Dissociation of Protein Kinase C Activation from Phorbol Ester-induced Maturation of HL-60 Leukemia Cells*

(Received for publication, December 24, 1984)

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The role of C-kinase in the induction of maturation of HL-60 promyelocytic leukemia cells was examined using two activators of this kinase, 12-O-tetradecanoylphorbol 13-acetate (TPA) and 1-oleoyl-2-acetylglyceryl (OAG). At 10^-8 M, a concentration that induced maturation, TPA effectively stimulated C-kinase activity in cell-free preparations by increasing the affinity of the enzyme for Ca2+. Similar activation was observed with 20 μg/ml of OAG. At these concentrations, addition of either compound to intact cells stimulated the phosphorylation of cellular proteins. Treatment with TPA resulted in an increased phosphorylation of 14 proteins, 9 of which also changed in response to OAG. In addition to the effects on protein phosphorylation, TPA and OAG both affected choline lipid metabolism. TPA at 10^-8 M stimulated the incorporation of [methyl-3H]choline into phosphatidylcholine, sphingomyelin, and lysophosphatidylcholine. OAG at 20 μg/ml had quantitatively similar effects on the labeling of the former two lipids, but did not affect incorporation of choline into lysophosphatidylcholine. Despite the similar biochemical effects of TPA and OAG, the diglyceride was unable to induce HL-60 cell maturation as measured by inhibition of cell growth, development of nonspecific esterase activity, phagocytosis, adherence of cells to plastic, and loss of transferrin receptor activity. The lack of effect is not due to metabolism of OAG; maturation could not be induced by treating cells with fresh OAG every 2 h for a period of 12 h. These results suggest a dissociation of the activation of C-kinase and the induction of HL-60 cell maturation by TPA.

The human promyelocytic cell line, HL-60, can be induced to mature into monocyte-like cells by exposure to the phorbol ester, TPA (1, 2). Biochemical events associated with maturation include protein phosphorylation (3–7), alterations in choline lipid metabolism (8, 9), and loss of transferrin receptors from the cell surface (10). Several lines of evidence have suggested a role for protein kinase C in this process. In HL-60 cells, as well as in other cell types, the TPA receptor and C-kinase activity copurify (11–13). TPA directly activates this kinase (14) and induces the phosphorylation of several proteins in HL-60 cells (3–6). In addition, cell adherence, a measure of maturation, is blocked by palmitoylcarnitine, an inhibitor of C-kinase (15).

The stimulation of C-kinase by phorbol esters appears to be due to the same mechanism as that for diacylglycerols, i.e. a decrease in the K_m of the enzyme for Ca2+ (15). In fact, both classes of molecules compete for the same binding site on the enzyme (16). Thus, TPA mimics the effect of the endogenous activator of C-kinase and can be used to study the effect of activation of this enzyme on various cellular processes. However, a potential drawback to this approach is the variety of cellular events in addition to C-kinase that are affected by TPA (17, 18). Recently, a diglyceride capable of permeating the plasma membrane and activating C-kinase in intact cells has been synthesized (19). This compound, 1-oleoyl-2-acetylglycerol (OAG), has many of the same biochemical effects as TPA. It induces an increase in the phosphorylation of the same proteins in platelets, hepatocytes, and neutrophils as does TPA (19–21). Both compounds have the same effect on the phosphorylation and binding activity of the epidermal growth factor receptor (22), and both stimulate the formation of phosphatidylinositol 4-phosphate in platelets (23). Thus, OAG is also a potent tool for studying the function of C-kinase in intact cells, and since it is structurally more similar to the endogenous activator than is TPA, it may be a more specific activator of this enzyme.

In the present study we have examined the role of C-kinase activation in the maturation of HL-60 cells using both TPA and OAG as activators of this kinase. Both compounds stimulated C-kinase, increased protein phosphorylation, and altered choline lipid metabolism, but only TPA induced cell maturation. These results suggest a dissociation of C-kinase activation from the initiation of maturation, and suggest that metabolic effects of TPA other than, or in addition to, activation of this enzyme are important for induction.

EXPERIMENTAL PROCEDURES

Materials—1-oleoyl-2-acetylglycerol was obtained from Avanti Polar Lipids, Inc. TPA and dieolein were from Sigma. Carrier-free 32P-O_4, and [γ-32P]ATP were from Amersham, and [methyl-3H]choline chloride (80 Ci/mmol) was from New England Nuclear.

Cell Growth and Maturation—HL-60 cells were obtained from Dr. Robert C. Gallo, National Cancer Institute, and grown in RPMI 1640 (Gibco) supplemented with 20% heat-inactivated fetal bovine serum (Gibco) in a humidified atmosphere of 95% air, 5% CO_2. Cells between passages 27 and 55 were seeded at 1 x 10^6/ml in the presence of TPA, OAG, or vehicle (0.1% dimethyl sulfoxide). At daily intervals, cell numbers were determined using a Coulter counter (Coulter Diagnostics, Hialeah, FL), and maturation was assessed by measurement of

*This work was supported by Grants AM19813, CA02817, and CA28852 and Fellowships AM07061 and CA07330 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: TPA, 12-O-tetradecanoyl phorbol 13-acetate; OAG, 1-oleoyl-2-acetylelcol; HEPES, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid.

Vol. 960, No. 10, Issue of May 25, pp. 5979-5984, 1985
Printed in U.S.A.
nonspecific esterase activity (24) and phagocytosis. Phagocytosis was assessed by counting the number of cells containing one or more fluorescent microspheres following a 24-h incubation with TPA or OAG. Transferin binding was measured using \(^{125}\)I transferin as previously described (25). For OAG repletion experiments, cells were incubated in polycarbonate tubes at 1-2 × 10^6/ml in RPMI 1640 + 20% fetal bovine serum with vehicle (0.1% dimethyl sulfoxide), OAG, or TPA. At 2-h intervals the cells were centrifuged at 100 × g for 5 min and resuspended in fresh media plus drug. At the end of 12 h the cells were centrifuged, resuspended in fresh media without drug, and plated in polycarbonate dishes. Adherence was assessed after 24 h.

**Choline Incorporation**—Cells were incubated at 5 × 10^6/ml in RPMI 1640 + 20% fetal bovine serum containing 5 μCi/ml of [methyl-\(^3\)H]choline chloride in the presence of TPA, OAG, or vehicle (0.1% dimethyl sulfoxide). At the indicated times, the cells were diluted in a 5-fold excess of phosphate-buffered saline and centrifuged at 100 × g for 5 min. The cells were resuspended in distilled water and lipids were extracted (26). Phospholipids were separated on Silica Gel H plates in a solvent system of chloroform/methanol/acetic acid/water (50/30/8, v/v). The spots corresponding to phosphatidylcholine, sphingomyelin, and lysophosphatidylcholine were scraped into scintillation vials and counted.

**Phosphorylation**—Cells were incubated at 1-2.5 × 10^6/ml for 30 min in phosphate-free RPMI 1640 (Flow Laboratories) containing 200 μCi/ml of carrier free \(^{32}\)PO_4 (TPA, OAG, or vehicle (0.1% dimethyl sulfoxide)) was added and incubation was continued for 1 h. Cells were washed in phosphate-free RPMI 1640 and pelleted in a microfuge. The pellet was resuspended in ice-cold lysis buffer (25 mM HEPES-Tri, 5 mM MgCl\(_2\), pH 7.4) and cells were disrupted by 15 passes through a 27-gauge needle. The homogenate was layered onto cold lysis buffer containing 1 M sucrose and centrifuged for 15 min to remove nuclei. The supernatant, containing particulate and soluble fractions, was prepared for and subjected to isoelectric focusing for 10,000 V-h as described by Garrison (27). Electrophoresis in the second dimension was on a 12% polyacrylamide gel (28).

**C-kinase Activation**—To determine the relative effectiveness of OAG and TPA as activators of C-kinase, we assayed this enzyme in cell-free preparations. As shown in Fig. 1, phosphatidylserine stimulated C-kinase activity at Ca\(^{++}\) concentrations greater than 10^{-6} M. TPA and OAG increased the sensitivity of the enzyme to Ca\(^{++}\), resulting in activation above 10^{-7} M Ca\(^{++}\). In the range of free Ca\(^{++}\) concentrations between 10^{-7} and 10^{-6} M, OAG and TPA activated C-kinase to the same extent. At 1.24 × 10^{-7}, 5.60 × 10^{-7}, 1.10 × 10^{-6}, and 5.60 × 10^{-6} M Ca\(^{++}\), 20 μg of OAG stimulated C-kinase 1.7-, 1.9-, 2.5-, and 2.3-fold, respectively. Under the same conditions, 10^{-4} M TPA stimulated the kinase 1.5-, 1.7-, 2.9-, and 2.6-fold, respectively. Only at the highest Ca\(^{++}\) concentration tested, 10^{-4} M, was TPA a better activator than OAG.

**Protein Kinase**—Cells were lysed and treated with Triton X-100 to solubilize membrane-bound C-kinase. After chromatography on DEAE-cellulose, C-kinase was assayed over a range of free Ca\(^{++}\) concentrations. Results represent mean ± S.E. of at least three experiments.

**RESULTS**

**C-kinase Activation**—To determine the relative effectiveness of OAG and TPA as activators of C-kinase, we assayed this enzyme in cell-free preparations. As shown in Fig. 1, phosphatidylserine stimulated C-kinase activity at Ca\(^{++}\) concentrations greater than 10^{-6} M. TPA and OAG increased the sensitivity of the enzyme to Ca\(^{++}\), resulting in activation above 10^{-7} M Ca\(^{++}\). In the range of free Ca\(^{++}\) concentrations between 10^{-7} and 10^{-6} M, OAG and TPA activated C-kinase to the same extent. At 1.24 × 10^{-7}, 5.60 × 10^{-7}, 1.10 × 10^{-6}, and 5.60 × 10^{-6} M Ca\(^{++}\), 20 μg of OAG stimulated C-kinase 1.7-, 1.9-, 2.5-, and 2.3-fold, respectively. Under the same conditions, 10^{-4} M TPA stimulated the kinase 1.5-, 1.7-, 2.9-, and 2.6-fold, respectively. Only at the highest Ca\(^{++}\) concentration tested, 10^{-4} M, was TPA a better activator than OAG.**
Protein Kinase C Activation and HL-60 Cell Maturation

FIG. 2. Two-dimensional gel electrophoresis of HL-60 cell proteins. Cells were incubated with carrier-free $^{32}$PO$_4$ for 30 min and then $10^{-8}$ M TPA or 20 $\mu$g/ml of OAG for an additional 60 min. Cells were lysed and centrifuged to remove nuclei. Proteins were electrophoresed in two dimensions and autoradiographed. Spots indicated by arrows have been consistently observed in at least three experiments. Proteins unique to TPA are indicated by arrowheads. A, control; B, $10^{-8}$ M TPA; and C, 20 $\mu$g/ml of OAG.

FIG. 3. Effect of TPA and OAG on the incorporation of choline into phosphatidylcholine, lysophosphatidylcholine, and sphingomyelin. HL-60 cells were treated with TPA or OAG at the indicated concentrations for 3 h, and [methyl-$^3$H]choline incorporation into phosphatidylcholine (PC), sphingomyelin (SPM), and lysophosphatidylcholine (LPC) was measured. Results represent mean ± S.E. of at least three experiments.

into sphingomyelin in a concentration-dependent manner (Fig. 3). TPA at $10^{-8}$ M and OAG at 20 $\mu$g/ml increased incorporation 40%. As observed for phosphatidylcholine, higher concentrations of TPA increased the incorporation into sphingomyelin, whereas 20 $\mu$g/ml of OAG was the maximally effective concentration of this agent. In contrast to the results observed with phosphatidylcholine and sphingomyelin, OAG had no effect on the incorporation of [methyl-$^3$H]choline into lysophosphatidylcholine, while TPA caused a concentration-dependent increase (Fig. 3).

Cell Growth and Maturation—The similarities between TPA and OAG in terms of C-kinase activation, protein phosphorylation, and choline lipid metabolism suggested that these two compounds should both induce maturation of HL-60 cells. As shown in Fig. 4, untreated HL-60 cells had a doubling time of approximately 24 h. OAG at 20 and 40 $\mu$g/ml had no effect on the rate of cell growth, whereas $10^{-8}$ and $3 \times 10^{-8}$ M TPA markedly reduced the rate of growth.

The loss of transferrin receptors from the cell surface has been correlated with maturation of HL-60 cells (10). The data in Fig. 5 illustrate important distinctions between the action of TPA and OAG with respect to both short- and long-term effects. Within 2–3 h of exposure to $10^{-8}$ TPA, the specific binding of transferrin to the cell surface was decreased by 50%. In contrast, 20 $\mu$g/ml of OAG induced an increase in transferrin binding during the same time frame. The long-
term effect of TPA was to suppress completely the binding of nonspecific esterase activity. In contrast, treatment with incubation, displayed phagocytic activity, whereas similar treatment with transferrin to the cell surface, which was observed between 6 and 70% was metabolized. Longer incubation periods did not increase the number of cells able to phagocytize microspheres was also unaffected by centrifugation. Lipids were extracted from the cell pellet and the amount of OAG was quantitated based on the gas chromatographic analysis of oleic acid.

The cell growth and transferrin receptor data suggest that OAG did not induce maturation of HL-60 cells. We measured the appearance of nonspecific esterase activity as an additional marker of maturation (24). After 2 days of treatment with $10^{-8}$ M TPA, 50–60% of the cell population demonstrated nonspecific esterase activity. In contrast, treatment with 20 $\mu$g/ml of OAG for up to 4 days did not increase the number of cells containing nonspecific esterase activity. The number of cells able to phagocytize microspheres was unaffected by OAG. After 24 h of exposure to TPA, 30% of the cells displayed phagocytic activity, whereas similar treatment with 20 $\mu$g/ml of OAG caused no increase above control in the number of phagocytic cells (2–4%).

**OAG Metabolism**—As illustrated in Table I, HL-60 cells metabolized OAG over a period of several hours. After 1 h of incubation, 30% of the OAG added was metabolized; by 6 h, 70% was metabolized. Longer incubation periods did not result in any greater loss of OAG. However, the lack of effect of OAG on cell maturation cannot be explained by metabolism of the diglyceride. Treatment of cells with a range of OAG concentrations for 12 h with addition of fresh media plus drug every 2 h failed to increase the number of cells that were adherent to plastic (Fig. 6). In contrast, treatment with TPA under the same conditions resulted in a concentration-dependent increase in the number of adherent cells. When choline lipid metabolism was examined using this protocol, 20 $\mu$g/ml of OAG had greater effects than $10^{-8}$ M TPA after the 12-h incubation (data not shown), indicating that this protocol supplied the cell with sufficient amounts of OAG to produce a long-term biochemical effect, but nonetheless, did not induce maturation.

**DISCUSSION**

The Ca$^{2+}$-dependent protein kinase, C-kinase, has been implicated in HL-60 maturation by extension of results from other cell types and tissues as well as by indirect evidence in HL-60 cells. The major impetus for investigating the involvement of C-kinase in TPA-induced events is the observation that phorbol esters bind to and activate this enzyme (11–14). The present study demonstrates that TPA and OAG, a diglyceride capable of activating C-kinase in intact cells as well as cell homogenates (18), induce similar biochemical effects in HL-60 cells. Both compounds activate C-kinase in *vitro* to the same extent over a range of physiological Ca$^{2+}$ concentrations (Fig. 1) and induce a similar pattern of HL-60 cell protein phosphorylation (Fig. 2), which is consistent with results in platelets (19), neutrophils (20), and hepatocytes (21). It is interesting to note that all 9 proteins phosphorylated in response to OAG are also phosphorylated in response to TPA, but that there are 5 additional proteins observed after treatment with the phorbol ester. It is unlikely that C-kinase is responsible for the phosphorylation of these 5 proteins since, at the concentrations employed in these experiments, OAG and TPA activate this enzyme to the same extent (Fig. 1). Rather, these changes more likely reflect the pleiotropic nature of TPA action (17, 18). A similar argument has been made by Feuerstein et al. (31) and Belnis et al. (32), who have suggested the involvement of kinases other than C-kinase in the action of TPA. We are currently investigating the role of other protein kinases in the response of HL-60 cells to TPA. A very early event in TPA-induced maturation is the stimulation of choline incorporation into phosphatidylcholine (8, 9). OAG elicits a similar, but not identical, response (Fig. 3). At 20 $\mu$g/ml, OAG stimulates choline incorporation into phosphatidylcholine and sphingomyelin to the same extent as $10^{-8}$ M TPA, a concentration that very effectively inhibits cell growth and induces maturation (Figs. 4 and 6). However,
concentrations of TPA greater than $10^{-8}$ M induce larger changes in choline incorporation, whereas 20 µg/ml of OAG is the maximally effective concentration of this agent. These effects are probably not mediated by C-kinase activation, since phosphorylation of cytidylyltransferase, the rate-limiting enzyme for phosphatidylcholine synthesis, results in a decrease in activity (33). The stimulation is more likely due to the translocation of cytidylyltransferase from the cytosol to the endoplasmic reticulum (34). A biochemical event that is not similarly affected in phorbol ester and diglyceride-treated cells is lysophosphatidylcholine metabolism. TPA stimulates the incorporation of choline into lysophosphatidylcholine (Fig. 3), presumably via phospholipase $A_2$ activation (35, 36). OAG is unable to mimic this effect, raising the interesting possibility that lysopidols or arachidonic acid metabolites derived from phospholipase $A_2$ action are part of the induction mechanism. This is supported by the observation that incubation of human macrophages with synthetic analogs of lysophosphatidylcholine increases their cytotoxicity toward U937 cells (37), which is very similar to what is observed in HL-60 cells, i.e., those cells that differentiate become cytotoxic toward the undifferentiated population (38).

Based on the similar biochemical effects of OAG and TPA, we anticipated that OAG would induce HL-60 maturation. This is clearly not the case. OAG has no effect on cell growth (Fig. 4), phagocytosis, or nonspecific esterase activity. In addition, OAG causes a transient increase in the specific binding of transferrin to the cell surface, whereas TPA induces a rapid reduction in the number of cell surface transferrin receptors (Fig. 5). This effect of OAG could be due to a recruitment of internal receptors to the surface, perhaps through a change in the degree of phosphorylation (39) or acylation (40). The lack of effect of OAG on cell maturation cannot be explained by metabolism of this compound. Repletion of OAG every 2 h for a total of 12 h does not induce the cells to mature (Fig. 6). Under these experimental conditions, OAG and TPA both have prolonged effects on choline incorporation into phosphatidylcholine, suggesting that this protocol provides the cells with a continuous supply of OAG for the 12-h period. In addition, the discrepancy between the early effects of TPA and OAG on the loss of transferrin receptors suggests a very basic difference between the biochemical action of these two agents.

The results of the present study demonstrate that, despite many similar biochemical responses elicited by OAG and TPA, the effects on cell maturation are distinct, suggesting that activation of C-kinase is either not required for the induction of maturation, or is not sufficient to induce maturation. This interpretation is contrary to that drawn by Nakaki et al. (15) based on studies with palmitoylcarnitine, an inhibitor of C-kinase. In that study, cell adhesion in response to TPA was inhibited, while the development of acid phosphatase activity was not. It is possible to envision that a cell surface related property such as adhesion would be affected by compounds with detergent-like properties without invoking inhibition of C-kinase. The results of our study are supported by investigations with another tumor promoter, teleocidin. This compound activates C-kinase and displaces [$H^3$]phorbol dibutyrate binding with $K_0$ of approximately 60 ng/ml and 25 ng/ml, respectively (41, 42). However, maturation of HL-60 cells is induced half-maximally by a much lower concentration of teleocidin (1.5 ng/ml (43), a concentration at which there is neither activation of C-kinase nor competition for phorbol dibutyrate binding (41, 42). Thus, the present work, demonstrating that it is possible to activate C-kinase without inducing maturation, and the work with teleocidin, demonstrating that maturation can be induced without activating C-kinase, provide complimentary evidence that a discrepancy exists between C-kinase activation and the induction of monocytic maturation of HL-60 cells. Therefore, we further conclude that an effect of TPA other than the activation of C-kinase is of critical importance to the induction process through which leukemic cells become committed to the differentiation pathway.

Acknowledgments—We gratefully acknowledge the support and helpful discussions of Drs. Howard Rasmussen and Alas Sartorelli.
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