Sphingomyelinase Action Inhibits Phorbol Ester-induced Differentiation of Human Promyelocytic Leukemic (HL-60) Cells*

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Prior studies showed that sphingomyelinase action and the free sphingoid bases inhibited protein kinase C (Kolesnick, R. N., and Clegg, S. (1988) J. Biol. Chem. 263, 6534-6537). The present studies investigated whether sphingomyelinase action also inhibited a biologic process mediated via protein kinase C, phorbol ester-induced differentiation of HL-60 promyelocytic cells into macrophages. The potent phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA) stimulated time- and concentration-dependent conversion of HL-60 cells into macrophages, ED50 = 5 x 10^-10 M. Differentiation involved growth inhibition, adherence of the suspended cells to tissue culture plastic, morphologic changes, and development of specific enzymatic markers. Sphingomyelinase, which increased the level of sphingoid bases and inactivated protein kinase C, prevented this event. In control incubations, cell number increased 2.10-fold over 24 h, and 2 ± 1% of the cells were adherent. In incubations with TPA (0.5 nm), cell number increased only 1.75-fold, and 30% were adherent. Sphingomyelinase (3.8 x 10^-8 unit/ml) restored growth to incubations containing TPA to 2.02-fold and reduced adherence to 15%. Sphingomyelinase (3.8 x 10^-2 unit/ml) also restored growth partially and reduced adherence to a maximal concentration of TPA (3 nm). Similar results were obtained with the sphingoid base sphingosine (3-4.5 μM). Sphingomyelinase antagonized the morphologic changes associated with conversion to the macrophage phenotype. Untreated HL-60 cells presented typical promyelocytic morphologic changes of nuclear and cell shape. TPA induced a larger cell population with abundant cytoplasm and unusual shape. Sphingomyelinase prevented these changes. Sphingomyelinase blocked TPA-induced increases in the macrophage marker enzymes, acid phosphatase and β-naphthyl acetate esterase. These studies indicate that the action of a sphingomyelinase, like the sphingoid bases, blocks phorbol ester-induced differentiation of HL-60 cells into macrophages and provides further support for the concept that sphingomyelinase action may be sufficient to comprise a physiologically relevant inhibitory pathway for protein kinase C.

Over the past 2 years, Bell and colleagues (1) have described a potential inhibitory pathway for protein kinase C involving free sphingoid bases. In a mixed micellar assay, the free sphingoid bases, sphingosine, erythro-sphingosine, and ω-sphingosine, all served as inhibitors of protein kinase C. Inhibition was competitive for Ca^2+, phorbol esters, 1,2-diacylglycerols, and probably phosphatidylserine. Further, addition of long chain sphingoid bases to intact cells blocked protein kinase C-mediated biologic events. These included thrombin-induced phosphorylation of the 40-kD protein (2) and second phase aggregation (3) in human platelets, phorbol ester-dependent differentiation of HL-60 (4) cells, and the N-formyl-L-methionyl-L-leucyl-L-phenylalanine-induced oxidative burst in human neutrophils (5, 6). Similarly, lysosphingolipids, which accumulate in the sphingolipidoses, were shown to inhibit protein kinase C in vitro (1). Based on these results, Hannun and Bell (1) postulated that build-up of these endogenous inhibitors of protein kinase C might be important to the pathogenesis of these diseases. Presently, however, no direct evidence has been presented to suggest that sphingolipids serve as physiologic inhibitors of protein kinase C.

Recent studies from this laboratory suggested that degradation of the sphingolipid sphingomyelin via a sphingomyelinase (sphingomyelin phosphodiesterase, EC 3.1.4.12) may initiate a physiologically relevant negative effector system for protein kinase C (7). This was based on the observation that 1,2-diacylglycerols, the physiologic activators of protein kinase C, stimulated a sphingomyelinase (8), and that sphingomyelinase action was sufficient to inactivate protein kinase C in GH3 pituitary cells (7). Inactivation was demonstrated as sphingomyelinase-induced redistribution of the active membrane-bound form of protein kinase C into the cytosol. Further, the tumor-promoting activators of protein kinase C, the phorbol esters, failed to activate a sphingomyelinase. Hence, it was suggested that some of the differences in the patterns of cellular activation between these agents may reflect activation by 1,2-diacylglycerols, but not phorbol esters of a potential inhibitory pathway for protein kinase C.

The present studies sought to establish whether sphingomyelinase action was sufficient to inhibit biologic events mediated via the protein kinase C pathway. Differentiation of HL-60 cells into macrophages has served as an intact cell model for this purpose (9-11). Under basal conditions, these cells are promyelocytes and grow in suspension. Rovera et al. (9, 10) showed that phorbol ester-stimulated conversion of these cells to macrophages was accompanied by numerous physical and biochemical changes. These included growth inhibition, adherence of the cells to tissue culture substrate, alterations of cellular morphology, and the development of specific enzymatic markers, such as acid phosphatase, α-naphthyl acetate esterase, and lysozyme. Further, Merrill et al. (4) have used this system to show that the sphingoid bases prevented these changes. The present studies demonstrate that sphingomyelinase stimulates the generation of sphingoid bases and inactivates protein kinase C in HL-60 cells, similar...
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to the effect in GH3 cells. Further, these studies show that sphingomyelinase, like the sphingoid base sphingosine, inhibits phorbol ester-induced conversion of HL-60 cells into macrophages.

EXPERIMENTAL PROCEDURES

Materials—Phorbol esters, phospholipids, sphingomyelinase from Staphylococcus aureus and Streptomyces species, phospholipase A2 from Naja naja. nocambique nocambique, sphingoid bases, lysine-rich hiones (type III-α), delipidated bovine serum albumin, and dimethyl sulfoxide were from Sigma. Reagents for acid phosphatase, α-naphthyl acetate esterase, and chloroacetate esterase assays were also from Sigma. [γ-32P]ATP (3000 Ci/mmol) was from Du Pont-New England Nuclear. UDP glucose dehydrogenase DE52 and P-1 exchange chromatography paper were from Whatman. McNeal's tetrasaccharide stain was from Polysciences Inc. Millex-GV 0.22-μm filters were obtained from Millipore Corp., and tissue culture plates were from Linbro. RPMI 1640, essential and nonessential amino acids, and fetal calf serum were from GIBCO. Liquid scintillation counting solution (Liquisint) was from National Diagnostics. Reagents were high performance liquid chromatography grade from Fisher.

Cell Culture—HL-60 cells were kindly supplied by Dr. Ellen Birn (Memorial Sloan-Kettering Cancer Center, New York). Stock suspensions of cells were grown in RPMI 1640 medium containing 10% fetal calf serum and essential and nonessential amino acids at 37 °C in 5% CO2 in air as described (10). Cell density never exceeded 1.0 × 10^5 cells/ml. Cells were subcultured on the day of an experiment at 1 × 10^5 cells/2 ml into single wells of 12-well tissue culture plates. Cell Studies—Phorbol esters were added in dimethyl sulfoxide (0.1%) and phospholipids as a 1:1 mol:complex with fatty acid-free bovine serum albumin as described (4). Control wells received diluent which was without effect. Sphingomyelinases and phospholipase A2 were sterilized through a 0.22-μm filter. For experiments with phospholipases, the media contained leupeptin, aprotinin, and soybean trypsin inhibitor (10 μg/ml each), which did not affect growth or adherence. At the indicated times, medium was collected, and the monolayer cells were harvested by trypsinization. Cell number was determined with a Coulter Counter. Acid phosphatase was assessed on portions of washed cells as described (12, 19). α-Naphthyl acetate esterase and chloroacetate esterase assays were performed on cells formalin fixed onto microscopic slides as described (12, 14). Morphotology was assessed with cells that had been stained with McNeal's tetrasaccharide stain. Cell viability was assessed by the exclusion of trypan blue.

Phospholipid Studies—Cells were labeled for 48 h in RPMI 1640 media containing 10% fetal calf serum and 21 μM [3H]serine (1 μCi/ml). Cells (5 × 10^5/ml) were resuspended in a buffered saline solution (135 mM NaCl, 4.5 mM KCl, 1.5 mM CaCl2, 0.5 mM MgCl2, 5.6 mM glucose, 10 mM HEPES, pH 7.4) without radiolabeled containing sphingomyelinase or glycerol phosphate buffer. Incubations were stopped by disruption of cells by 50 strokes of a tight-fitting Dounce homogenizer in a buffer containing 5 mM NaCl, 5 mM Tris-HCl, pH 7.5, 5 mM EGTA, 1 mM MgCl2, and the protease inhibitors, leupeptin and soybean trypsin inhibitor (10 μg/ml each), at 4 °C as described (17). Homogenates were centrifuged at 1,200 × g for 2 min to remove cell debris and nuclei and thereafter at 100,000 × g for 60 min to separate the cytosolic and particulate fractions as previously described (17). The pellet was resuspended in homogenization buffer containing 1% Triton X-100. Protein kinase C activities in the

cytosolic and particulate compartments were obtained by DAAE-52 column chromatography by elution with buffer containing 0.1 M NaCl, 20 mM Tris-HCl, pH 7.5, 5 mM EGTA, 2 mM EDTA, and 2 mM dithiothreitol, as described (7). Protein kinase C activity was measured by the transfer of 3P from [γ-32P]ATP to lysine-rich hiones using a standard reaction mixture containing 20 mM HEPES buffer, pH 7.5, 10 mM MgCl2, 2 mM dithiothreitol, 1 mg/ml lysine-rich hiones, 100 μM [γ-32P]ATP (1000 dpm/pmol), phosphatidylserine (80 μg/ml), 1,2-diolein (8 μg/ml), and portions of the enzyme solution to be assayed. The final free Ca2+ was set at 0.5 mM with CaCl2 and EGTA. Ca2+- and phospholipid-independent activity was measured in the absence of EGTA/phosphatidylinositol/diolein and was subtracted from the total activity. Incubations were terminated after 5 min at 4 °C. 32P-Labeled hiones (3 × 10^6 dpm/assay) were isolated by transfer of portions of the reaction mixture to phosphocellulose paper and quantitated by liquid scintillation counting as described (7).

Statistics—Statistical analysis was performed by f test and linear regression analysis by the method of least squares. For the acid phosphatase assays, 95% confidence intervals for the geometric mean ratios were calculated.

RESULTS

Initial studies investigated whether sphingomyelinase exhibited the same effects in HL-60 cells as observed in GH3 cells. Fig. 1 demonstrates the effect of sphingomyelinase (1.0 × 10^-3 unit/ml) on the degradation of sphingomyelin in cells labeled to isotopic equilibrium with [3H]serine (n = 3). The upper panel shows that sphingomyelinase stimulated a 38% decrease in sphingomyelin content from 560 pmol/10^6 cells to 350 pmol/10^6 cells after 30 min of stimulation. The level of ceramides increased concomitantly from 186 pmol/10^6 cells to 370 pmol/10^6 cells. Hence, sphingomyelinase induced quantitative conversion of sphingomyelin to ceramide. The bottom panel shows that sphingomyelinase also increased the level of sphingosine (1.51-fold). The basal level of sphingoid bases was 12.0 pmol/10^6 cells and increased to 18.0 pmol/10^6 cells (p < 0.025). These events were detectable by 5 min and maximal by 15 min of stimulation. As little as 3.8 × 10^-3 unit/ml of sphingomyelinase was effective, and a maximal effect was achieved with 3.8 × 10^-2 unit/ml; ED50 = 1 × 10^-3 unit/ml.

Similar data have been reported previously in GH3 rat pituitary cells (6).

Additional studies were performed to determine if sphingomyelinase inactivated protein kinase C in HL-60 cells, like the effect in GH3 cells (7). Table I demonstrates, as in other cells types, that the large majority (88%) of the total protein kinase C activity exists in the inactive cytosolic form (112 pmol-min^-1·10^6 cells^-1), whereas a small portion (12%, 15 pmol-min^-1·10^6 cells^-1) is in the active membrane-bound form. Sphingomyelinase (3.8 × 10^-4 unit/ml and 3.8 × 10^-4 unit/ml) reduced cytosolic activity minimally to 102 and 93 pmol-min^-1·10^6 cells^-1, respectively. In contrast, sphingomyelinase (3.8 × 10^-3 unit/ml) markedly reduced membrane-bound activity to 60% of control to 9 pmol-min^-1·10^6 cells^-1, and sphingomyelinase (3.8 × 10^-4 unit/ml) further reduced protein kinase C activity to 20% of control to 3 pmol-min^-1·10^6 cells^-1. Higher concentrations of sphingomyelinase (3.8 × 10^-3·3.8 × 10^-3·3×10^-3 unit/ml) did not further enhance this effect. Hence, as in GH3 cells, sphingomyelinase action generates sphingoid bases and specifically inactivates the membrane-bound form of protein kinase C.

Subsequent studies measured the effect of phorbol esters on differentiation of HL-60 cells into macrophages. The potent phorbol ester TPA stimulated time- and concentration-dependent adherence of the cells to the tissue culture plastic and inhibition of cell growth. Under basal conditions, 5 ± 1% of the cells were adherent, and the doubling time was 1.09 ± 0.05 days (n = 46, mean ± S.E.). In three studies, as little as 0.3 nm TPA increased adherence to 17%, and maximal ad-

1 The abbreviations are: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TPA, 12-O-tetradecanoylphorbol 13-acetate; EGTA, ethyleneglycolbis(2aminoethyl ether)tetraacetic acid.
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**FIG. 1.** The effect of sphingomyelinase (SMase) on sphingolipids. Upper panel, sphingomyelin; middle panel, ceramide; lower panel, sphingoid bases. Cells, labeled for 48 h with [3H]serine, were resuspended in a buffered saline solution containing 1 x 10^{-3} unit/ml or diluent. After 30 min, incubations were stopped by extraction of phospholipids into chloroform:methanol:concentrated HCl (100:100:1, v/v/v) containing 10 mM EDTA as described (8). Glycerophospholipids were saponified in methanolic KOH (0.1 M for 1 h at 37 °C). Sphingolipids were resolved by sequential one-dimensional TLC using chloroform:benzene:ethanol (80:40:75, v/v/v) followed by chloroform:methanol:28% ammonia (65:25:5, v/v/v) as solvents as described (8). [3H]Sphingomyelin contained 2866 ± 202 dpm/10⁶ cells. These data (mean ± S.E.) represent triplicate determinations from three experiments.

Adherence was achieved with 3 nM TPA to 60% by 24 h; EC⁵₀ = 5 x 10^{-10} M. It should be noted, however, that there was some variance in this response. Hence, the effect of 3 nM TPA on adherence, as reported below, varied between 40 and 65%. An inverse relationship was seen with cell growth, whereas 0.5 nM TPA reduced growth by only 30%, 3 nM TPA reduced growth by 80%. These results are very similar to those originally reported by Rovera and colleagues (9, 10).

Preliminary studies assessed the direct effects of sphingomyelinase on growth and adherence of HL-60 cells. For these studies, 1 x 10⁶ cells were incubated for 24 h with varying concentrations of sphingomyelinase derived from S. aureus. After 24 h, the media were collected, and the monolayer was obtained by trypsinization. The combined fractions were counted by a Coulter Counter. These data (mean ± S.E.) represent duplicate determinations from five experiments.

![Graph showing the effect of sphingomyelinase (SMase) on cell number.](image)

**FIG. 2.** The effect of sphingomyelinase (SMase) on growth of HL-60 cells. Cells were subcultured at 1 x 10⁶ cells/2 ml in RPMI 1640 containing 10% fetal calf serum, essential and nonessential amino acids, protease inhibitors, and various concentrations of sphingomyelinase derived from S. aureus. After 24 h, the media were collected, and the monolayer was obtained by trypsinization. The combined fractions were counted by a Coulter Counter. These data (mean ± S.E.) represent duplicate determinations from five experiments.

**Table 1**

<table>
<thead>
<tr>
<th>Activity</th>
<th>Cytosol</th>
<th>Membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>pmol [³²P]histone/min⁻¹⋅10⁶ cells⁻¹</td>
<td>112 (100)</td>
<td>15 (100)</td>
</tr>
<tr>
<td>SMase (3.8 x 10⁻⁵ units/ml)</td>
<td>102 (91)</td>
<td>9 (63)</td>
</tr>
<tr>
<td>SMase (3.8 x 10⁻⁴ units/ml)</td>
<td>93 (83)</td>
<td>3 (20)</td>
</tr>
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</table>

**TABLE I**

Effect of sphingomyelinase on protein kinase C activities in HL-60 cells

Cells (0.5 x 10⁶/ml) were incubated in RPMI 1640 media containing 10% fetal calf serum and various concentrations of sphingomyelinase or glycerol phosphate buffer. After 24 h, cells were homogenized at 4 °C. Protein kinase C activities in the cytosolic and particulate compartments were eluted from DEAE-cellulose columns with buffer containing 0.1 M NaCl as described (7). Protein kinase C activity was measured by transfer of ³²P from [γ⁻³²P]ATP to lysine-rich histones using a standard reaction mixture. Ca²⁺- and phospholipid-independent activity was measured in the absence of Ca²⁺/phosphatidylinerine/diolein and was subtracted from total activity. Incubations were terminated after 5 min, and ³²P-labeled histone (3 x 10⁴ dpm/assay) was quantitated by transfer to phosphocellulose paper and liquid scintillation counting as described (7). Activity is also presented as a percent of control in parentheses. These data represent duplicate determinations from two experiments.
inhibited growth. The lowest concentrations affected growth minimally, whereas the higher concentrations more effectively limited growth. Similar data have been reported for sphinganine (4) and have also been observed for sphingosine (see below). Removal of sphingomyelinas resulting in restoration of growth. However, at the highest concentrations, restoration of growth was delayed for as long as 24 h. Sphingomyelinas did not alter basal adherence of cells at any concentration. These studies demonstrate that sphingomyelinas, like the sphingoid bases, has direct effects on cell growth. Therefore, to limit these effects, subsequent studies utilized 24-h incubations.

Further studies measured the effect of sphingomyelinas treatment on phorbol ester-induced growth inhibition and adherence of HL-60 cells. Table II (A) demonstrates the effect of sphingomyelinas (3.8 × 10^{-5} unit/ml) on preventing growth inhibition and adherence induced by a submaximal concentration of TPA (0.5 nM). In these studies (n = 5), the control population doubled from 1.0 to 2.1 × 10^6 cells by 24 h. As above, 3.8 × 10^{-5} unit/ml of sphingomyelinas did not directly affect growth, and 0.5 nM TPA partially inhibited growth to 1.75 × 10^6 cells. Co-incubation with TPA and sphingomyelinas restored cell growth. In these incubations, cell number increased to 2.02 × 10^6 cells, a value not different from control. Similar results were obtained in 11 additional studies with these agents (see below). In these studies, TPA (0.5 nM) inhibited growth by almost 50%, and sphingomyelinas restored growth completely. Sphingomyelinas also prevented TPA-induced adherence of HL-60 cells to the tissue culture plates. As above, in control incubations and incubations containing sphingomyelinas, only 2% and 3% of the cells were adherent, respectively. TPA increased the percentage of adherent cells to 30%, and sphingomyelinas reduced this effect by half to 15% of the total. Similar effects on both cell growth and adherence were obtained with 7.8 × 10^{-5} unit/ml of sphingomyelinas. Concentrations greater than 1 × 10^{-4} unit/ml less effectively restored growth in these studies due to direct effects of these higher concentrations of sphingomyelinas on this parameter. Studies were also performed with a maximal concentration of TPA (3 nM, Table II). Similar results were obtained with some minor differences. In these studies (N = 6), the control population increased from 1.0 × 10^6 cells to 1.86 × 10^6 cells by 24 h. This maximal concentration of TPA reduced growth by 80% to 1.17 × 10^6 cells. The low concentrations of sphingomyelinas utilized above did not prevent the growth arrest by 3 nM TPA. However, higher concentrations of sphingomyelinas were effective. The most effective concentration of sphingomyelinas, 3.8 × 10^{-2} unit/ml, partially restored growth to incubations containing TPA to 1.55 × 10^7 cells. This is the same level to which this high concentration of sphingomyelinas directly reduced growth in these studies. In contrast, this concentration of sphingomyelinas effectively prevented adherence. In these studies, 4% of the control and 46% of the TPA-treated cells were adherent. Again, sphingomyelinas did not directly affect adherence, but reduced TPA-induced adherence to 8% of the total. These studies indicate that sphingomyelinas can prevent phorbol ester-induced differentiation of HL-60 cells as measured by growth inhibition and adherence.

Studies were performed to assess the specificity of these effects of sphingomyelinas. Fig. 3 demonstrates the effect of boiled sphingomyelinas on inhibition of phorbol ester action. These studies used the high concentration of sphingomyelinas (3.8 × 10^{-2} unit/ml) to markedly reduce cell growth. As above, cell number (upper panel) nearly doubled in control incubations, and TPA decreased cell growth and stimulated adherence (lower panel). Sphingomyelinas also directly reduced cell growth, although to a lesser extent than TPA. Again, sphingomyelinas restored growth and prevented adherence in incubations containing TPA. Boiled sphingomyelinas, however, failed to inhibit cell growth and did not antagonize phorbol ester-induced growth inhibition or cell adherence. Hence, the action of sphingomyelinas appears to be intrinsic to the enzymatic activity contained in the commercial enzyme preparation. Two other sets of studies were performed to assess the specificity of the sphingomyelinas effect. In the first, the action of sphingomyelinas from S. aureus was compared to another sphingomyelinas derived from Streptomyces species. Although the concentrations required were somewhat different, in two separate studies, both sphingomyelinas blocked phorbol ester-induced inhibition of cell growth and adherence. In contrast, phospholipase A2 action did not antagonize the phorbol ester action. In these studies (N = 2), phospholipase A2 derived from N. mocambique mocambique did not directly inhibit cell growth and was ineffective over the concentration range 0.039–9.45 units/ml to alter TPA (0.5 nM) action. It should be noted that this is the maximally effective range for the generation of lysophosphatidylcholine in corresponding studies (N = 3). These studies indicate by three separate approaches that this is a specific effect of sphingomyelinas to inhibit phorbol ester action.

Merrill et al. (4) reported similar findings with the sphingoid base sphinganine. These results were confirmed in this laboratory using the sphingoid base sphingosine. Table III (A) demonstrates that sphingosine, like sphingomyelinas, re-

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**Table II**

**Effect of sphingomyelinas on growth and adherence of TPA-induced HL-60 cells**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Sphingomyelinas</th>
<th>TPA</th>
<th>TPA + sphingomyelinas</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Cells/dish (× 10^4)</td>
<td>2.10 ± 0.05</td>
<td>2.11 ± 0.05</td>
<td>1.75 ± 0.08^*</td>
<td>2.02 ± 0.04^*</td>
</tr>
<tr>
<td>% adherence</td>
<td>2 ± 1</td>
<td>3 ± 1</td>
<td>30 ± 3^*</td>
<td>15 ± 2^*</td>
</tr>
<tr>
<td>B. Cells/dish (× 10^6)</td>
<td>1.86 ± 0.05</td>
<td>1.55 ± 0.07^a</td>
<td>1.17 ± 0.08^a</td>
<td>1.55 ± 0.06^a</td>
</tr>
<tr>
<td>% adherence</td>
<td>4 ± 1</td>
<td>6 ± 1</td>
<td>46 ± 2^*</td>
<td>8 ± 1^*</td>
</tr>
</tbody>
</table>

^* Significantly different from control (p < 0.005).
^a Significantly different from TPA (p < 0.025).
^c Significantly different from TPA (p < 0.005).
stored cell growth and reversed cell adherence induced by TPA. In these incubations (N = 4), the cells doubled over 24 h, and growth was not altered by sphingosine (3 μM). As above, a maximal concentration of TPA (3 nM) reduced growth markedly to 1.25 × 10^6 cells, and sphingosine restored growth to 55% of control to 1.57 × 10^6 cells. Similarly, 3 μM sphingosine reduced TPA-induced adherence from 45% to 30% of the total cells. Table III (B) demonstrates the effect of a higher concentration of sphingosine (4.5 μM) on 3 nM TPA-stimulated events. Again, this concentration of sphingosine did not directly affect cell growth and restored growth in TPA-treated incubations to 75% of control (N = 5). This concentration of sphingosine was also slightly more effective than 3.0 μM to reverse TPA-induced cellular adherence. These concentrations of sphingosine did not affect cell viability as assessed by exclusion of trypan blue. However, concentrations greater than 4.5 μM increased cell permeability to the dye. Further, as reported by Merrill et al. (4) and as demonstrated for sphingomyelinase, sphingosine directly affected cell growth after 24 h. At all concentrations that were effective in inhibiting phorbol ester-stimulated events, sphingosine delayed growth after 24 h. This occurred even if cells were resuspended in medium without the sphingoid base beginning at 24 h. Thereafter, cell growth recovered to the usual level. These studies indicate that sphingomyelinase and the sphingoid base sphingosine have very similar effects on growth and adherence of resting and phorbol ester-stimulated HL-60 cells.

Additional studies measured the effects of sphingomyelinasen on the morphologic changes associated with phorbol ester-induced conversion of these cells into macrophages. As shown in the left-hand panel of Fig. 4, unstimulated HL-60 cells have the typical appearance of promyelocytes. These cells have a large nucleus to cytoplasmic ratio of 3–4:1, a uniform appearance, and a comparatively small size. The cell shape is usually round as is the shape of the nucleus. One or two nucleoli are often apparent as are small azurophilic granulations. Sphingomyelinase had little effect on cell morphology. However, TPA (0.5 nM) treatment for 24 h resulted in dramatic alterations in morphology. As exemplified in the middle panel, a second population, representing a little less than half of the cells, developed with a different morphology. These cells have significantly more cytoplasm, often with a nuclear to cytoplasmic ratio of 1:1 or 1:2. This population is also larger and has greater variability in cell shape. Many cells have one or more cytoplasmic projections. The nuclei are often eccentric and invaginated. Further, the cytoplasm is less basophilic and contains few azurophilic granulations. The right-hand panel of Fig. 3 demonstrates the predominant morphology of HL-60 cells co-incubated with TPA and sphingomyelinase. As is apparent from the photograph, these cells are morphologically indistinguishable from the original resting cells shown in the left-hand panel. Hence, sphingomyelinase treatment prevented the phorbol ester-induced development of the macrophage morphology.

**Table III**

| Effect of sphingosine on growth and adherence of TPA-stimulated HL-60 cells |
|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
|                               | Control                        | Sphingosine                   | TPA                          | TPA + sphingosine             |
| A. Cells/dish (× 10^4)         | 2.04 ± 0.14                    | 1.90 ± 0.15                   | 1.25 ± 0.07                   | 1.57 ± 0.09                   |
| % adherence                   | 4 ± 1                          | 6 ± 1                         | 45 ± 1                       | 30 ± 4                       |
| B. Cells/dish (× 10^4)         | 1.82 ± 0.10                    | 1.72 ± 0.05                   | 1.23 ± 0.09                   | 1.82 ± 0.12                   |
| % adherence                   | 3 ± 1                          | 8 ± 1                         | 46 ± 3                       | 25 ± 3                       |

* Significantly different from control (p < 0.001).
* Significantly different from TPA (p < 0.025).
* Significantly different from TPA (p < 0.005).
Conversion of HL-60 cells into macrophages is also accompanied by the development of specific enzymatic markers including acid phosphatase and α-naphthyl acetate esterase. TPA (0.5 nM) stimulated a 4-fold increase in acid phosphatase activity from 0.72 to 2.85 units/10^6 cells over 24 h (N = 4). Sphingomyelinase (0.75 × 10^{-4} unit/ml) did not affect the basal acid phosphatase activity. However, this concentration of sphingomyelinase partially prevented the increase induced by TPA. In incubations containing both TPA and sphingomyelinase, the level of acid phosphatase activity increased to 2.05 units/10^6 cells, which is 40% less of an increase than with TPA alone (p < 0.05 versus TPA alone). In two studies, a higher concentration of sphingomyelinase (3 × 10^{-4} unit/ml), which incompletely restored cell growth, further reduced the effect of TPA to 1.39 units/10^6 cells. Similarly, sphingomyelinase treatment prevented the TPA-induced development of α-naphthyl acetate esterase activity (N = 2). In control and sphingomyelinase-treated incubations, there was minimal staining for α-naphthyl acetate esterase activity. In TPA (0.5 nM)-treated incubations, there was a marked staining for this activity, which was mostly blocked by sphingomyelinase. It should be noted that these enzymatic markers as well as the morphologic changes were confined to the cells of the monolayer under all conditions employed. D-Chloroacetate esterase activity, a marker for conversion of HL-60 cells via the myeloid pathway, was not enhanced by any of these manipulations. These studies demonstrate that sphingomyelinase treatment prevents the development of the enzymatic markers as well as the morphologic changes, growth inhibition, and cellular adherence associated with phorbol ester-induced conversion of these cells into macrophages.

**DISCUSSION**

Prior studies performed in GH3 pituitary cells demonstrated that the action of a sphingomyelinase, presumably via generation of sphingoid bases, was sufficient to inactivate protein kinase C. Similarly, sphingomyelinase generated sphingoid bases and inactivated protein kinase C in HL-60 cells. The present studies also demonstrate that sphingomyelinase inhibited phorbol ester-induced differentiation of HL-60 cells into macrophages. This was measured in four different ways. Sphingomyelinase blocked the growth inhibition, adherence to the substratum, morphologic changes, and the development of the enzymatic markers associated with the macrophage phenotype. This effect of sphingomyelinase appeared to be specific since sphingomyelinases from different sources stimulated similar effects, and this effect was not mimicked by a potent phospholipase A2. Further, this action appears to be intrinsic to the enzymatic activity in the commercial sphingomyelinase preparation since boiling abolished the effect. These studies also corroborate the inhibition by the sphingoid bases of phorbol ester-induced conversion of HL-60 cells into macrophages as observed by Merrill et al. (4). Thus, sphingomyelinase and the free sphingoid bases not only similarly inactivate protein kinase C (7), but also an event known to be mediated via the protein kinase C pathway.

Prior studies demonstrated that phorbol ester-induced differentiation of HL-60 cells into macrophages was mediated via protein kinase C (9-11, 15). This was evidenced by the specificity of this effect for those phorbol esters that are direct activators of protein kinase C (10). Further, phorbol esters did not induce differentiation in HL-60 cell variants in which they failed to stimulate translocation of the inactive cytosolic form of protein kinase C to the membrane (15). The studies of Merrill et al. (4) and the present studies that demonstrate that inhibitors of protein kinase C prevent the phorbol ester effect also support this notion. However, it has often been reported that the other class of protein kinase C activators, the 1,2-diacylglycerols, failed to mimic phorbol ester-induced conversion of HL-60 cells into macrophages (16-18). In the past, it had been suggested that this resulted from rapid metabolism of 1,2-diacylglycerols. However, addition of 1,2-diacylglycerols as often as every 2 h failed to remedy this problem (17, 18). Whether the failure of 1,2-diacylglycerols to mimic phorbol ester-induced differentiation of HL-60 cells may result from sphingomyelinase activation is currently under examination. In this regard, preliminary studies demonstrate that 1,2-diacylglycerols stimulate degradation of cholesterol-containing phospholipids in HL-60 cells, similar to their effect in GH3 cells (8).

In summary, these studies suggest that the action of a sphingomyelinase, perhaps via the generation of free sphingoid bases, may be sufficient to comprise a physiologically relevant inhibitory pathway for protein kinase C. Whether sphingomyelinase action may be regulated during physiologic stimulation and conceivably function as a negative feedback mechanism for the protein kinase C pathway is presently under investigation.

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**REFERENCES**

10. 1,2-Diacylglycerols stimulate degradation of phosphatidycholine and sphingomyelin in GH3 cells by a phospholipase A2 and a sphingomyelinase, respectively (8). Similarly, the 1,2-diacylglycerol, 1,2-diacetylamlgycerol (diCa), stimulated the coordinate degradation of these phospholipids and the release of metabolites into the medium of HL-60 cells. A maximal concentration of diCa (30 μg/ml) reduced phosphatidycholine and sphingomyelin levels to 60% of control by 1 h; EC50 = 5 μg/ml. Studies are in progress to determine if the mechanisms involved in HL-60 cells are the same as those reported for GH3 cells (R. N. Kolesnick and S. Clegg, unpublished results).