Inhibition by Palmitoylcarnitine of Adhesion and Morphological Changes in HL-60 Cells Induced by 12-O-Tetradecanoylphorbol-13-acetate

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ABSTRACT

Effects of DL-palmitoylcarnitine (PC), an inhibitor of calcium-activated, phospholipid-dependent protein kinase (protein kinase C), on 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced cell differentiation were investigated in human promyelocytic leukemia cells (HL-60). TPA caused HL-60 cell adhesion concomitant with morphological changes, and an increase in acid phosphatase activity. The median effective concentration was 1 nM, which corresponded well to the dissociation constant of [3H]TPA binding to the cell extract. [3H]TPA binding to the cell extract was saturable and reversible. The maximal number of [3H]TPA-binding sites was 1.5 pmol/mg protein and a Hill coefficient was unity, indicating noncooperative interactions. PC, but neither palmitic acid nor DL-carnitine, inhibited the TPA-induced cell adhesion and morphological changes with the median inhibitory concentration of 1 μM, whereas a TPA-induced increase in acid phosphatase activity was not affected by 3 μM PC. Addition of PC 1 or 2 days after the addition of TPA was also effective in inhibiting the cell adhesion. Among various acylcarnitines, PC had the largest effect. [3H]TPA binding to the cell extract was not inhibited by PC at the concentration which was effective in inhibiting the TPA-induced cell adhesion. These results indicate that protein kinase C possibly mediates HL-60 cell differentiation induced by TPA.

INTRODUCTION

A tumor promoter, TPA, produces various biological effects (2, 3). It has been suggested that the initial event involved in TPA action is the binding with cell surface receptors (8, 28). Although the signal transduction after the receptor occupancy is largely unknown, a recent hypothesis states that the effects of TPA are mediated through protein kinase C; TPA stimulates the protein kinase C in a cell-free system (6), the TPA receptor copurifies with the protein kinase C (20), and TPA translocates the protein kinase C from the cytosol to the plasma membrane (16).

HL-60 cells, human promyelocytic leukemia cells, are a useful system for investigation into TPA actions; Huberman and Callahan (12) demonstrated that TPA induces terminal differentiation in HL-60 cells. The receptors for phorbol esters are identified in HL-60 cells by use of [3H]phorbol-12,13-dibutyrate (30), and TPA produces a large number of morphological and functional changes concomitant with the HL-60 cell differentiation (4, 5, 13, 22).

We have examined whether or not TPA actions on HL-60 cells are mediated through the protein kinase C by using acylcarnitines, including PC, which inhibit protein kinase C activity in bovine heart (14, 31, 32) and HL-60 cells (10). The data show that PC inhibited the TPA-induced HL-60 cell adhesion and morphological changes but had no effects on TPA-induced increase in acid phosphatase activity.

MATERIALS AND METHODS

Materials. Drugs were purchased from the following sources: TPA, DL-acetylcarnitine, DL-hexanoylcarnitine, DL-lauroylcarnitine, DL-myristoylcarnitine, PC, DL-steaoylcarinitine, DL-carnitine, palmitic acid, and BSA. Sigma Chemical Co. (St. Louis, MO); [3H]TPA (20.0 Ci/mmol), New England Nuclear (Boston, MA); Roswell Park Memorial Institute Medium 1640 and fetal calf serum, Grand Island Biological Co. (Grand Island, NY); Gibco’s solution, Merck-Japan (Tokyo, Japan); trypsin, Difco Laboratories (Detroit, MI). The purity of [3H]TPA was checked by thin-layer chromatography on silica gel plates with a solvent system, methylene chloride:acetone (3:1, v/v). The purity was greater than 95%.

Acylcarnitines were dissolved in distilled water for use.

Cells. HL-60 cells were kindly supplied by Professor Tatsuo Suda (Department of Biochemistry, School of Dentistry, Showa University, Tokyo, Japan). They were cultured in tissue culture plastic dishes (Nunc, Denmark) in Roswell Park Memorial Institute Medium 1640 plus 10% heat-inactivated fetal calf serum without any antibiotics. For experiments, 1 x 10^6 cells were seeded in 1 ml of the medium in 35-mm plastic dishes, and were added with various drugs dissolved in 5 μl ethanol or distilled water. Ethanol at a concentration up to 1% produced no detectable effects on HL-60 cell growth or on adhesion induced by TPA.

Cell Adhesion Assay. The suspended cells and the adhesive cells were counted 48 hr after the addition of TPA and other chemicals, unless otherwise indicated. The plastic dishes were rotated, gently shaken, and the medium was removed. After adding 1 ml fresh medium, the same procedure was repeated. The cell number in the combined 2 ml of medium was referred to as suspended cells. The cells in the dishes were treated with 1 ml of a solution containing 137 mM NaCl, 2 mM KCl, 2 mM NaH2PO4, 0.27 mM KH2PO4, 11.2 mM glucose, and 0.05% trypsin, and then incubated at 37°C for 15 min, followed by forceful pipetting. This procedure could detach all the cells from the plastic dishes. The number of the cells detached by this procedure was referred to as adhesive cells.

[3H]TPA-binding Assay. Cells were collected by centrifugation at 1000 rpm for 5 min. They were washed twice with a phosphate-buffered saline containing 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, and 1.3 mM KH2PO4; they were suspended in a buffer (1 x 10^6 cells/ml) containing 50 mM Tris-HCl (pH 7.5), and were homogenized twice with a sonicator (Ohtake Works, Tokyo, Japan) for 10 sec in ice. The homogenate was centrifuged at 800 x g for 5 min, and the resultant supematant (about 1 mg protein/ml) was stored at -80°C until use for binding assay. The storage for at least 2 weeks did not lose the [3H]TPA-binding activity. After thawing the cell extract, the Tris-HCl buffer was added to adjust the protein concentration to the appropriate level. For separating bound from free [3H]TPA, a filtration technique was performed with use of cold acetone (1, 11). In brief, the cell extract (200 μg of protein/ml) was...
incubated with 50 mM Tris-Cl buffer (pH 7.5), containing 4 mg BSA per ml, and various concentrations of [3H]TPA in a total volume of 950 μl at 39° for 20 min. Incubations were terminated by rapid filtration through Whatman (Whatman, Ltd., Maidstone, Kent, England) GF/C glass fiber filters. The filters were rapidly washed 4 times with 2.5 ml of cold acetone cooled in a dry ice bath. We ensured that further washing up to 8 times did not reduce the remaining counts. The filters were placed in scintillation vials with the addition of 7.5 ml of emulsion scintillator (ACS, Amersham, Arlington Heights, IL), and were counted with a Beckman Model LS3800 liquid scintillation spectrometer at an efficiency of 45%. Nonspecific binding was defined as those counts observed in the presence of 1 μM TPA. Specific binding was defined as total binding minus nonspecific binding, and was approximately 80% of the total binding with 1 nm [3H]TPA, under our experimental conditions.

We found that without BSA, the glass filters (Whatman) themselves showed a small specific binding of [3H]TPA. Increase in number of washing times failed to reduce the artifact created by GF/C. The inclusion of BSA in the reaction mixture circumvented the artifact.

Other Experimental Conditions. Activity of acid phosphatase (EC 3.1.3.2) was determined by the method of Schnyder and Baggiolini (27), which utilizes hydrolysis of p-nitrophenylphosphate to p-nitrophenol. Amounts of protein was assayed according to Lowry et al. (19). For the determination of cell morphology, cells were smeared on a slide glass and stained with Giemsa. At least 300 cells were counted under a light microscope.

RESULTS

[3H]TPA Binding and HL-60 Cell Adhesion. Specific binding of 1 nm [3H]TPA was linear, with the concentration of the cell extract in the range of 0 to 200 μg protein per ml. Therefore, all assays were conducted at 200 μg protein per ml. The binding of [3H]TPA reached apparent equilibrium within 20 min at 39° and was reversible (data not shown). Nonspecific binding of [3H]TPA increased linearly to 13 nm [3H]TPA (Chart 1, top), the highest concentration examined. The specific binding was apparently saturable (Chart 1, bottom). According to Scatchard analysis (26), dissociation constant (Kd) of the specific binding was 1.2 nm. The maximal number of binding sites (Bmax) was 1.5 pmol/mg protein (Chart 1). A Hill plot (29) revealed a straight line with a Hill coefficient of 1.0, indicating noncooperative interactions (data not shown).

Untreated HL-60 cells continued to grow in suspension (Chart 2, see curve labeled none). After the addition of 1 nm TPA, HL-60 cells were adhesive in 2 days (Chart 2, see curve labeled TPA only). The treatment with TPA also inhibited cell growth in agreement with the results obtained by other investigators (12, 25). TPA caused HL-60 cell adhesion in a dose-dependent manner. The apparent median effective concentration was about 1 nm. The Kd for [3H]TPA binding was consistent with the median effective concentration for the cell adhesion. However, the [3H]TPA binding was a much faster process (20 min for equilibration) than the detectable adhesion (2 days).

HL-60 cell adhesion appeared to require the continuous activation by TPA under the conditions we used. We observed that the cells which had been adhered by 48-hr treatment with 1 nm TPA were detached by replacement with a fresh medium containing no TPA (Table 1). Table 1 also indicates that total cell number at 72 hr correlates with the duration after the removal of TPA, indicating that HL-60 cells proliferated after the TPA removal. Therefore, the effect of 1 nm TPA on the HL-60 cell adhesion and cell growth appeared reversible under our experimental conditions. In contrast to the cell adhesion, the morphological changes appeared irreversible if the cells were exposed to 1 nm TPA during more than 19 hr.

Effect of PC on the TPA-treated Cells. PC inhibited the TPA-induced cell adhesion in a dose-dependent manner, with approximate IC50 of 1 μM (Chart 3). The effect of PC was not due to nonspecific inhibition of cell adhesion, because we observed that PC at 3 μM failed to prevent the adhesion of Chinese hamster fibroblast cells (V79) or Chinese hamster ovary cells (CHO-K1).‡ PC was also effective in inhibiting the TPA-induced adhesion
of the enzyme activity (TPA + 3.0 mM PC; 10.1 ± 0.4 mmoles/mg of PC (0.3 to 3.0 mM) with 1 nM TPA failed to reduce the induction of TPA, 12.1 ± 1.3 mmoles/mg protein/60 min). Simultaneous addition of PC about 7-fold [control, 1.8 ± 0.1 (S.E.) mmoles/mg protein/60 min; cells with 1 nM TPA for 2 days increased the enzyme activity by 1910%]. Concentrations higher than 3 mM PC were not effective.

The percentage of macrophage-like cells was increased from 1% to 29% by treatment of TPA for 2 days. PC apparently inhibited the TPA-induced increase in acid phosphatase activity of HL-60 cells (12,25). However, treatment of HL-60 cells with TPA in the presence of 16 to 160 nM TPA, whereas the HL-60 cells we tested were not effective when added 1 or 2 days after the TPA addition (Chart 2). Palmitic acid or DL-carnitine had no effects on the cell adhesion at 3 μM. The 3 compounds at the concentrations used had no detectable effects on HL-60 cell growth (data not shown). Other acylcarnitines, when the dose that had no detectable effects on the cell growth was used (data not shown), were less effective than PC in inhibiting the TPA-induced HL-60 cell adhesion (Table 2).

Untreated HL-60 cells contained mainly myeloblasts and promyelocytes. However, treatment of HL-60 cells with TPA increased the number of macrophage-like cells, which had a smaller ratio of nucleus to cytoplasm and a reniform nucleus. The percentage of macrophage-like cells was increased from 1 to 29% by treatment of TPA for 2 days. PC apparently inhibited the TPA-induced morphological changes; simultaneous addition of PC with TPA decreased the percentage of macrophage-like cells to 14%

However, not all responses induced by TPA were inhibited by PC. It has been shown that binding sites of phorbol esters in HL-60 cells are identified by [3H]phorbol-12,13-dibutyrate (30). We identified TPA-binding sites by the use of [3H]TPA itself. The PC inhibited the binding of TPA, but this could not explain the inhibition of HL-60 cell adhesion, because cell adhesion was inhibited at much lower concentrations than those of other acylcarnitines.

### Table 1

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>No. of cells x 10^6</th>
<th>% of adhesive</th>
<th>Morphological changes</th>
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<tr>
<td>+TPA</td>
<td>-TPA</td>
<td>+TPA</td>
<td>-TPA</td>
</tr>
<tr>
<td>0-72</td>
<td>7.9</td>
<td>&lt;0.03</td>
<td>&lt;1</td>
</tr>
<tr>
<td>0-1.5</td>
<td>1.5-72</td>
<td>7.6</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>0-4.5</td>
<td>4.5-72</td>
<td>6.3</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>0-19</td>
<td>19-72</td>
<td>2.8</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>0-30</td>
<td>30-72</td>
<td>2.6</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>0-46</td>
<td>46-72</td>
<td>1.6</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>0-72</td>
<td>0.3</td>
<td>0.90</td>
<td>75</td>
</tr>
</tbody>
</table>

### Table 2

<table>
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<th>Treatment</th>
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<th>Adhesive</th>
<th>% of adhesive</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2.9</td>
<td>&lt;0.03</td>
<td>&lt;2</td>
</tr>
<tr>
<td>TPA</td>
<td>0.32</td>
<td>0.64</td>
<td>67</td>
</tr>
<tr>
<td>TPA + myristoylcarnitine (3 μM)</td>
<td>0.39</td>
<td>0.38</td>
<td>49</td>
</tr>
<tr>
<td>TPA + stearoylcarnitine (3 μM)</td>
<td>0.6</td>
<td>0.17</td>
<td>22</td>
</tr>
</tbody>
</table>

### Chart 3

Reversibility of the TPA-induced cell adhesion

HL-60 cells (1 x 10^6/ml) were cultured for 72 hr with the addition of 1 nM TPA at Time 0. After various time intervals, the medium was removed, and cells were washed twice with fresh medium and cultured in the fresh medium with no TPA until 72 hr. The cell number was counted at 72 hr. Values are means of duplicate determinations.

### Chart 4

[3H]TPA Binding and PC. Unlabeled TPA inhibited [3H]TPA binding with IC50 of 1.8 nM (Chart 1), which is consistent with Kd derived from the saturation experiment (Chart 1). PC inhibited [3H]TPA binding only at concentrations higher than 10 μM with IC50 of 30 μM (Chart 4).

**DISCUSSION**

HL-60 cells were established by Collins et al. in 1977 (7). We confirmed that TPA caused morphological changes, cell adhesion, and an increase in acid phosphatase activity of HL-60 cells (12, 25).

Rovera et al. (25) noted that adhesion of HL-60 cells induced by TPA was irreversible and independent of the continuous presence of 16 to 160 nM TPA, whereas the HL-60 cells we used were apparently dependent on the presence of 1 nM TPA. These apparently differing results are possibly due to the much lower concentrations we used, although the precise reason is unknown.

It has been shown that binding sites of phorbol esters in HL-60 cells are identified by [3H]phorbol-12,13-dibutyrate (30). We identified TPA-binding sites by the use of [3H]TPA itself. The maximal binding sites obtained from these 2 studies are consistent. Although PC inhibited the binding of TPA, this could not explain the inhibition of HL-60 cell adhesion, because cell adhesion was inhibited at much lower concentrations than those of other acylcarnitines.
required for the inhibition of TPA binding. Moreover, if PC inhibits the TPA binding to the cells, and if the TPA-binding sites consist of a single component, all responses produced by TPA should be inhibited. But this is not the case, because PC failed to reduce the TPA-induced increase in acid phosphatase activity.

TPA may affect cyclic nucleotide-dependent protein kinase activities in some tissues. However, inconsistent observations have been reported, as reviewed by Blumberg (2, 3). In HL-60 cells, PC produces no significant effect on cyclic AMP-dependent or cyclic GMP-dependent protein kinase activity (12).

It has been shown that TPA affects phospholipid metabolism in various cells (15, 17, 20, 21, 23, 24), as well as in HL-60 cells (4, 5). Two enzymes involved in phospholipid metabolisms are considered here. The MDCK cell line is the first demonstration that TPA activates phospholipase A₂ (EC 3.1.1.4) (18). However, we could not detect any activation of phospholipase A₂ of HL-60 cells by 1 nm TPA (data not shown). The other enzyme lysophospholipase is inhibited competitively by PC with a Kᵢ of 10 μM (9). Therefore, we could not rule out the possibility that the effect of PC was due to the inhibition of lysophospholipase, although it is unknown whether TPA stimulates lysophospholipase in HL-60 cells. TPA failed to stimulate the lysophospholipase activity in a cell-free system. ¹

It is known that Na⁺-K⁺-ATPase (EC 3.6.1.3) is inhibited by PC with a IC₅₀ of 44 to 48 μM (33). However, these concentrations of PC are much higher than those used in this study. Moreover, TPA stimulates the enzyme only at concentrations higher than μM order (2). Since the HL-60 cell adhesion was realized by a nM concentration, it is difficult to attribute the TPA-induced cell adhesion to Na⁺-K⁺-ATPase activation.

Protein kinase C is activated by TPA at a reasonably low concentration (7), and is inhibited by PC in bovine heart (14, 31, 32) and HL-60 cells (10). The effective concentrations required for the enzyme inhibition (IC₅₀, 8 μM) (10) are of the same order as those required for the inhibition of cell adhesion in this study (IC₅₀, 1 μM). Furthermore, the effects of acylcarnitines on protein kinase C (32) show a pattern similar to that of HL-60 cell adhesion.

These results agree with the view that some TPA actions on HL-60 cells are mediated through protein kinase C. However, the TPA-induced increase in acid phosphatase activity was not inhibited by 3 μM PC, which at this concentration was effective in inhibiting HL-60 cell adhesion and morphological changes. There might be 2 explanations: (a) a higher concentration (more than 3 μM) could inhibit the increase in acid phosphatase activity; and (b) the TPA-induced increase in acid phosphatase activity is unrelated to protein kinase C. The first possibility could not be tested because of its inhibition of cell growth. Therefore, we could not exclude the possibility that protein kinase C is also involved in the TPA-induced increase in acid phosphatase activity, although if the first possibility is true, it would indicate that the inhibition of an increase in acid phosphatase activity is less sensitive to PC than that of cell adhesion and morphological changes.

In summary, these results indicate that protein kinase C possibly mediates the HL-60 cell differentiation induced by TPA.

ACKNOWLEDGMENTS

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