells to melphanal and CP but not to mitomycin C. In addition, it has been shown (44) that phorbol ester treatment restores L-system amino acid transport in chronic lymphocytic leukemia. Interestingly, the enhancement in amino acid transport required prolonged treatment with TPA (10 h) and was blocked by cycloheximide. Furthermore, cells with acquired resistance to CP have been found to have a reduced amino acid transport system (45). Therefore, it may be worthwhile to examine whether the pretreatment of HeLa cells with TPA involves an altered carrier. It may also be interesting to determine if selective phosphoproteins influence CP transport and cytotoxicity. Given the low therapeutic indices currently available with antitumor agents, our observation that phorbol esters cause marked sensitization to CP is intriguing and may have potential therapeutic importance.

\textbf{Acknowledgments}—We thank Beth E. Reffner and Vrinda S. Khandekar for excellent technical assistance.

\textbf{REFERENCES}


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Cellular Sensitization to cis-Platinum by Phorbol Esters

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