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Translocation of protein kinase C in human leukemia cells susceptible or resistant to differentiation induced by phorbol 12-myristate 13-acetate

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ABSTRACT **We investigated the possible relationship between the susceptibility of cells to differentiation induced by phorbol 12-myristate 13-acetate (PMA) and the subcellular translocation of calcium- and phospholipid-dependent protein kinase (protein kinase C) activity from the cytosol to the membrane. These two events were analyzed in a number of human leukemia cell lines, including four cell variants of the promyelocytic cell line HL-60 that exhibit different degrees of susceptibility to PMA-induced differentiation. The phenotype of the differentiated cells was characterized by increased reactivity with monoclonal antibodies against maturation-specific cell surface antigens, increased nonspecific esterase activity, and acquisition of morphological cell maturation. Analysis of the subcellular distribution of protein kinase C activity in each of these cell types revealed that 90% of the kinase activity was present in the cytosolic fraction, with the remaining activity in the membrane fraction. Treatment of the differentiation-susceptible cells with 160 nM PMA resulted, within 5 min after treatment, in a >60% decrease in protein kinase C activity in the cytosolic fraction and a >1500% increase in the activity in the membrane fraction. No such subcellular redistribution of protein kinase C activity was found after treatment of the differentiation-resistant cells. On the basis of these findings, we suggest that the process of subcellular translocation of protein kinase C activity, initiated after the binding of PMA to this kinase, is required for the induction of cell differentiation by this phorbol diester.**

Tumor-promoting phorbol diesters, including phorbol 12-myristate 13-acetate (PMA), can bring about morphologic, antigenic, biochemical, and functional changes in some cultured mammalian cells (1). In a number of these cell types, PMA can inhibit cell replication and induce cell differentiation (2–8); for example, in cells derived from human HL-60 promyelocytic leukemia (9), PMA induces the cells to acquire a mature phenotype that resembles that of macrophages (4, 5, 10, 11).

The biological activity of the phorbol diesters begins when they bind to specific cellular receptors (12–14). Several lines of evidence have demonstrated that this receptor is a calcium- and phospholipid-dependent protein kinase termed protein kinase C (15–18). Kraft and coworkers (19, 20) reported that treatment of intact cells with phorbol diesters results in a translocation of this protein kinase from the cytosolic to the membrane fraction of the treated cells. Other studies have shown that phorbol diesters with active tumor-promoting activity induce this intracellular translocation, whereas inactive derivatives do not (21). These studies implicate translocation of protein kinase C in the facilitation of the biological effects of phorbol diesters and a number of growth factors and hormones whose biological activity is mediated by protein kinase C (22–26).

To study the mechanism of PMA-induced cell differentiation, and to analyze the role of protein kinase C translocation in this event, it is instrumental to use cell variants resistant to PMA-induced cell differentiation. These types of cell variants have been isolated from the HL-60 cell line (10, 11, 27). Cells from these PMA-resistant variants differ from the susceptible parental cells in a number of biochemical and biophysical cell functions including membrane fluidity (28), down-regulation of specific binding of phorbol esters (13), and PMA-dependent protein phosphorylation (29).

In the present studies, we have examined the relationship between cell susceptibility to PMA-induced differentiation and the subcellular distribution of protein kinase C activity in a series of human leukemia cell lines, including three stable PMA-resistant HL-60 cell variants.

MATERIALS AND METHODS

Chemicals and Reagents. Adenosine 5′-[γ-^{32}P]triphosphate (4000 Ci/mmol; 1 Ci = 37 GBq) was purchased from ICN. Phosphatidylserine, diolein, ATP, histone type III-S, EGTA, phenylmethylsulfonyl fluoride, and dithiothreitol were purchased from Sigma. PMA was purchased from Chemicals for Cancer Research (Eden Prairie, MN), and phorbol 12,13-dibutyrate (PBt_2) and [^{3}H]PBt_2 were purchased from Life System (Newton, MA). 1,25-Dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] was a gift from M. Uskokovic (Hoffman–La Roche). Glass-fiber filters (GF/C) were from Whatman. The murine OKM1 and OKT monoclonal antibodies were obtained from Ortho Pharmaceutical.

Cells, Culture Conditions, and Induction of Cell Differentiation. Clone HL-205 was isolated from the human leukemia HL-60 cells originally provided by R. C. Gallo (National Cancer Institute, Bethesda, MD). HL-60 cells designated HL-525 and HL-534 were obtained by cloning HL-60 cells that had been subcultured 102 times in the presence of increasing concentrations (up to 3 μM) of PMA at 5- to 8-day intervals. Cell variants used in previous studies (10, 28, 29) were obtained from the same HL-60 cells, but after fewer subcultures and without cell cloning. Another HL-60 cell clone, HL-402, was isolated from B-II cells (30) obtained from D. W. Kufe (Dana-Farber Cancer Institute, Harvard Medical School, Boston). These variant HL-60 cell clones exhibited stable phenotypes in regard to their susceptibility or resistance to induction of cell differentiation by either PMA or 1,25-$(OH)_2D_3$ for at least 50–60 subcultures (200–300 cell generations).

The human monocytic THP-1 and the myeloblastic-promyelocytic ML-2 human leukemia cells (31, 32) were

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Abbreviations: PMA, phorbol 12-myristate 13-acetate; PBt_2, phorbol 12,13-dibutyrate; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3.

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obtained from J. Minowada (Veteran's Administration Medical Center, Hines, IL). Human T-lymphoid CEM-2 leukemia cells (8) were cloned from CEM cells originally obtained from the American Type Culture Collection.

Cells for the differentiation studies were inoculated into 60-mm tissue culture dishes and for protein kinase C determinations into 100-mm dishes (Falcon) at 1.5×10^5 cells per ml of RPMI 1640 medium supplemented with 20% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) (GIBCO) and were incubated at 37°C in an atmosphere of 8% CO_2 in air in a humidified incubator. Fresh PMA and 1,25-$(OH)_2D_3$ solutions were prepared for each experiment from a stock solution (1 mg/ml in dimethyl sulfoxide or ethanol) stored at −20°C. Dimethyl sulfoxide or ethanol at a final concentration of 0.2% in the growth medium did not affect protein kinase C activity or the expression of the various differentiation markers.

Induction of cell differentiation in the treated cells was confirmed by reaction with either OKM1 or OKT monoclonal antibodies [which recognize maturation-specific cell surface antigents (8, 11, 33, 34)], the presence of nonspecific esterase (35), or morphological cell maturation (11).

Subcellular Fractionation. This was a modification of the procedures described by Elias and Stuart (36). Prior to subcellular fractionation, $2–8 \times 10^7$ cells for each time point were washed three times with Dulbecco's phosphate-buffered saline (without Ca^{2+} and Mg^{2+}). The cells were then ruptured in 2 ml of homogenization buffer (20 mM Tris·HCl, pH 7.5/0.25 M sucrose/2 mM EDTA/0.5 mM EGTA/0.2 mM phenylmethylsulfonyl fluoride) by Dounce homogenization and centrifuged immediately at $2000 \times g$ for 10 min. The supernatant was collected and centrifuged at $105,000 \times g$ for 1 hr to separate cytosolic and membrane fractions. The membrane fraction was subsequently resuspended in extraction buffer and treated with 1% (vol/vol) Nonidet P-40. All procedures were carried out at 4°C. Of the total cellular protein, the cytosolic fraction contained 15% and the membrane fraction 10% (e.g,. 170 and 130 μg of protein per 10^7 HL-205 cells were in the two fractions, respectively).

PBt_2 Binding to Cellular Receptors. PBt_2 binding was assayed by a fiberglass filtration method (37). The reaction mixture, containing 25 mM Tris·HCl buffer (pH 7.3), 10 mM $Mg(OAc)_2$, 1.4 mM $CaCl_2$, 4 mg of bovine serum albumin per ml, 100 μg of phosphatidylserine per ml, 20 nM [^{3}H]PBt_2 (with or without 3 μM PMA), and 40 μl of cell homogenate in a total volume of 440 μl, was incubated for 2 hr on ice. Bound [^{3}H]PBt_2 was separated from free [^{3}H]PBt_2 by addition of 1 ml of 20 mM Tris·HCl, pH 7.5/10 mM $Mg(OAc)_2$/1 mM $CaCl_2$ and passage of the mixture through a Whatman GF/C glass filter. The filters were washed with the buffer and were assayed for radioactivity in a Packard Tricarb 300 scintillation spectrometer. Specific binding was calculated as total binding minus nonspecific binding observed in the presence of 3 μM PMA.

Protein Kinase Assay. Protein kinase C activity was assayed after a 5-min incubation at 30°C of a reaction mixture containing 25 mM Tris·HCl (pH 7.5), 200 μg of histone type III-S per ml, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 20 μM ATP, 1 μCi of [γ-^{32}P]ATP, and 0.1 μM phenylmethylsulfonyl fluoride, with or without phosphatidylserine (50 μg/ml) and diolein (0.5 μg/ml), in a total volume of 100 μl. Prior to their use in the assay, the solvents for the phosphatidylserine and diolein were evaporated under nitrogen gas and the residue was dispersed in water by sonication. The reactions were terminated by the addition of 1 ml of 10% (wt/vol) trichloroacetic acid/5 mM Na_2HPO_4 (stopping solution), followed by 50 μl of 1% bovine serum albumin. Precipitates were sedimented by centrifugation, redissolved in 1 M NaOH, reprecipitated with stopping solution, collected, and washed onto glass fiber filters for measurement of radioactivity in a Packard Tricarb 300 scintillation spectrometer. All samples were assayed in duplicate.

RESULTS

PMA-Induced Cell Differentiation in Cultured Human Leukemia Cells. Four HL-60 cell clones (variants) that exhibit different susceptibilities to PMA-induced cell differentiation were isolated to study the relationship between the susceptibility of cells to PMA-induced cell differentiation and the subcellular translocation of protein kinase C. Treatment of cells from the HL-205 variant with either 3 nM or 3 μM PMA for 6 days caused the cells to acquire a mature phenotype resembling that of macrophages, whereas treated cells from the other three cell variants (HL-525, HL-534, and HL-402) remained undifferentiated (Table 1). The mature phenotype in the HL-205 cells was defined by an increase in cell reactivity with OKM1 monoclonal antibody, staining for nonspecific esterase activity, and acquisition of a morphologically mature phenotype (Table 1), as well as by attachment of the cells to the surface of tissue culture dishes (data not shown). All of these properties are typical of peripheral macrophages and monocytes (11, 33, 35). We also tested the response of the four cell variants to another inducer of cell differentiation, 1,25-$(OH)_2D_3$. Treatment of cells from the PMA-susceptible HL-205 variant and from the PMA-resistant HL-525 and HL-534 variants with 1,25-$(OH)_2D_3$ caused the cells to acquire a monocyte-like phenotype (Table 1). These results indicate that the HL-525 and HL-534 cells have not lost their ability to differentiate but rather were resistant specifically to differentiation induced by PMA. In contrast, cells from the PMA-resistant HL-402 variant were also resistant to the induction of cell differentiation by 1,25-$(OH)_2D_3$.

We tested another four human leukemia cell lines, in addition to the four HL-60 cell variants, for their susceptibility to PMA-induced cell differentiation. These cell lines included the parental HL-60, the myelocytic ML-2, the monocytic THP-1, and the T-lymphoid CEM-2 leukemia cell lines. Treatment of the HL-60, ML-2, and THP-1 cell lines with 3 nM PMA for 6 days caused the cells to acquire a mature phenotype resembling that of macrophages, whereas this treatment caused the CEM-2 cell line to acquire a phenotype resembling that of mature suppressor T lymphocytes (8, 11, 13, 32).

PMA-Mediated Translocation of Specific Binding of [^{3}H]PBt_2 from the Cytosol to the Membrane Fraction. In

Table 1. Induction of differentiation markers in HL-60 cell variants, measured 6 days after treatment with either PMA or 1,25-$(OH)_2D_3$

Inducer	Conc.	% positive cells			
		HL-205	HL-525	HL-534	HL-402
Reactivity with OKM1 antibody					
None	—	8	≤5	≤5	≤5
PMA	3.0 nM	≥95	≤5	≤5	≤5
PMA	3.0 μM	≥95	≤5	≤5	≤5
1,25-$(OH)_2D_3$	0.3 μM	≥95	82	90	≤5
Morphological differentiation					
None	—	≤10	≤10	≤10	≤10
PMA	3.0 nM	83	≤10	≤10	≤10
PMA	3.0 μM	88	≤10	≤10	≤10
1,25-$(OH)_2D_3$	0.3 μM	≥95	≥95	≥95	≤10
Staining for nonspecific esterase					
None	—	12	≤5	≤5	≤5
PMA	3.0 nM	≥95	≤5	≤5	≤5
PMA	3.0 μM	≥95	≤5	≤5	≤5
1,25-$(OH)_2D_3$	0.3 μM	≥95	76	62	≤5

preliminary experiments, we tested the distribution of specific binding of [^{3}H]PBt$_2$ to its receptor, protein kinase C, in the cytosolic and membrane fractions obtained from the four HL-60 cell variants that are either susceptible or resistant to PMA-induced cell differentiation. The subcellular fractions were prepared both from control cells and from cells treated for 5 min with 160 nM PMA. In the absence of PMA, all four types of cells exhibit a similar value of total specific binding of [^{3}H]PBt$_2$ to its receptor in both the cytosolic and membrane fractions, with the binding in the cytosolic fraction ranging from 2.4 to 2.9 pmol of bound PBt$_2$ per mg of protein and the binding in the membrane fraction ranging from 0.6 to 0.8 pmol per mg of protein (Table 2). PMA treatment of the differentiation-susceptible HL-205 cells decreased the specific binding of [^{3}H]PBt$_2$ in the cytosolic fraction by about 70% and increased this binding in the membrane fraction by more than 300%. No such changes in specific binding of [^{3}H]PBt$_2$ were observed after PMA treatment in either of the subcellular fractions obtained from the PMA-resistant HL-525, HL-534, or HL-402 cells (Table 2).

These results indicate that PMA causes a translocation of the PBt$_2$ receptor from the cytosol to the membrane in the differentiation-susceptible cells but not the resistant cells.

PMA-Mediated Translocation of Protein Kinase C Activity from the Cytosol to the Membrane Fraction. Analysis of the subcellular distribution of the activity of protein kinase C showed that 90% of the activity was in the soluble fraction of the various leukemia cells. The cytosolic fractions obtained from the HL-60 cell variants catalyzed the formation of similar amounts of products, ranging from 2.4 to 2.9 nmol of phosphorylated residues per min per mg of protein (Table 3).

Incubation of the differentiation-susceptible HL-205 cells with 160 nM PMA resulted in a time-dependent reduction in the activity of protein kinase C in the cytosolic fraction. This activity began to decrease within 2 min after the beginning of PMA treatment and reached its lowest level (40% of control level) 5–10 min after treatment (Table 3, Fig. 1). The decrease in the cytosolic kinase C activity after 5 min of treatment with 1.6–160 nM PMA was dose-dependent (data not shown). In contrast, PMA caused little or no decrease in the cytosolic kinase C activity in the PMA-resistant HL-525, HL-534, and HL-402 cells (Table 3, Fig. 1).

Concomitant with the decrease in the cytosolic protein kinase C activity in the PMA-treated differentiation-susceptible cells, we observed a significant increase in this activity in the membrane fraction. Treatment of HL-205 cells with 160 nM PMA for 5 min resulted in a >15-fold increase in protein kinase C activity in the membrane fraction (Table 3, Fig. 1). This increase in membrane-associated protein kinase C was also time- (Fig. 1) and dose-dependent (data not shown). In contrast, no significant increase in PMA-induced membrane-associated protein kinase C was observed in the HL-525, HL-534, or HL-402 cells, which are resistant to PMA-induced cell differentiation (Table 3, Fig. 1).

We also investigated the possibility that the differences between the susceptible and resistant cells in the subcellular distribution of protein kinase C activity after PMA treatment may result from the presence of enzyme inhibitors. We added cytosolic and membrane fractions from either untreated or PMA-treated HL-525 cells to those derived from either untreated or PMA-treated HL-205 cells, and vice versa, and then tested for protein kinase C activity. The results indicated that adding the fractions from the resistant cells to those of the susceptible cells and, reciprocally, adding fractions from the susceptible to those from the resistant cells did not alter the protein kinase C activities in the different cell fractions but rather yielded the expected mean values for the different mixtures. The PMA-induced reduction of the cytosolic and increase of the membranous protein kinase C activity in the susceptible HL-205 cells was also not caused by a direct effect of PMA on the enzyme activity, because addition of 160 nm PMA to the reaction mixture containing either fraction did not alter the kinase C activity.

We also analyzed the subcellular translocation of protein kinase C in HL-60, ML-2, THP-1, and CEM-2 cells, which are also susceptible to the induction of cell differentiation by PMA. The results indicated that treatment of these cells for

Table 2. Subcellular distribution of specific binding of [^{3}H]PBt$_2$ to its receptors in control and PMA-treated HL-60 cell variants

Fraction	PMA	Specific binding, pmol/mg of protein			
		HL-205	HL-525	HL-534	HL-402
Cytosol	−	2.9	2.7	2.6	2.4
	+	0.9	2.4	2.4	2.2
Membrane	−	0.6	0.8	0.8	0.7
	+	1.9	0.9	1.0	0.7

Specific binding of [^{3}H]PBt$_2$ to its receptors in the subcellular fractions was measured 5 min after treatment with or without 160 nM PMA.

Table 3. Subcellular distribution of protein kinase C activity in control or PMA-treated cells

Fraction	PMA	Kinase activity, nmol per min per mg of protein			
		HL-205	HL-525	HL-534	HL-402
Cytosol	−	2.9 ± 0.6	2.4 ± 0.4	2.6 ± 0.5	2.4 ± 0.3
	+	1.1 ± 0.4*	1.8 ± 0.3	2.2 ± 0.3	2.1 ± 0.3
Membrane	−	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.1
	+	3.1 ± 0.7*	0.5 ± 0.3	0.3 ± 0.2	0.2 ± 0.1

Protein kinase C activity was assayed in cytosolic and solubilized membrane fractions of cells treated for 5 min with or without 160 nM PMA. In the absence of phosphatidylserine and diolein the protein kinase activity in the cytosolic fractions was 1.8 ± 0.4 nmol per min per mg of protein, and in the membranous fractions, 5.9 ± 1.2 nmol per min per mg of protein. The results are the mean value of at least three independent experiments ± SD.
*Statistically significant compared with control ($P < 0.05$).

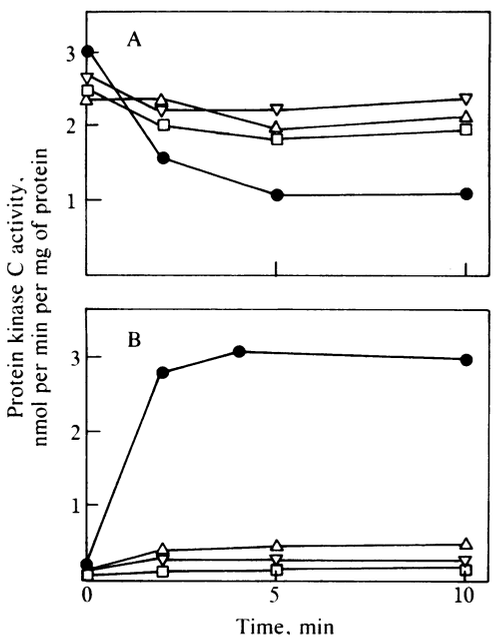

FIG. 1. Protein kinase C activity in the cytosolic (*A*) and membrane (*B*) fractions of susceptible (●, HL-205) and resistant HL-60 variant cells (△, HL-525; ▽, HL-534; □, HL-402) treated with 160 nM PMA for various times.

5 min with 160 nM PMA resulted in a translocation of protein kinase C similar to that observed in the HL-205 cells, which are also susceptible to PMA-induced cell differentiation. In brief, our results indicate that PMA induces a rapid translocation of protein kinase C activity from the cytosol to the membrane fraction in cells susceptible to PMA-induced cell differentiation but not in cells resistant to such an induction.

DISCUSSION

Induction of cell differentiation by phorbol diesters and some related chemicals begins when they bind to a high-affinity and saturable receptor (11–14), which is a calcium- and phospholipid-dependent kinase (protein kinase C) (15–18). This binding is believed to activate the protein kinase, resulting in the phosphorylation of various cellular proteins (38–40), including some that reside in or around the nucleus (29). On the basis of these results, it was suggested (29) that induction of cell differentiation by PMA may require the migration of protein kinase C (PMA receptor) to the vicinity of the nucleus (nuclear membrane) where, through phosphorylation of regulatory proteins, the kinase causes the appropriate alterations in gene expression. These speculations can be tested by analyzing the subcellular distribution of protein kinase C in cells that are either susceptible or resistant to induction of differentiation by the phorbol esters.

We have, therefore, included in the present studies a series of human leukemia cells that differ in their susceptibility to induction of cell differentiation by PMA. PMA treatment of five different cell types, including HL-205 cells, all of which are susceptible to PMA-induced cell differentiation, resulted in a translocation of protein kinase C activity from the cytosol to the membrane fraction. In contrast, none of the three HL-60 cell variants that are resistant to PMA-induced cell differentiation exhibited such a translocation after PMA treatment.

The change in the subcellular distribution of protein kinase C may represent either a true translocation of the enzyme or the conversion of a loose association of protein kinase C with the membrane to a tight association (41, 42). The translocation of protein kinase C could be detected within 2 min after PMA treatment, a time shorter than that required to saturate the phorbol diester receptors by its ligands (13). These results suggest that the binding of phorbol ester to its receptor is coupled with translocation of protein kinase C to the membrane fraction that presumably also contains nuclear components. This association with this membrane fraction, which is rich in phospholipids, may be a key factor in enzyme activation.

On the basis of our results, we suggest that the translocation of protein kinase C activity, which occurs shortly after PMA treatment, is required, although not necessarily sufficient (43), for the induction of cell differentiation by PMA, as well as for other biological activities induced by phorbol diesters in these and various other cell types.

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