Effect of Retinoic Acid on Phorbol Ester-stimulated Differentiation and Protein Kinase C-dependent Phosphorylation in the U937 Human Monoblastoid Cell

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ABSTRACT

Phorbol esters stimulate differentiation of certain human leukemic cell lines. Although activation of protein kinase C may mediate certain effects of phorbol esters, controversy exists as to the role of protein kinase C activation in phorbol ester-induced differentiation. Retinol acid modulates responses to phorbol esters in several cell types. Retinoic acid has also been found to alter protein kinase C-dependent phosphorylation in leukemic cells. We correlated the effects of retinoic acid on protein kinase C-dependent phosphorylation and differentiation stimulated by 12-O-tetradecanoylphorbol-13-acetate (TPA), a phorbol ester, in the human monoblastoid U937 cell line. At concentrations <1 nM, which were 100-fold less than those directly stimulating differentiation, retinoic acid potentiated TPA-induced differentiation of the U937 cell as assessed by enhanced adherence to plastic and acquisition of nonspecific esterase activity. TPA-stimulated decreases in cellular proliferation were not affected by retinoic acid treatment. Without altering the sensitivity to TPA, retinoic acid increased the maximal response to this agent. Retinoic acid enhanced TPA-stimulated phosphorylation of a Mr, 48,000 substrate in intact 32P-labeled U937 cells and also increased the protein kinase C-dependent phosphorylation of a similar Mr, 48,000 substrate and a Mr, 80,000 substrate in cellular extracts. In cellular extracts the retinoic acid-induced enhancement of protein kinase C-dependent phosphorylation was predominantly localized to the cytosolic fraction. Increases in protein kinase C-dependent phosphorylation were evident within a 12-h exposure to 1 nM retinoic acid and were observed at retinoic concentrations of 0.01 to 1 nM. A retinoic acid-induced increase in the protein kinase C-dependent phosphorylation of an exogenous substrate, histone, was observed following diethylaminoethyl extraction of cytosol, but not a solubilized particulate fraction. The conditions of retinoic acid treatment increasing protein kinase C activity and enhancing protein kinase C-dependent phosphorylation of endogenous substrates were similar to those conditions potentiating phorbol ester-induced differentiation. Thus, the retinoic acid-induced amplification of phorbol ester signal transduction at the level of protein kinase C activation could mediate the effects of this vitamin on phorbol ester-induced differentiation.

INTRODUCTION

Retinoic acid stimulates differentiation of epithelial cells and other cell types (1–5). Retinoic acid stimulates the human monoblastoid cell line, U937, and the promyelocytic cell line, HL-60, to express phenotypic characteristics of more terminally differentiated cells (6–9). In addition to this direct effect, retinoic acid synergistically enhances U937 and HL-60 cell differentiation in response to dibutyryl cyclic AMP and lymphokines (7, 8).

Phorbol esters are another group of agents which induce differentiation of human leukemic cells (10–13). In the mouse epidermis tumor promotion model, retinoic acid inhibits phorbol ester-induced increases in ornithine decarboxylase activity and inhibits tumor promotion (14, 15). The inability of retinoic acid to inhibit tumor formation in response to non-phorbol ester tumor promoters suggests that retinoic acid interacts in a specific manner with the mechanisms responsible for phorbol ester-stimulated tumor production (16, 17). Retinoic acid also inhibits the effects of phorbol esters in other cell types (18, 19). In certain cell types, rather than inhibiting, retinoic acid enhances phorbol ester-stimulated cellular events (20, 21). Thus, in a variety of cells, retinoic acid modulates the effects of phorbol esters.

In response to treatment with phorbol esters, the U937 cell differentiates into a monocyte-macrophage-like cell (22, 23). One possible mechanism by which phorbol esters exert an effect on cellular function is through activation of the calcium-phospholipid-dependent protein kinase, protein kinase C. By substituting for diacylglycerol, an endogenous protein kinase C activator, phorbol esters activate protein kinase C (24). As evidenced by the ability of exogenously added diacylglycerol analogues to mimic certain effects of phorbol esters, protein kinase C activation may mediate at least a portion of the effects of phorbol esters on cellular function (25–31). However, controversy exists as to whether activation of protein kinase C is a sufficient stimulus to induce leukemic cell differentiation. Most studies (32–35), but not all (36), have demonstrated that the sole addition of diacylglycerol analogues does not stimulate leukemic cell differentiation. The inability of diacylglycerol analogues to mimic all of the effects of phorbol esters has also been demonstrated in other cell types (37–43).

To gain insight into the role of protein kinase C activation in mediating phorbol ester-induced leukemic cell differentiation, we examined the effects of retinoic acid on protein kinase C-dependent phosphorylation and phorbol ester-induced differentiation in the U937 leukemic cell line.

MATERIALS AND METHODS

Materials. DMEM: Ham’s F-12, Eagle’s minimal essential medium without glucose and phosphates, fetal bovine serum, penicillin, and streptomycin were purchased from Grand Island Biological Co. [γ-32P]ATP and [35S]poly(A)-containing RNA were purchased from New England Nuclear. P-81 papers and DEAE-cellulose were obtained from Whatman. Scintiverse and molecular weight standards for gel electrophoresis were purchased from Fisher and Bio-Rad, respectively. l-1-Tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin was obtained from Worthington. Trans-retinoic acid and all other chemicals were purchased from Sigma.

Cell Culture Conditions. Characteristics of the U937 cell, derived from a patient with histiocytic lymphoma, have been described previously (44). Cells were passaged every 2 to 3 days in medium consisting of DMEM:Ham’s F-12 medium (1:1) supplemented with 5% fetal bovine serum, 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 100 μM L-phenylalanine, and 100 μg/ml streptomycin. Cells were passaged every 2 to 3 days in medium consisting of DMEM:Ham’s F-12 medium (1:1) supplemented with 5% fetal bovine serum, 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 100 μM L-phenylalanine, and 100 μg/ml streptomycin. Cells were

Received 3/21/88; revised 7/12/88; accepted 7/15/88.

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1 This work was supported by a Charles E. Culpepper Foundation Fellowship and grants from the USPHS (AM 31683) and the National Cancer Institute (CA 43823).

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3 The abbreviations used are: DMEM, Dulbecco’s modified Eagle’s minimal essential medium; EGTA, ethylene glycol bis-[β-aminoethyl ether]-N,N,N’,N’-tetraacetic acid; SDS, sodium dodecyl sulfate; PMSF, phenylmethylsulfonyl fluoride; TPA, 12-O-tetradecanoylphorbol-13-acetate; MeSO2, dimethyl sulfoxide; ED50, 50% effective dose.

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incubated at 37°C in humidified 5% CO2:95% air. Viability was deter-
mimed by trypsin blue exclusion. Cells were greater than 90% viable
prior to all experimental treatments. When added, retinoic acid was
dissolved in ethanol, while TPA was dissolved in Me2SO. The maximal
concentrations of ethanol and Me2SO exposed to the cells were <0.01% 
and <0.001%, respectively. These solvent concentrations did not affect 
cellular growth, protein kinase C activity, or endogenous substrate 
phosphorylation.

Assessment of Differentiation. Measurement of growth and adherence 
was performed as described elsewhere (45). U937 cells (107/well) were 
cultured in 24-well plates in 1 ml of DMEM/Ham’s F-12 medium 
supplemented with 5% (v/v) fetal bovine serum and where indicated retinoic acid and TPA. After a 72- or 96-h incubation period, the
nonadherent cells contained in the medium and the cells adherent to 
the plastic well were separated. After lysis, the adherent and non-
adherent nuclei were counted using a Coulter D2N counter. The addi-
tion of adherent and nonadherent nuclei gave the total cell number. 
Using reagents obtained in a kit from Sigma, nonspecific esterase 
activity was measured according to previously described methods (46).

Preparation of Cellular Fractions. Cells were pelleted by centrifuga-
resuspended in a buffer containing 20 mM Tris-HCl (pH 7.4), 0.25 mM sucrose, 2 mM EDTA, and 0.5 mM EGTA with 50 μg/ml of PMSF and disrupted with 50 strokes of a tight fitting glass homoge-
nizer. The homogenate was centrifuged at 100,000 × g for 60 min. The
cytosol was removed and the pellet was resuspended in a buffer con-
taining 20 mM Tris-HCl (pH 7.4), 2 mM EDTA, 0.5 mM EGTA, 50 
μg/ml of PMSF, and 0.1% Triton X-100. After a 45-min incubation
at 4°C, the sample was centrifuged at 100,000 × g for 60 min. The
supernatant containing the solubilized particulate fraction was saved. 
In certain experiments cells were homogenized in a buffer containing 
20 mM Tris-HCl (pH 7.4), 2 mM EDTA, 0.5 mM EGTA, 50 μg/ml of PMSF, and 0.1% Triton X-100. After a 45-min incubation at 4°C, the
homogenate was centrifuged at 100,000 × g for 60 min. The superna-
tant, termed the total cell-solubilized extract, was saved. This extract
contained both cytosolic and solubilized particulate fractions. Protein 
content was determined by the method of Lowry et al. (47).

In certain experiments, cellular extracts were applied to a 2- x 0.75-
cm DEAE-cellulose column that had been previously equilibrated with
a buffer containing 20 mM Tris-HCl (pH 7.4), 2 mM EDTA, 200 
μM EDTA, 50 μM EGTA, 1.5 mM calcium chloride, and 1 
μM [γ-32P]ATP (5 μCi/tube) in a total volume of 50 μl. Where indicated,
phosphatidylycerine and diolein were prepared and added at the same
concentrations described for determination of protein kinase C depend-
ent histone phosphotransferase activity. After incubation for 1 min at
27°C, the reaction was terminated by adding 25 μl of a 10% (w/v) SDS
stop solution followed by heating to 110°C for 3 min. One-dimensional
polyacrylamide electrophoresis was performed as described by Laemmli
(50). The stacking and running gels were 3 and 10% acrylamide, respectively. Autoradiography was done utilizing Kodak XAR film at 
−70°C. Concurrent electrophoresis of molecular weight standards al-
lowed assignment of approximate molecular weights to the phospho-
rylated substrates. In certain experiments, 32P incorporation into en-
dogenous substrates was quantitated by densitometric analysis of the 
autoradiogram. The autoradiograms were scanned at 600 nm using a
Gilford spectrophotometer. Autoradiograms, when used for densito-
metric analysis, were derived from gels exposed to X-ray film for a
shorter period of time than the autoradiograms used as figures.

Intracellular Phosphorylation. Cells were suspended in Eagle’s minimal essential medium without glutamine and phosphates for 30 min. 32P,
0.25 μCi/3 × 106 cells, was added for 2 h, after which the cells were
centrifuged and the supernatant was removed. The cells were resus-
pended in fresh phosphate-free medium at a concentration of 3 × 106/
ml.

After a 60-min incubation of the 32P-labeled cells with 0.001% 
Me2SO or TPA at the indicated concentration, the reaction was ter-
minated by adding one-third volume of a 10% (w/v) SDS stop solution
followed by heating to 110°C for 3 min. Electrophoresis, using a 12%
polyacrylamide running gel, was performed. After staining and destain-
ing, the gels were treated with 1 N potassium hydroxide for 1 h at 56°C. 
The KOH treatment was followed by exposure to the destaining solu-
tion for 1 h. The gels were dried and autoradiography was performed.
Alkaline treatment was used to facilitate visualization of TPA-dependent 
substrate phosphorylation.

Tryptic Phosphopeptide Mapping. Protein kinase C-dependent phos-
phorylation was stimulated in 32P-labeled intact cells and cellular ex-
tacts. The reactions were terminated by adding one-third volume of a 
10% (w/v) SDS stop solution followed by heating to 110°C for 3 min.
After electrophoresis, using a 0.75-mm slab gel, autoradiography
was performed on the wet gel. Using the autoradiogram as a template, the
phosphorylated M, 48,000 substrate was excised from the gel. Gel slices 
were dehydrated with 4 changes of acetone (15 min each) and reswollen
in 50 mM ammonium bicarbonate, pH 7.0, for 10 min. After two quick
washes with ammonium bicarbonate, the gel piece was incubated over-
night with tumbling in 0.5 ml of 50 mM ammonium bicarbonate 
containing 1 mg/ml 1-L-lysino-2-phenyl ethyl chloromethyl ket-
one-treated trypsin. Supernatants were collected and gel pieces were
washed once with additional ammonium bicarbonate. The combined
supernatants contained greater than 85% of the 32P. Tryptic phospho-
peptide fragments were separated using a C18-MBondapak column (Waters). The samples were eluted with a linear 0–60% acetonitrile
gradient in 0.1% trifluoroacetic acid, pH 2.0. The protein pattern of the
added trypsin was monitored at 214 nm. One hundred 0.5-ml fractions
were collected at a flow rate of 0.5 ml/min. 32P associated with the
phosphopeptides was detected on line using a Radiometric Flo-<wbr/>one with a BD flow cell (500 μl volume). Data were plotted using Dysc-
Application software.

RESULTS

At micromolar concentrations retinoic acid stimulates U937
differentiation as assessed by increases in the number of cells 
adhering to plastic surfaces and decreases in cellular prolifera-
Fig. 1). However, when added at concentrations less than

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100 nM, retinoic acid did not significantly stimulate U937 differentiation as evidenced by a lack of an effect on adherence to plastic or cellular growth (Fig. 1).

At concentrations of 0.1 to 10 nM, TPA stimulated U937 differentiation as demonstrated by an increased number of cells adherent to plastic (Fig. 2). Exposure to 1 nM retinoic acid for 24 h prior to and during the 72-h incubation with TPA enhanced the phorbol ester-induced increases in cellular adherence (Fig. 2). While retinoic acid increased the maximal response approximately 4-fold, the approximate ED50 for TPA-stimulated adherence to plastic was similar, 0.3 nM, in both vehicle- and retinoic acid-treated cells. Increases in TPA-stimulated adherence to plastic were observed at retinoic acid concentrations of 0.03 to 1 nM (Fig. 3). Maximal effects on TPA-stimulated differentiation were observed after a 24-h preexposure followed by coincubation of retinoic acid with the phorbol ester. However, continuous exposure to retinoic acid was not required to enhance phorbol ester-induced differentiation since preincubation alone significantly enhanced TPA-stimulated differentiation.

Retinol, a vitamin A analogue having 1/1000th the potency of retinoic acid in other biologic systems (9), was analyzed for effects on TPA-stimulated differentiation. While ineffective at concentrations up to 1 µM when evaluated alone (data not shown), retinol enhanced TPA-stimulated differentiation over a concentration range of 10 to 1000 nM. Retinol potentiated TPA-stimulated differentiation to the same maximal extent as did retinoic acid. The approximate ED50 for potentiating TPA-induced differentiation was 100 nM for retinol as compared to 0.1 nM for retinoic acid. Thus, retinol was approximately 1/1000th as potent as retinoic acid in enhancing TPA-stimulated differentiation.

Under conditions that maximally increased TPA-stimulated adherence to plastic, retinoic acid also enhanced TPA-induced increases in expression of another indicator of differentiation, nonspecific esterase activity (Table 1).

Unlike the synergistic enhancement of other parameters of TPA-induced differentiation, retinoic acid did not potentiate the TPA-induced decrease in cellular proliferation. In four separate experiments total cell number was 12.5 × 10⁴ ± 2.7 (SEM) in cells exposed for 72 h to 10 nM TPA and 18.0 × 10⁴ ± 2.0 in cells exposed to 1 nM retinoic acid for 24 h prior to and during a 72-h incubation with 10 nM TPA (0.05 < P < 0.1, Student's t test). The slight increase in total cell number in retinoic acid-treated cells was due to an increase in the adherent cell number (control, 1.2 × 10⁴ ± 0.3; retinoic acid, 4.7 × 10⁴ ± 1.6). The nonadherent cell numbers were 11.3 × 10⁴ ± 2.9 and 13.3 × 10⁴ ± 3.4 in control and retinoic acid-treated cells, respectively (P > 0.1, Student's t test).

Since activation of protein kinase C and subsequent protein phosphorylation may mediate the cellular responses stimulated by phorbol esters (24), the effect of retinoic acid on TPA-stimulated phosphorylation was examined. Exposure of intact ¹⁵P-labeled cells to 10 nM TPA for 60 min stimulated phosphorylation of a M, 48,000 substrate (Fig. 4A). Prior exposure to 1
Retinoic acid alters protein kinase C.

Fig. 3. Effect of varying retinoic acid concentrations on TPA-stimulated adherence to plastic. U937 cells were incubated with 0.01% ethanol or varying concentrations of retinoic acid as indicated on the abscissa. After a 24-h incubation, 10^7 cells were resuspended in 1 ml of fresh medium containing the same additives to which TPA was added at a concentration of 3 nM. After a 72-h incubation with TPA, the adherent cell number was determined. Points, mean of 6 determinations; bars, SE. Similar results were obtained in a separate experiment. *, P < 0.01 versus vehicle-treated cells. Student’s t test, N = 6.

Table 1 Effect of retinoic acid on TPA-stimulated acquisition of nonspecific esterase activity

Cells were preincubated for 24 h with the additions indicated in Column 1. Following this preincubation, the cells were resuspended in fresh medium containing the additives listed in Column 2. At the end of this incubation, nonspecific esterase activity was determined in duplicate as described in "Materials and Methods."

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Expression of nonspecific esterase activity (% of total cell no.)</th>
</tr>
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<tbody>
<tr>
<td>24 h preincubation</td>
<td>72 h final incubation</td>
</tr>
<tr>
<td>0.01% ethanol</td>
<td>0.001% Me2SO +</td>
</tr>
<tr>
<td>1 nM retinoic acid</td>
<td>0.001% Me2SO + 1 nM retinoic acid</td>
</tr>
<tr>
<td>0.01% ethanol</td>
<td>3 nM TPA + 0.01% ethanol</td>
</tr>
<tr>
<td>1 nM retinoic acid</td>
<td>3 nM TPA + 1 nM retinoic acid</td>
</tr>
</tbody>
</table>

* Mean ± SEM.

** mean 0.01 versus cells preincubated with 0.01% ethanol followed by a 72-h exposure to 3 nM TPA; N = 6 determinations pooled from three separate experiments.

nm retinoic acid for 48 h enhanced TPA-stimulated phosphorylation of the M, 48,000 substrate (Fig. 4A). In 7 separate experiments, TPA-stimulated phosphorylation of the M, 48,000 substrate was increased by a 48-h exposure to 1 nm retinoic acid to 146% of vehicle-treated cells. The basal TPA-independent phosphorylation of the M, 48,000 substrate was not altered by pretreatment with retinoic acid. Pretreatment with retinoic acid increased the maximal phosphorylation of the M, 48,000 substrate stimulated by TPA (Fig. 4B). The approximate ED_{50} for TPA-stimulated phosphorylation of the M, 48,000 substrate was similar, 2 nm, in vehicle- and retinoic acid-treated cells. Following a 48-h preincubation with retinoic acid, an enhancement in TPA-stimulated phosphorylation of the M, 48,000 substrate was clearly discernible at an retinoic acid concentration of 0.3 nM and maximal by a concentration of 1 nM (data not shown).

The effect of retinoic acid on protein kinase C-dependent endogenous substrate phosphorylation in cellular extracts was examined. Addition of protein kinase C activators (calcium, phosphatidylserine, and diolein) to a cellular extract containing cytosolic and solubilized particulate fractions stimulated phosphorylation of several substrates, the most prominent being a substrate with a molecular weight of 48,000 (Fig. 5). Substrates with molecular weights of 80,000, 140,000, and 170,000 were also phosphorylated in response to protein kinase C activation. Pretreatment for 48 h with 1 nM retinoic acid enhanced the protein kinase C-dependent phosphorylation of the M, 48,000 and M, 80,000 substrates but did not alter the protein kinase C-dependent phosphorylation of the M, 140,000 and M, 170,000 substrates (Fig. 5). The direct addition of 0.1 μM retinoic acid to cellular homogenates did not alter the protein kinase C-dependent phosphorylation of endogenous substrates (data not shown).

Increases in the protein kinase C-dependent phosphorylation of the M, 48,000 and M, 80,000 substrates were discernible at a retinoic acid concentration of 0.01 nM and maximal by a concentration of 0.3 nM (Fig. 6A). Increases in the protein kinase C-dependent phosphorylation of the M, 48,000 and M, 80,000 substrates were observed after a 24-h incubation with 1 nM retinoic acid and were maximal after 48- to 72-h exposure (Fig. 6B).

While the basal phosphorylation of the M, 48,000 and M, 80,000 substrates was not affected, retinoic acid did enhance the basal protein kinase C-independent phosphorylation of M, 56,000 and M, 64,000 substrates (Fig. 5).

Retinoic acid-induced increases in protein kinase C-dependent and independent phosphorylations were predominantly localized to the cytosolic fraction (data not shown).

A decrease in phosphatase activity responsible for dephosphorylation of the M, 48,000 and M, 80,000 substrates could produce an apparent increase in the protein kinase C-dependent phosphorylation of these substrates. To examine whether retinoic acid decreased the rate of substrate dephosphorylation, the following experiment was done. 32P incorporation into the substrates was stimulated by adding protein kinase C activators to the cytosolic extracts. Following a 1-min period of 32P incorporation 100-fold excess of unlabeled ATP, 0.1 mM, was added. Sixty s after the addition of unlabeled ATP, the decline in 32P associated with the M, 48,000 and M, 80,000 substrates was minimal and similar between vehicle- and retinoic acid-treated cells (Table 2).

To determine if an identical M, 48,000 substrate in intact cells and cellular extracts was phosphorylated in response to protein kinase C activation, tryptic phosphopeptide maps of the M, 48,000 substrates were compared. Phosphorylation of the M, 48,000 substrate in cellular extracts was stimulated by adding calcium, phosphatidylserine, and diolein. The M, 48,000 substrate phosphorylation was stimulated in the 32P-labeled, intact cells by a 60-min exposure to 10 nM TPA. Following termination of the phosphorylation reactions, the substrates were separated by polyacrylamide gel electrophoresis. The phosphorylated M, 48,000 substrates were identified by autoradiography, excised from the gel, and digested with trypsin. The tryptic digests were analyzed by reverse phase chromatography.
Preincubation with: Vehicle  Vehicle  Retinoic Acid
Exposure to 100nM TPA: −  +  +

Fig. 4. Effect of retinoic acid on phorbol ester-stimulated phosphorylation in intact 32P-labeled U937 cells. In A, cells were treated with 0.01% ethanol or 1 nM retinoic acid as shown above the autoradiogram. After a 48-h incubation, cells from each treatment group were labeled with 32P. As indicated above the autoradiogram, 3 x 10⁶ 32P-cells were exposed to 0.001% Me2SO or 10 nM TPA. After 60 min the reaction was terminated and the samples were subjected to polyacrylamide gel electrophoresis followed by autoradiography. The TPA-dependent phosphorylation of a M₄ 48,000 substrate is indicated to the side of the autoradiogram. In B, cells were incubated with 0.01% ethanol (O) or 1 nM retinoic acid (A). After a 48-h incubation the cells were labeled with 32P, 32P-labeled cells (3 x 10⁶) were incubated with vehicle or varying concentrations of TPA as shown on the abscissa. After a 60-min incubation the reaction was terminated and the samples were analyzed using polyacrylamide gel electrophoresis followed by autoradiography. Quantitation of M₄ 48,000 substrate (p 48) phosphorylation was determined by densitometric analysis of the autoradiogram. This experiment was repeated with similar results.

In both cellular extracts and intact cells, tryptic digestion of the phosphorylated M₄ 48,000 substrate yielded three peaks of radioactivity with nearly identical retention times (Fig. 7).

The retinoic acid-induced increases in protein kinase C-dependent phosphorylation could have been due to an increase in the amount of protein kinase C available for activation or an increase in the amount of endogenous substrate phosphorylated by protein kinase C. Using exogenous histone as a phosphate acceptor, protein kinase C activity was directly examined. Since the most pronounced alterations in protein kinase C-dependent endogenous substrate phosphorylation occurred in the cytosol, protein kinase C activity in this fraction was initially evaluated. Prior to measurement of activity, protein kinase C was extracted by DEAE chromatography. Retinoic acid treatment increased cytosolic protein kinase C activity eluting from a DEAE resin (Fig. 8). Kinase activity specifically stimulated by calcium, phosphatidyserine, and diolein was measured in cytosolic fractions which had been previously batch extracted using DEAE resin (see “Materials and Methods”). Protein kinase C activity contained in DEAE-extracted cytosol derived from retinoic acid-treated cells was 236 ± 19% of vehicle-treated cells (P < 0.01 versus control, Student’s t test, N = 6). Retinoic acid treatment did not alter protein kinase C activity in DEAE-extracted solubilized particulate fraction (90 ± 12% of vehicle-treated cells, P > 0.1, Student’s t test, N = 6).

The following experiments were done to determine whether the conditions of retinoic acid treatment inducing changes in protein kinase C activity were similar to those increasing protein kinase C-dependent phosphorylation of endogenous substrates and potentiating TPA-stimulated differentiation. The duration of exposure and concentration of retinoic acid altering protein kinase C activity were examined (Fig. 9). After exposure to 1 nM retinoic acid an effect was discernible by 12 h and maximal by 36 h. Following a 48-h exposure, the retinoic acid effect was evident at 0.03 nM and maximal at a concentration of 1 nM.

DISCUSSION

Retinoic acid potentiated TPA-stimulated differentiation of the human U937 monoblastoid cell. The more mature monocytic phenotype was manifested by increased adherence to plastic surfaces and the acquisition of nonspecific esterase activity. While retinoic acid potentiated the effects of TPA on certain parameters indicative of a more differentiated phenotype, cellular growth was not affected by retinoic acid treatment. In fact, total cell number was slightly, but not significantly, greater in cells treated with retinoic acid and TPA than in those treated with only TPA. The increase in total cell number following
Fig. 5. Effect of retinoic acid on protein kinase C-dependent phosphorylation of endogenous U937 substrates. Cells (15 x 10⁶/50 ml of medium) were incubated with 0.01% ethanol or 1 nM retinoic acid (indicated above the autoradiograms). After a 48-h incubation a cellular extract containing both cytosolic and solubilized particulate fractions was prepared. Endogenous substrate phosphorylation in the presence (+) and absence (−) of protein kinase C activators (shown above the autoradiograms) followed by polyacrylamide gel electrophoresis with autoradiography was performed as described in “Materials and Methods.” Molecular weight estimates of substrates are indicated on the ordinates of the autoradiogram.

exposure to both agents was due to an increase in the number of more differentiated, adherent cells. The ability of agents inducing U937 differentiation to affect certain parameters of differentiation more so than others has been documented previously (51, 52).

The similar conditions of retinoic acid treatment increasing the protein kinase C-dependent phosphorylation of histone and endogenous substrates suggested that the increase in endogenous substrate phosphorylation could have been due either to an increase in the quantity of kinase available for activation or to a facilitation of preexisting kinase activation. In addition to the increase in protein kinase C-dependent phosphorylation in cellular homogenates, retinoic acid enhanced TPA-stimulated phosphorylation in intact ³²P-labeled cells. The similar tryptic phosphopeptide maps of the M, 48,000 substrate suggested that a similar mechanism mediated the enhanced phosphorylation of this substrate in the intact cell and cellular homogenates. Retinoic acid selectively increased the protein kinase C-dependent phosphorylation of certain endogenous substrates (M, 48,000 and M, 80,000) but not in others (M, 140,000 and M, 170,000). Possible mechanisms explaining the preferential enhancement in phosphorylation of the M, 48,000 and M, 80,000 substrates would include increased synthesis of these substrates or a selective increase in expression of a specific protein kinase C isozyme. Protein kinase C is a member of a gene family (53).

Three protein kinase C isozymes have been isolated (54). Protein kinase C isozymes thus far characterized exhibit differences in substrate specificity (55). Thus, retinoic acid could potentiate phosphorylation of the M, 48,000 and M, 80,000 substrates by enhancing the expression of a protein kinase C isozyme which preferentially phosphorylates these substrates.

Using the human promyelocytic HL-60 cell, retinoic acid treatment has also been shown to increase protein kinase C-dependent phosphorylation (56, 57). In this cell line, the increases in protein kinase C-dependent phosphorylation have been observed at retinoic acid concentrations which directly stimulate differentiation (58). Protein kinase C-dependent phosphorylation is also increased following treatment with
Table 2 Comparison in the rates of dephosphorylation of the M, 48,000 and M, 80,000 substrates between vehicle- and retinoic acid-treated cells

<table>
<thead>
<tr>
<th>Time after addition of unlabelled ATP</th>
<th>M, 48,000</th>
<th></th>
<th>M, 80,000</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle Retinoic acid Vehicle Retinoic acid</td>
<td>8.7 17.2</td>
<td>5.8 9.4</td>
<td>8.4 16.2</td>
<td>5.0 8.9</td>
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</tbody>
</table>

Fig. 7. Tryptic phosphopeptide mapping of the M, 48,000 substrate. Cells (15 × 10⁶/50 ml of medium) were treated with 1 nM retinoic acid. After a 48-h incubation a cytosolic extract was prepared and protein kinase C-dependent phosphorylation was stimulated. In addition, a portion of the cells were labeled with 32P and exposed to 10 nM TPA for 60 min. Phosphorylation in the cytosolic extract and the intact cell was terminated and the samples were subjected to polyacrylamide gel electrophoresis. A gel piece containing the M, 48,000 substrate, identified by autoradiography, was excised from the gel, treated with ammonium bicarbonate, and digested with trypsin. Equal cpm of the digests were analyzed by reverse phase chromatography. The phosphorylated substrates were identified by autoradiography. 35P associated with the substrates was quantitated by densitometric analysis and is expressed in arbitrary units. This experiment was repeated with similar results.

Fig. 8. DEAE-cellulose chromatography of cytosol derived from vehicle- or retinoic acid-treated cells. After a 48-h incubation with 0.01% ethanol or 1 nM retinoic acid, cytosolic fractions were prepared. Equal amounts of the extracts, 1 mg of protein, were applied to and eluted from the DEAE columns as described in "Materials and Methods." Protein kinase C activity in the vehicle (O)- and retinoic acid (△)-treated cells was determined. ---, conductivity of the eluate. Similar elution profiles of protein kinase C activity were obtained in four separate experiments.

Fig. 9. Effects of the duration of exposure and concentration of retinoic acid on protein kinase C activity. Cells (30 × 10⁶/50 ml of medium) were exposed to 1 nM retinoic acid for varying periods of time (A) or to varying concentrations of retinoic acid for 48 h (B). At the end of the treatment periods, cytosolic fractions were prepared and the extracts, 1 mg of protein, were applied to DEAE-cellulose resin. Protein kinase C activity was eluted from the resin in a batchwise fashion. Protein kinase C activity was determined in the DEAE eluates. Each point is the mean of two determinations within a single experiment. Each experiment was repeated on a separate occasion with similar results.
without significantly altering the ED₉₀ for TPA. The similar concentrations of retinoic acid enhancing protein kinase C-dependent phosphorylation and potentiating TPA-stimulated differentiation were also consistent with a possible relationship between these events. Thus, by enhancing TPA-stimulated phosphorylation retinoic acid amplified phorbol ester signal transduction. Such an amplification could mediate the potentiation of phorbol ester-stimulated cellular events such as differentiation. However, byrostatin and diacylglycerol derivatives which like phorbol esters activate protein kinase C fail to stimulate leukemic cell differentiation (32, 34, 59). These findings suggest that the sole activation of protein kinase C may not directly stimulate differentiation. Despite the controversy as to whether protein kinase C activation solely mediates TPA-stimulated differentiation, the close correlation between the effects of retinoic acid on differentiation and protein kinase C-dependent phosphorylation suggests that activation of this kinase is involved in the signal transduction mediating TPA-stimulated differentiation.

REFERENCES

Effect of Retinoic Acid on Phorbol Ester-stimulated Differentiation and Protein Kinase C-dependent Phosphorylation in the U937 Human Monoblastoid Cell


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