Phospholipase C (PLC)-mediated hydrolysis of membrane phospholipids results in the production of diacylglycerol, inositol phosphates, and choline metabolites. Inositol triphosphate increases calcium levels, while diacylglycerol activates protein kinase C. The present studies demonstrate that exogenous PLC generates inositol phosphates, releases choline metabolites, and activates protein kinase C in human HL-60 promyelocytic leukemia cells. PLC also induced monocytic differentiation of HL-60 cells as manifested by adherence, growth inhibition, and appearance of monocytic cell surface antigens. Furthermore, PLC treatment of HL-60 PROMYELOCYTIC leukemia cells differentiate into monocyte-like cells following exposure to phorbol esters such as 12-0-tetradecanoyl-phorbol-13-acetate (TPA). The differentiated HL-60 phenotype is characterized by growth inhibition, increased adherence, loss of cell surface transferrin receptors, an increase in monocyte surface markers, induction of alpha-naphthyl acetate esterase (nonspecific esterase) staining, and certain patterns of protein phosphorylation. TPA-induced PLC activation is further associated with down-regulation of c-myc expression and induction of c-fos, c-fms, and tumor necrosis factor (TNF) transcripts. While c-fos and c-fos code for cell nucleus-associated products, c-fms codes for the macrophage colony-stimulating factor receptor, and TNF is expressed by activated monocytes. Although TPA activities the calcium- and phospholipid-dependent protein kinase C (PKC), the role of this enzyme in initiating events associated with monocytic differentiation has remained unclear.

Phospholipase C (PLC) is activated by cell membrane signal transduction pathways that probably involve a guanine nucleotide–binding protein. Activation of PLC results in the hydrolysis of phosphatidylinositol bisphosphate (PIP2) and phosphatidylinositol trisphosphate (PIP3) to produce diacylglycerol (DG). Both DAG and calcium play a role in the activation of PKC as measured by the translocation of enzyme activity from cytosol to membrane fractions. Activation of PKC results in the phosphorylation of a number of proteins, some of which, such as topoisomerase II, may be involved in gene regulation. PKC activity copurifies with the TPA receptor, and TPA directly activates this enzyme. Whether TPA-induced activation of PKC is sufficient to explain the induction of HL-60 differentiation is complicated by additional cellular effects of TPA. Therefore, other activators of PKC have been studied in an attempt to more precisely understand the role of this enzyme in the induction of monocytic differentiation.

In the present study we employed purified PLC from Clostridium perfringens to induce hydrolysis of PI and phosphatidylycholine. Activation of PKC was determined as translocation of PKC activity from cytosol to membrane fractions. We demonstrate that PLC treatment of HL-60 cells leads to changes in inositol phosphate concentrations, release of choline metabolites, and PKC activation. We also demonstrate that PLC induces phenotypic changes as well as changes in gene expression that are consistent with induction of monocytic differentiation. These results support the role of PLC-mediated activation of PKC in the induction of monocytic differentiation.

MATERIALS AND METHODS

Cell culture. HL-60 cells were grown in RPMI 1640 medium (Hazleton Laboratories, Vienna, VA) containing 4 mmol/L l-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.1 mmol/L nonessential amino acids, and 10 mmol/L sodium pyruvate (GIBCO, Grand Island, NY) in 15% heat-inactivated fetal bovine serum (PBS; Hazelton) at a density of 2 × 10³/mL in a 5% CO₂ humidified atmosphere at 37°C. Viable cells were determined by trypan blue exclusion. PLC (C perfringens, Cooper Biomedical, Malvern, PA) was dissolved in phosphate-buffered saline (PBS, Ca²⁺ and Mg²⁺ free) to a stock concentration of 0.1 U/μL. Phospholipase A₂ (PLA₂; porcine pancreas; Sigma Chemical Co, St Louis; 600 U/mg) was supplied as a 3.2 mol/L solution in ammonium sulfate and added directly to culture media.

Induction of monocytic differentiation. Cell adhesion was determined by calculating the percentage of nonadherent cells as compared with the total cells (after adherent cells were scraped from plastic culture flasks).

Monoclonal antibodies Mo1, Mo2, and My4, which react with cell surface antigens expressed on monocytes, were used as previously described in the laboratory of Dr James Griffin (Division of Tumor Immunology, Dana-Farber Cancer Institute). ID3, a monoclonal antibody that reacts with granulocytes, was also employed.

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Results are expressed as the percentage of fluorescent cells above background with a control antibody.

**RNA extraction and hybridization.** Total cellular RNA was purified by the guanidine thiocyanate-cesium chloride method and analyzed by gel electrophoresis through 1% agarose-formaldehyde gels followed by Northern blot transfer to nitrocellulose. Hybridization conditions were as previously described.

Probes included the 1.6 kilobase (kb) ClaI/EcoRI fragment of the human c-myc 3' exon purified from the pM C41-3 RC plasmid, the 2.7-kb Xho-I/Nco-I fragment of the pc-fos (human)-1 plasmid, the 2.0-kb Pst I fragment of the chicken beta-actin gene purified from the pA1 plasmid, the 1.3-kb PstI fragment of the v-fms gene isolated from the plasmid PSM3, and the pE4 plasmid containing a 1.1-kb Pst fragment of the TNF cDNA.

**PKC activity.** Cells (4 x 10^6) were washed twice with ice-cold PBS (Ca^2+ and Mg^2+ free) containing 2 mmol/L dithiothreitol and lysed by ten aspirations through a 25-gauge needle in a 0.4 mL column buffer (20 mmol/L Tris-HCl, pH 7.5, 2 mmol/L EDTA, 2 mmol/L dithiothreitol) containing 25 μg leupeptin. Homogenates were sedimented at 12,000 g for 15 minutes to yield cytosol and particulate fractions. The pellet was suspended in column buffer containing 1% Triton X-100, sonicated for 15 seconds, and incubated with 10 mmol/L LiCl to inhibit inositol phosphatase. The cells were homogenized by cytosol and particulate fractions were suspended at a concentration of 0.5 mL per 5 x 10^7 cells/mL in buffer containing 25 μg/mL leupeptin. Cytosol and particulate fractions were partially purified on 0.4 mL DES2 cellulose columns at 4°C via elution with 1 mL of column buffer containing 80 mmol/L NaCl. The enzyme was assayed by a modification of a previously described procedure measuring the incorporation of 32P from γ-[32P]adenosine triphosphate (ATP) (Amersham Corp., Arlington Heights, IL) into III-I histone (Sigma). PKC activity is reported as 32P incorporation per microgram of protein per minute in the presence of 1.5 mmol/L Ca^2+, 100 μg/mL phosphatidylserine, and 10 μg/mL diolein minus that measured in the presence of 0.8 mmol/L added EDTA, 1 mmol/L ethylene glycol tetracetic acid, and 100 μg/mL phosphatidylserine. Protein was measured on duplicate samples by the Bradford assay using gamma globulin as a standard.

**[3H]inositol phosphate measurements.** Inositol phosphate pools were measured according to previously described methods. HL-60 cells in logarithmic growth were grown for 48 hours in isositol-free RPMI 1640 medium (Gibco) supplemented with 15% heat-inactivated FBS (Hazelton) and 5 μCi/mL [3H]inositol (New England Nuclear, Boston; 10 Ci/mmol). After 48 hours, the cells were pelleted, washed twice with medium, suspended at a concentration of 5 x 10^6 cells/mL in buffer containing 5 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 5 mmol/L KCl, 5.5 mmol/L glucose, 0.8 mmol/L MgSO4, 1 mmol/L CaCl2, and 0.1% bovine serum albumin. The cell suspension (0.5 mL) was incubated for 30 minutes in the presence of 10 μmol/L L-arginine to inhibit inositol phosphatase. The cells were then incubated with 0.5 U/mL PLC for three minutes at 37°C. The reaction was stopped by the addition of 1.88 mL of chloroform-methanol-HCl (100:100:2). and 0.62 mL each of chloroform and H2O were added to separate the phases. After centrifugation at 1,200 g for five minutes, a 1.8-mL aliquot of the upper aqueous phase was mixed with 3.2 mL of water and placed on a column of Dowex AG1-X8-formate. The column was washed with 8 mL of water and then with 20 mL of 60 mmol/L ammonium formate/5 mmol/L disodium tetrahydrate to elute [3H]inositol and [3H]glycerophosphorylinositol. [3H]inositol-1-monophosphate (IP) was eluted with 8 mL of 200 mmol/L ammonium formate/100 mmol/L formic acid. The [3H]inositol-1,4-bisphosphate (IP2) was then eluted with 8 mL of 400 mmol/L ammonium formate/100 mmol/L formic acid and the [3H]inositol-1,4,5-trisphosphate (IP3) was eluted with 8 mL of 1 mmol/L ammonium formate/100 mmol/L formic acid. Fractions of 2 mL were counted after the addition of 8 mL Hydrofluor.

**Choline metabolite release.** Release of [methylcholine metabolites was determined according to previously described techniques. HL-60 cells in logarithmic growth phase were grown for 48 hours in the presence of 0.2 μCi/mL [3H]methylcholine (80 Ci/mmol; Amersham). The cells were then washed three times with PBS, resuspended in fresh media, and incubated with PLC for 60 minutes at 37°C. Aliquots of the supernatant were then collected and centrifuged at 150 g for five minutes, and the cell free supernatant was dissolved in Hydrofluor for scintillation counting.

**RESULTS**

**Cell growth and differentiation.** The induction of terminal HL-60 cell differentiation is associated with growth inhibition. The cytostatic effects induced with 33 nmol/L TPA is mimicked by treatment with 0.5 U/mL PLC but not by a lower concentration (0.1 U/mL) of this enzyme (Fig 1). Differences in counts between cells treated with 0.5 U/mL PLC and control cells or cells treated with 0.1 U/mL PLC were significant on the second and third days of the experiment.

Inhibition of HL-60 growth by 0.5 U/mL PLC was associated with increased Mo1, Mo2, and My4 antigen expression, whereas 0.1 U/mL PLC had no significant effect on the expression of these markers of monocytic differentiation (Table 1). In contrast, PLC treatment had no detectable effect on expression of ID3, a marker associated with granulocytic differentiation (data not shown). Moreover, in concert with induction of monocytic differentiation, 0.5 U/mL but not 0.1 U/mL PLC induced HL-60 cell adherence to the culture dishes (Table 1).

**RNA Expression.** Previous studies have demonstrated that TPA treatment of HL-60 cells is associated with a decrease in c-myc RNA levels and an induction of c-fos and c-fms expression. Similar findings were obtained following PLC treatment. Thus, the level of c-myc RNA was slightly increased at one hour but declined to undetectable levels by three days of treatment.
six hours of treatment with 0.5 U/mL PLC (Fig 2). A longer time course is shown in Fig 3. c-fos transcripts reappeared at 36 and 48 hours (Fig 3). c-fms transcripts were not induced by 24 hours (data not shown) or 48 hours of treatment with 0.2 U/mL PLA2 (Fig 4). This concentration is a threefold excess over that shown to release tritium from [3H] oleic acid–labeled Escherichia coli membranes. Treatment of cells with PLC that had been boiled for 15 minutes resulted in an 80% reduction of the c-fms signal as determined by optical densitometry. The stability of actin transcripts during PLC treatment (data not shown) implied that the observed changes in gene expression were unrelated to nonspecific effects or to differences in the amount of RNA on the hybridization filters.

Phosphoinositol formation. IP1, IP2, and IP3 were monitored in control and PLC-treated cells. Approximately 1 μCi [3H]inositol was incorporated per 10⁷ cells. IP1, IP2, and IP3 represented 0.95%, 0.14%, and 0.067% of the total incorporated [3H]inositol in control cells, respectively. The level of each of the phosphoinositol species was significantly increased as compared with that in untreated cells after three minutes of treatment with 0.5 U/mL PLC (Table 2).

Choline metabolite release. Choline metabolite release was monitored in control and PLC-treated cells preincubated for 48 hours with [3H]methylcholine. Treatment of cells with 0.5 U/mL PLC for 60 minutes produced a sevenfold increase in recoverable counts in cell-free supernatants as compared with untreated cells (Table 2).

PKC activation. Activation of PKC was accompanied by translocation of enzyme activity from the cytosolic to the membrane fractions. A statistically significant decrease was detected in the level of PKC activity in the cytosol of

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**Table 1. Effects of PLC on Monocyte Antigen Expression and Adherence**

<table>
<thead>
<tr>
<th>Cell Surface Markers</th>
<th>Adherence†</th>
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</thead>
<tbody>
<tr>
<td>Mo1</td>
<td>Mo2</td>
</tr>
<tr>
<td>Control</td>
<td>3.8±2.2</td>
</tr>
<tr>
<td>PLC, 0.1 U/mL</td>
<td>17.0±10.5</td>
</tr>
<tr>
<td>PLC, 0.5 U/mL</td>
<td>24.8±6.5‡</td>
</tr>
</tbody>
</table>

*Percent positive cells above background after 36 hours of treatment with 0.5 U/mL PLC. Values are means ± SE for at least three independent experiments.
†Measured at 36 hours of treatment and expressed as the mean percentage adhered ± SE for five independent experiments.
‡Denotes value significantly greater than control (Student’s t test, P < .05).
cells treated for 20 minutes with 0.5 U/mL PLC. This effect was associated with a corresponding increase in the PKC activity in the membrane. The total activity in each case was similar (approximately 0.45 pmol ATP/µg/min) in the control and untreated cells, thus indicating no loss of activity in preparing the cell fractions or during treatment with the inducer (Table 3).

**DISCUSSION**

Previous evidence suggests that activation of PKC is involved in signaling mechanisms that induce HL-60 differentiation. TPA, teleocidin, and debromoapsyliatoxin induce HL-60 differentiation and activate PKC.34 Furthermore, leukemic cell lines resistant to phorbol ester–induced differentiation do not exhibit translocation of PKC from cytosol to membrane fractions.35 Moreover, sphingoid bases36 and palmitoyl carnitine,37 inhibitors of PKC, block phorbol ester–induced HL-60 differentiation. However, bryostatin 1, another activator of PKC, induced HL-60 differentiation in one study38 but not in another.39 The evidence is also perhaps conflicting with regard to synthetic DAGs that activate PKC. Thus, 1-oleoyl-2-acetylglycerol is ineffective as an inducer of HL-60 maturation,40 while dioctanoylglycerol induces these cells along the monocytic lineage.41

The present results demonstrate that treatment of HL-60 cells with PLC is associated with induction of a monocytic phenotype. Furthermore, these results demonstrate that the effects of PLC are not mediated through nonspecific membrane perturbation. The PLC-induced monocytic phenotype was characterized by growth inhibition, adherence, and cell surface expression of monocyte antigens. PLC also induced specific changes in gene expression. Thus, PLC treatment was associated with an early (one hour) increase in c-myec RNA levels and then a down-regulation in the level of these transcripts by three to six hours. Similar results have been obtained following treatment of these cells with TPA.6 In contrast, c-fos expression underwent biphasic changes in PLC-treated HL-60 cells. Similar findings have been observed following treatment of these cells with bryostatin 1,38 but not with TPA.6 The basis for this difference is unclear, although c-fos RNA levels in monocytes are induced by agents that either activate PKC or increase intracellular cAMP.42 Thus, PLC, bryostatin 1, or TPA may differ in their effects on certain metabolic pathways. Finally, the induction of c-fms and TNF transcripts in PLC treated HL-60 cells was similar to that observed after treatment of these cells with TPA.6

The present study also supports the involvement of both PI and phosphatidylycholine turnover in the induction of HL-60 monocytic differentiation. DAG, the endogenous activator of PKC, is released by hydrolysis of both of these membrane phospholipids. In this regard, PLC treatment of HL-60 cells was associated with the release of both inositol phosphates and choline metabolites from membrane phospholipids in association with the activation of PKC. PLC hydrolyzes PI, phosphatidylinositol-4-phosphate (PIP), and phosphatidyl-inositol-4,5-biphosphate (PIP2) to yield DAG and IP1, IP2, and IP3, respectively.43 PIP2 is the preferred substrate for PLC, although PI and IP3 are more readily hydrolyzed by PLC when IP3 release increases intracellular calcium levels.41 An increase in PI and PIP hydrolysis further amplifies the generation of a DAG and activation of PKC. In the present studies, PLC treatment had a significant effect on the translocation of PKC activity.

Phorbol esters stimulate hydrolysis of phosphatidylycholine through a pathway dependent on PKC activation.32 In contrast, factors such as serum or platelet-derived growth factor stimulate hydrolysis of phosphatidylycholine through a pathway that is at least partially independent of PKC. These factors may act through activation of PLC.32 Of interest, treatment of mouse epidermal cells with PLC was associated with an increase in choline and DAG levels as well as phenotypic changes similar to those induced by phorbol

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**Table 2. Effects of PLC on Inositol Phosphate Production and Choline Metabolite Release**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PLC (0.5 U/mL)</th>
<th>Significance*</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP1</td>
<td>2.19 ± 0.19</td>
<td>2.935 ± 154</td>
<td>P &lt; .005</td>
</tr>
<tr>
<td>IP2</td>
<td>2.17 ± 0.20</td>
<td>776 ± 196</td>
<td>P &lt; .08</td>
</tr>
<tr>
<td>IP3</td>
<td>181 ± 21</td>
<td>383 ± 62</td>
<td>P &lt; .04</td>
</tr>
<tr>
<td>Choline</td>
<td>12,147 ± 704</td>
<td>89,882 ± 5,512</td>
<td>P &lt; .002</td>
</tr>
</tbody>
</table>

*Values represent the mean counts per minute of five (inositol phosphate) or three (choline) separate experiments.

*Comparisons made by paired Student's t test.
ester.** PLC treatment of HL-60 cells could thus result in the generation of choline either by acting directly on phosphatidylcholine or indirectly via activation of PKC. The present findings demonstrate that treatment of HL-60 cells with exogenous PLC results in a significant release of both labeled phosphoinositols and choline metabolites. However, the relative contribution of these two pathways toward DAG production remains unclear. Nonetheless, the results support the hypothesis that PLC is involved in inducing HL-60 cell differentiation and that phosphatidylcholine metabolites as well as inositol phosphates may function as second messengers in this system.

In summary, PLC induced phenotypic changes in HL-60 cells, as well as changes in c-myc, c-fos, c-fms, and TNF expression, that are consistent with differentiation along the monocytic lineage. This induction of monocytic differentiation was associated with production of inositol phosphates, release of choline metabolites, and translocation activation of PKC into cell membranes. Although the precise role of PLC in inducing monocytic differentiation remains unclear, physiological agents such as TNF* or γ-interferon** that induce HL-60 cells along this lineage may transduce signals that activate PLC.

REFERENCES


30. Bradford MM: A rapid and sensitive method for quantitation...


35. Homma Y, Henning-Chubb CB, Huberman E: Translocation of protein kinase C in human leukemia cells susceptible or resistant to differentiation induced by phorbol 12-myristate 13-acetate. Proc Natl Acad Sci USA 83:7316, 1986


Phospholipase C activates protein kinase C and induces monocytic differentiation of HL-60 cells

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