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Diacylglycerols mimic phorbol diester induction of leukemic cell differentiation

(endogenous phorbol/tumor promotion/protein kinase C/phosphoprotein/macrophage)

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ABSTRACT **Activation of cellular protein kinase C appears to be involved in the mechanism by which phorbol diesters induce differentiation of human myeloid leukemia cells (HL-60). Protein kinase C is thought to be physiologically activated by diacylglycerol derived from receptor-mediated phosphatidylinositol hydrolysis. *sn*-1,2-diacylglycerols with short saturated acyl side chains (C_4–C_{10}) were synthesized and found to be potent activators of protein kinase C partially purified from HL-60 cells. These diacylglycerols were also competitive inhibitors of [^{3}H]phorbol dibutyrate binding to the soluble phorbol diester receptor. The most potent diacylglycerol, *sn*-1,2-dioctanoylglycerol, displaced >90% of [^{3}H]phorbol dibutyrate from the phorbol diester receptor of intact HL-60 cells. Because of probable cellular metabolism of *sn*-1,2-dioctanoylglycerol, hourly doses were required to maintain persistent occupancy of the phorbol diester binding site. Treatment of HL-60 cells with either phorbol 12-myristate 13-acetate or *sn*-1,2-dioctanoylglycerol produced identical phosphoprotein changes. Finally, *sn*-1,2-dioctanoylglycerol induced differentiation of the HL-60 cells into cells with morphologic characteristics of macrophages. Substitution of the hydroxyl group at position 3 with a hydrogen, chloro, or sulfhydryl moiety inactivated *sn*-1,2-dioctanoylglycerol. These data strengthen the hypothesis that protein kinase C activation plays a role in macrophage differentiation.**

The phorbol diester tumor promoters are plant products that enhance tumor formation in two-stage models of skin carcinogenesis (1). Paradoxically, these same compounds will rapidly induce most myeloid leukemia cells (cell lines and fresh explants from patients) to cease proliferation and to differentiate into cells with the morphologic, antigenic, biochemical, and functional properties of macrophages (2, 3). The phorbol diesters exert these effects via a specific receptor (4, 5). Data from several laboratories indicate that this receptor is the Ca^{2+}–phospholipid-dependent protein kinase, termed protein kinase C (6–9). We have recently presented data that suggest activation of protein kinase C is involved in differentiation of human promyelocytic leukemia cells (HL-60) induced by phorbol diesters (10).

Protein kinase C is regulated *in vitro* by Ca^{2+} and diacylglycerol, and is presumably activated *in vivo* in response to receptor-mediated phosphatidylinositol turnover, which gives rise to diacylglycerol, and/or Ca^{2+} flux (11). *In vitro*, the phorbol diesters activate the kinase by interaction at the diacylglycerol site (12). In intact cells, the phorbol diesters bypass the receptor–phosphatidylinositol-mediated regulatory mechanisms and activate the kinase directly (11). Discrete and sustained changes in cellular phosphoproteins have been shown to occur during phorbol diester-induced differentiation of HL-60 cells, although evidence that these phosphorylation events are protein kinase C dependent or are involved in the process of differentiation has not been presented (13, 14).

These data suggest that diacylglycerols may be the "endogenous phorbol" and may have a role in the physiological regulation of myeloid differentiation. To test this hypothesis, we have synthesized a series of *sn*-1,2-diacylglycerols with fatty acids 4–10 carbons long. These compounds have been tested for their ability (*i*) to compete for binding to the soluble HL-60 phorbol diester receptor and to the receptor of intact cells; (*ii*) to activate partially purified protein kinase C from HL-60 cells; (*iii*) to induce phosphoprotein changes in intact HL-60 cells; and (*iv*) to induce macrophage differentiation of the HL-60 cells. The results of these studies suggest that *sn*-1,2-diacylglycerol is the endogenous phorbol.

MATERIALS

HL-60 cells were from American Type Culture Collection; RPMI-1640 medium was from GIBCO; Pentex bovine serum albumin was from Miles; HA filters were from Millipore; Pharmalyte pH 3.5–10.0 was from Pharmacia; molecular weight standards M_r 14,000–220,000 were from Bio-Rad; Lightning Plus screens were from DuPont; DE52 and DE81 filters were from Whatman; and histone III-S, bovine serum albumin fraction V and 1,2-diolein were from Sigma. The phosphatidylserine was from Avanti Biochemicals. Phorbol 12-myristate 13-acetate and phorbol dibutyrate were from L. C. Services (Woburn, MA); [^{3}H]phorbol 12,13-dibutyrate ([^{3}H]PDB) (17 Ci/mmol; 1 Ci = 37 GBq) was from New England Nuclear; [γ-^{32}P]ATP and [^{32}P]orthophosphate were from ICN. *Crotalus adamanteus* venom was purchased from Miami Serpentarium Laboratories (Miami, FL). Phospholipase C (from *Bacillus cereus*), octanoic acid, dioleoylphosphatidylcholine, dicyclohexylcarbodiimide, and *S*(+)-1,2-propanediol were from Sigma. Other short chain symmetric diacylphosphatidylcholines were purchased from Avanti Biochemicals. Octanoic anhydride was prepared by the procedure of Selinger and Lapidot (15). Acyl esters were quantified by the hydroxamate assay of Stern and Shapiro (16). Thin-layer chromatography was performed using Silica Gel 60 plates (Merck) obtained from American Scientific Products (Stone Mountain, GA). Purity of diacylglycerols and analogs was determined by thin-layer chromatography

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Abbreviations: diC_4, *sn*-1,2-dibutyrylglycerol; diC_6, *sn*-1,2-dihexanoylglycerol; diC_8, *sn*-1,2-dioctanoylglycerol; diC_{10}, *sn*-1,2-didecanoylglycerol; $diC_{18:1}$, *sn*-1,2-dioleoylglycerol; OAG, *sn*-1-oleoyl-2-acetylglycerol; deoxy-diC_8, 1,2-dioctanoyl-3-chloro propanediol; thio-diC_8, 1,2-dioctanoyl-3-thioglycerol; PMA, phorbol 12-myristate 13-acetate; PDB, phorbol 12,13-dibutyrate.

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in heptane/ethyl ether/acetic acid. Spots were located by spraying with 33% sulfuric acid and charring at 180°C.

METHODS

Preparation of *sn*-1,2-Diacylglycerols. Phosphatidylcholine (100 μmol) of the appropriate acyl chain length was digested in a two-phase ether/water system using 25 units of phospholipase C (17). After 2 hr of intermittent agitation, the ether was removed under a stream of nitrogen, and the aqueous phase was extracted with chloroform/methanol (18). The lipids were dissolved in 2 ml of chloroform and loaded on a 3-cm minicolumn of silica gel in a Pasteur pipette equilibrated with chloroform. This column was washed with 6 ml of chloroform and the combined chloroform eluates were dried under nitrogen, dissolved in chloroform, and stored at −20°C.

Preparation of *sn*-1-Oleoyl-2-acetylglycerol (OAG). Dioleoylphosphatidylcholine (100 mg) was digested with 10 mg of *Crotalus adamanteus* venom (19). After 30 min of agitation at room temperature, the ether was evaporated and the aqueous phase was extracted as described above. The organic phase of the extract was concentrated to ≈0.2 ml. This residue was dissolved in 5 ml of chloroform and loaded on a 1 × 10 cm column of silica gel in chloroform. The column was washed with 5 ml of chloroform and four 5-ml vol of chloroform/methanol (9:1) to elute the oleic acid. The lysophosphatidylcholine was then eluted with 5-ml portions of chloroform/methanol/water (1:2:0.8) until thin-layer chromatography in chloroform/methanol/water/acetic acid (25:15:4:2) indicated that elution was complete. Solvents were removed by rotary evaporation until only water remained, which was removed by lyophilization.

The lysophosphatidylcholine was dissolved in 2 ml of chloroform to which was added 0.25 ml each of pyridine and acetic anhydride. This mixture was incubated overnight at room temperature in the dark. Thin-layer chromatography indicated that no lysophosphatidylcholine remained. Methanol (1 ml) was added to consume excess acetic anhydride. After 3 hr, the solvents were largely removed under nitrogen, and residual acetic acid and pyridine were removed by chloroform/methanol extraction at neutral pH followed by washing of the lower phase with 3 portions of neutral and 3 portions of acidic preequilibrated upper phase.

The chloroform phase was dried under nitrogen, and the 1-oleoyl-2-acetylphosphatidylcholine was converted to the diacylglycerol as described above for the symmetric diacylphosphatidylcholines. Recoveries by this procedure are ≈75% based on dioleoylphosphatidylcholine.

Synthesis of 1,2-Dioctanoylpropanediol (Deoxy-diC$_8$), 1,2-Dioctanoyl-3-chloropropanediol (Chloro-diC$_8$) and 1,2-Dioctanoyl-3-thioglycerol (Thio-diC$_8$). To 1 ml of heptane was added 0.25 ml of pyridine, 600 μmol of octanoic anhydride, and 150 μmol of either *S*(+)-1,2-propanediol, 3-chloro-1,2-propanediol or 1-thiomethyl-1-thioglycerol. After 20 hr at room temperature in the dark, the solvents were removed under nitrogen and residual pyridine was removed by acidic chloroform/methanol extraction. The lower phase was dried and the lipids were dissolved in 2 ml of heptane, which was washed with 50 mM NaOH in 50% ethanol until the aqueous ethanol phase was basic to pH paper. The heptane phase was then applied to a 0.8 × 5 cm column of silica gel in heptane and was eluted with chloroform/heptane (2:3). Fractions of 2 ml were collected and those containing the diacyl product were pooled and dried. The diacyl analogs eluted before the monoacyl and other products. The thiomethyl group was removed from the thio analog by treatment with a 4-fold excess of dithiothreitol in methanol at 75°C for 24 hr. Dithiothreitol and thio-diC$_8$ were separated by partitioning between heptane and water, and the heptane phase was chromatographed as described above.

Enzyme/Receptor Preparation and Assays. Protein kinase C was partially purified from the cytosol of HL-60 cells and was assayed with histone III-S or H1 as the ^{32}P-labeled acceptor substrate, as described (10). The phorbol diester receptor assay of intact HL-60 cells was performed by the method of Goodwin and Weinberg using 10 nM [^{3}H]PDB (20). The standard soluble phorbol diester receptor assay was performed as follows: 10 μl of soluble receptor was added to 230 μl of 10 nM [^{3}H]PDB in 20 mM Tris·HCl, pH 7.5/25 μM Ca^{2+}/10 mM Mg^{2+}/10 μM ATP/100 μg of phosphatidylserine per ml. Nonsaturable binding was determined in the presence of 5 μM unlabeled PDB. Receptor-bound and free [^{3}H]PDB were separated by rapid filtration through a DE81 filter and by washing with 5 ml of 20 mM Tris·HCl, pH 7.5/0.25 mM Ca^{2+}/20% methanol at 4°C. The soluble receptor assay was modified to include histone, 10 μM $CaCl_2$, and 25 nM [^{3}H]PDB for the correlation with kinase activation (see legend to Fig. 1). The filters were dried, suspended in 10 ml of Ultrafluor, and ^{3}H was measured in a Beckman LS6800 with 40% efficiency. HL-60 phosphoproteins were analyzed by the 2-dimensional method of O'Farrell with a pH 3.5–10 gradient in the first dimension and a 5%–15% acrylamide gradient in the second dimension (14).

RESULTS

***In Vitro* Effects of Diacylglycerols.** Several diacylglycerols were prepared and tested for their ability to activate partially purified protein kinase C from HL-60 cells (Fig. 1*A*). *sn*-1,2-dioctanoylglycerol (diC$_8$), *sn*-1,2-didecanoylglycerol (diC$_{10}$), and *sn*-1,2-dioleoylglycerol (diC$_{18:1}$) were approximately equipotent in their ability to stimulate protein kinase C. *sn*-1,2-dihexanoylglycerol (diC$_6$) and OAG were one order of magnitude less potent, and *sn*-1,2-dibutyrylglycerol (diC$_4$) was ≈3 orders of magnitude less potent than diC$_8$, diC$_{10}$, or diC$_{18:1}$. The diC$_8$ analog lacking the hydroxyl at position 3 (deoxy-diC$_8$) was inactive as a kinase activator. PDB was 3 orders of magnitude more potent than the best diacylglycerols.

To further characterize the interaction of these diacylglycerols with protein kinase C, their ability to inhibit binding of [^{3}H]PDB to the soluble kinase preparation was determined using incubation conditions identical to those used for the kinase assays (Fig. 1*B*). diC$_{18:1}$, diC$_8$, and diC$_{10}$ yielded indistinguishable inhibition curves; OAG was slightly less potent. diC$_6$ was again ≈1 order of magnitude less potent, while diC$_4$ was inactive at <1 mM. Deoxy-diC$_8$ did not inhibit [^{3}H]PDB binding. Similar rank orders of potency were evident for diacylglycerol-dependent kinase activation and diaylglycerol-dependent [^{3}H]PDB binding inhibition (diC$_8$ = diC$_{10}$ = diC$_{18:1}$ > OAG = diC$_6$ >> diC$_4$), suggesting that affinity for the phorbol diester activation site on the enzyme determined the potencies of the various diacylglycerols as kinase activators. However, because the concentration of [^{3}H]PDB used in the receptor assay was well above the K_d for [^{3}H]PDB, higher concentrations of unlabeled PDB or diacylglycerol were necessary for binding inhibition than for kinase activation. It should be noted that both histone H1 and histone III-S partially inhibit [^{3}H]PDB binding.

We characterized the mechanism of this binding inhibition using OAG as a representative diacylglycerol. In a double reciprocal plot of our binding inhibition data (Fig. 2), lines derived in the presence of OAG intersected at the *y* axis, indicating that the inhibition was primarily competitive. However, the line derived in the absence of OAG consistently intersected below the others, suggesting that ≈20% of

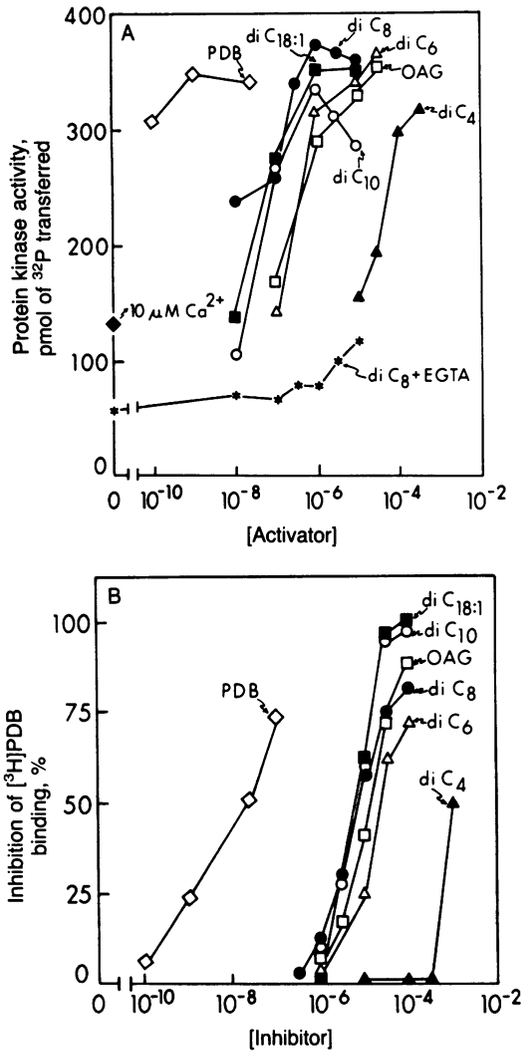

FIG. 1. (*A*) Activation of protein kinase C by various diacylglycerols. Ten-microliter samples of partially purified soluble protein kinase C–phorbol diester receptor were assayed for protein kinase activity in the presence of 10 μM $CaCl_2$/5 mM $MgCl_2$/10 μM [γ-^{32}P]ATP/phosphatidylserine (200 μg/ml)/histone H1 (200 μg/ml), in addition to one of the following: diC_4 at 10–300 μM (▲); diC_6 at 0.1–30 μM (△); diC_8 at 0.01–10 μM (●); diC_{10} at 0.01–10 μM (○); $diC_{18:1}$ at 0.01–10 μM (■); OAG at 0.1–30 μM (□); PDB at 0.1–25 nM (◇). Control values [10 μM Ca^{2+}, no activator (◆); diC_8 and 10 mM EGTA (*)] are also shown. Data points are averages of duplicate values. (*B*) Inhibition of [^{3}H]PDB binding by various diacylglycerols. Ten-microliter samples of partially purified soluble protein kinase C–phorbol diester receptor were incubated with 25 nM [^{3}H]PDB/5 mM $MgCl_2$/10 μM ATP/10 μM $CaCl_2$/phosphatidylserine (200 μg/ml)/histone H1 (200 μg/ml), in addition to one of the following: diC_4 at 10–1000 μM (▲); diC_6 at 1–100 μM (△); diC_8 at 0.3–100 μM (●); diC_{10} at 1–100 μM (○); $diC_{18:1}$ at 1–100 μM (■); OAG at 1–100 μM (□); PDB at 0.1–100 nM (◇). Data points are averages of duplicate values.

the inhibition may be noncompetitive, perhaps due to an OAG-induced alteration in the phosphatidylserine vesicle structure. The K_i for inhibition of binding by OAG was 0.5×10^{-6} M; expressed relative to the concentration of phosphatidylserine, the K_i was 0.2% (wt/wt), which compares well with a previously reported value of 0.38% of $diC_{18:1}$ (12). Previous data from our laboratory indicated a K_d of 8.1×10^{-9} M for PDB binding to the soluble HL-60 receptor (10). Using the DE81 binding assay, we found that the K_d value varied depending on the concentration of phosphatidylserine. With phosphatidylserine at 200 μg/ml, the K_d was $<1 \times 10^{-9}$ M, while at 20 μg/ml, the K_d was ≈1

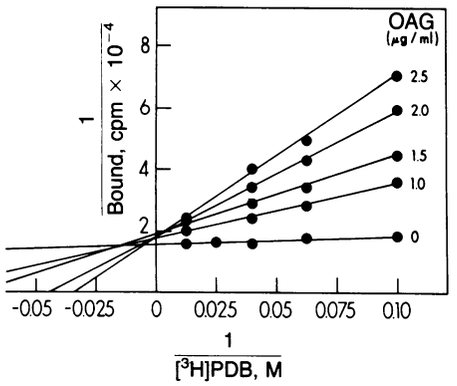

FIG. 2. Lineweaver–Burk analysis of inhibition of [^{3}H]PDB binding by OAG. Ten-microliter samples of partially purified soluble protein kinase C–phorbol diester receptor assayed for [^{3}H]PDB binding in the presence of 25 μM $CaCl_2$/phosphatidylserine (100 μg/ml), and various concentrations of OAG and [^{3}H]PDB. Data points are averages of two experiments performed in triplicate; lines were determined by linear regression analysis.

order of magnitude greater. Furthermore, in agreement with Sharkey *et al.* (12), we found that the K_i value for OAG varied as a function of the concentration of phosphatidylserine in the assay. Therefore, the degree of binding inhibition was dependent on the molar ratio of diacylglycerol to phosphatidylserine, not on the absolute concentration of diacylglycerol (data not shown).

Effects of Diacylglycerols on Intact HL-60 Cells. Having shown that diacylglycerol effectively competed with phorbol diester for binding to the protein kinase C–phorbol diester receptor partially purified from HL-60 cells, we next examined the effects of the various diacylglycerols on [^{3}H]PDB binding to intact HL-60 cells. The ability of a single dose of the various diacylglycerols to displace [^{3}H]PDB bound to intact HL-60 cells was tested (Fig. 3). The importance of the fatty acid chains present in the diacylglycerols became readily apparent. $DiC_{18:1}$, effective in both activating protein kinase C and in inhibiting [^{3}H]PDB binding in the *in vitro*

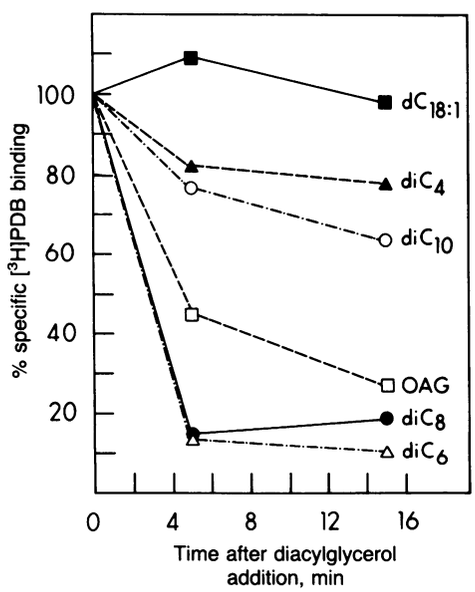

FIG. 3. Displacement of [^{3}H]PDB bound to intact HL-60 cells by various diacylglycerols as a function of time. Intact HL-60 cells at 1×10^6 cells per ml were incubated for 20 min with 10 nM [^{3}H]PDB. Reaction mixture was then made 200 μM with one of the following: diC_4 (▲), diC_6 (△), diC_8 (●), diC_{10} (○), $diC_{18:1}$ (■), and OAG (□); 2×10^5 cell samples were assayed for [^{3}H]PDB binding at the indicated times after diacylglycerol addition.

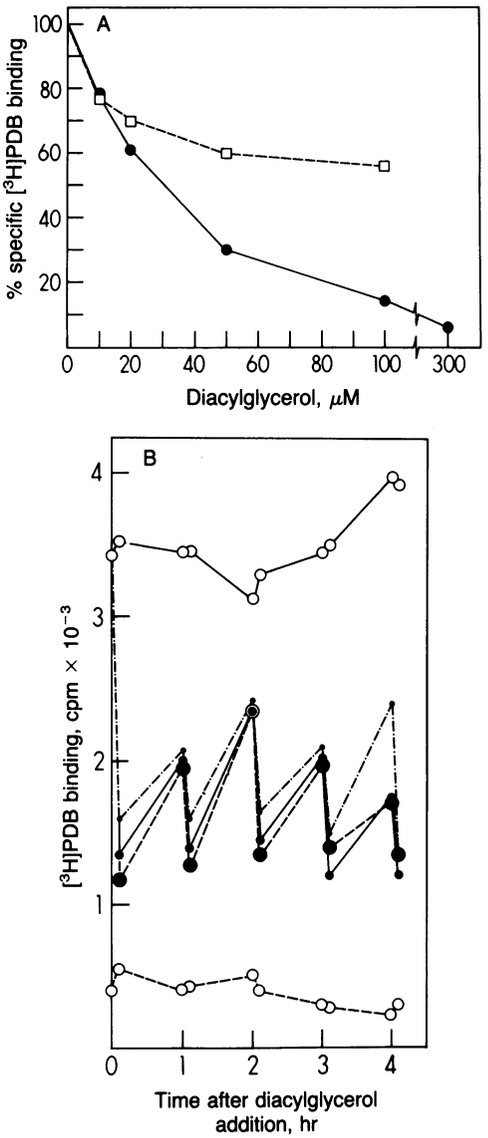

FIG. 4. (*A*) Inhibition of [^{3}H]PDB binding to intact HL-60 cells by various diacylglycerols as a function of diacylglycerol concentration. Intact HL-60 cells at 1 × 10^6 cells per ml were incubated for 10 min in the presence of 10 nM [^{3}H]PDB and various concentrations of diC_8 (●) and OAG (□). Data points are averages of duplicate values. (*B*) Inhibition of [^{3}H]PDB binding to intact HL-60 cells by diC_8 as a function of time. Intact HL-60 cells at 1 × 10^6 cells per ml were incubated for 20 min with 10 nM [^{3}H]PDB. Reaction mixtures were then made 100 μM (– –), 75 μM (—), and 50 μM (–·–) with diC_8, with further hourly diC_8 additions of 20 μM, 15 μM, and 10 μM, respectively. Cell samples (2 × 10^5) were assayed for [^{3}H]PDB binding at the indicated times. Total [^{3}H]PDB bound in the absence of diC_8 (○–○) and nonsaturable binding (○– – –○) are also depicted.

protein kinase C–phorbol diester receptor assays, was completely ineffective in displacing [^{3}H]PDB from intact cells. DiC_{10} was also highly effective in the *in vitro* assays; however, it was only moderately effective in displacing [^{3}H]PDB from intact cells. We believe that the long side chains of these derivatives prohibit them from efficiently reaching the regulatory site on cellular protein kinase C–phorbol diester receptor. DiC_6 and diC_8, with shorter side chains, were the most efficacious diacylglycerols we tested in our intact cell [^{3}H]PDB displacement assay. Since diC_8 was also a highly effective *in vitro* protein kinase C activator/[^{3}H]PDB binding inhibitor, we chose to examine the ability of diC_8 to mimic the phorbol diesters in inducing HL-60 cells to differentiate into macrophages. Deoxy-diC_8, chloro-diC_8, and thio-diC_8 did not displace [^{3}H]PDB from intact cells at concentrations up to 300 μM.

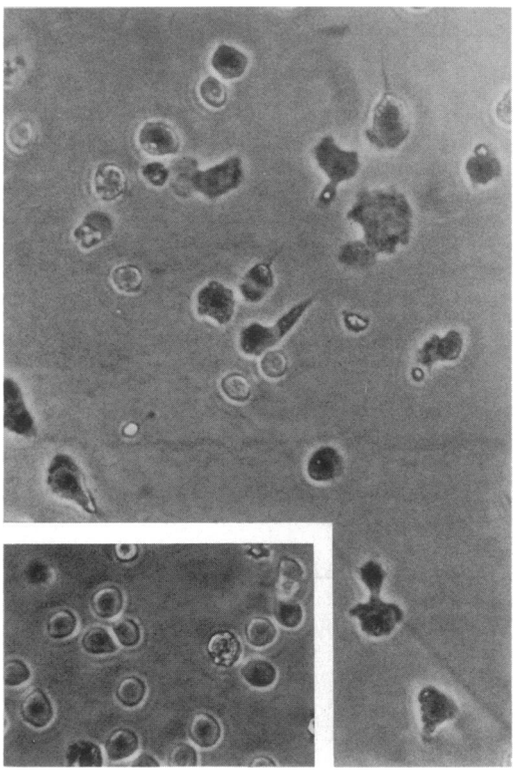

FIG. 5. Induction of differentiation of HL-60 cells by diC_8. HL-60 cells at 1 × 10^6 cells per ml, maintained for the duration of the experiment in RPMI-1640 medium/0.1% bovine serum albumin were treated with an initial dose of 20 μM diC_8 and with subsequent bihourly doses of 10 μM for 14 hr. Forty-eight hours after the initial diC_8 addition, the cells were photographed with a ×40 phase objective. (*Inset*) Untreated controls.

In preparation for attempting to induce cell differentiation with diacylglycerol, we examined the concentration dependence and time course of diC_8 inhibition of [^{3}H]PDB binding to intact HL-60 cells (Fig. 4 *A* and *B*). In Fig. 4*A*, diC_8 is compared to OAG, which has been used by others as a cell-permeable diacylglycerol (11). Inhibition of binding by both compounds was dose dependent; however, diC_8 was significantly more efficacious and inhibited >90% of [^{3}H]PDB binding at concentrations >100 μM. OAG appeared to have access to only 50% of the total [^{3}H]PDB sites. Fig. 4*B* shows that diC_8 at 50, 75, and 100 μM caused a rapid displacement of [^{3}H]PDB at 37°C. We believe that the subsequent rebound in [^{3}H]PDB binding with time is a result of metabolism of the diC_8 by the cells. Thus, we added diC_8 at hourly intervals to achieve a steady-state level of binding inhibition, and we tested different loading and maintenance doses. A loading dose of 50 μM with hourly 10 μM maintenance doses was approximately as effective as loading at 75 μM and maintenance of 15 μM or loading at 100 μM and maintenance of 20 μM. The 50 μM/10 μM regimen produced the least cellular toxicity. Subsequent experiments demonstrated that 20 μM pulses every 2 hr for 14–16 hr without a loading dose were equally effective.

Phorbol diester-responsive HL-60 cells were induced to differentiate by treatment with either diC_8 (50 μM loading dose and 12 hourly 10 μM maintenance doses or 7 bihourly 20 μM doses) or 1 nM PDB for 13 hr. The cells were then washed and cultured for an additional 36 hr free of inducers. The percentage of cells that had committed to the differentiated phenotype was determined by spontaneous adherence and macrophage morphology at 48 hr (Fig. 5). In four

experiments, <5% of the control cells became adherent, whereas 60% of the cells induced with phorbol dibutyrate and 50% of the cells induced with diC_8 became adherent with macrophage morphology. Mortality was ≈10% in both treatment groups at 24 hr, >25% at 48 hr, and >50% at 60 hr. At 48 hr, neither the diC_8 nor the PDB-treated cells would ingest 1-μm latex beads.

Finally, to lend further support for a common mechanism of action of diC_8 and phorbol diesters, we compared phosphoprotein changes induced in the HL-60 cells by diC_8 to those induced by phorbol diester. Cellular phosphoproteins were analyzed by isoelectric focusing two-dimensional gel electrophoresis. Exposure of ^{32}P-labeled equilibrated cells to either 50 nM phorbol 12-myristate 13-acetate (PMA) or 50 μM diC_8 brought about similar phosphoprotein alterations (data not shown). With both stimuli, proteins of M_r 55,000, 80,000, and 21,000 with pI 5.0, 5.4, and 5.5, respectively, demonstrated increased phosphorylation, while a protein of M_r 21,000 and pI 6.3 showed decreased phosphorylation. These changes were sustained for at least 30 min and were identical to our previous results with PMA as a stimulus (14).

DISCUSSION

Evidence from several laboratories suggests that the phorbol diester receptor is a cellular enzyme, protein kinase C, and that activation of this kinase may be involved in the pleiotropic cellular effects of the phorbol diesters (6–11). However, as yet, genetic defects in this enzyme have not been described, so that evidence in support of a causal role for protein kinase C in any specific cellular process must remain correlative. In a previous paper, we presented data that suggested phorbol diester activation of protein kinase C played a role in the differentiation of leukemia cells into macrophages (10). Data presented here extend these correlative observations in several ways.

First, for a series of synthetic diacylglycerols, the rank order of potencies for inhibition of phorbol diester binding to the soluble receptor correlated with the order of potencies for kinase activation (Fig. 1). Second, diacylglycerols were competitive inhibitors of *in vitro* phorbol diester binding, indicating that both classes of compound interact with the same form of the enzyme, perhaps at a common site (Fig. 2). Third, cell-permeable diacylglycerols (diC_8, diC_6, and OAG) effectively competed for the phorbol diester receptor of intact HL-60 cells (Figs. 3 and 4). DiC_8 was the most effective and was able to displace >90% of specifically bound [^{3}H]PDB, demonstrating that most phorbol diester receptors of HL-60 cells recognize diacylglycerols and, therefore, that protein kinase C may be the only biologically relevant phorbol diester receptor in these cells. Finally, the fact that treatment with diC_8 and phorbol diesters led to identical changes in HL-60 phosphoproteins and cellular differentiation lends further support to the hypothesis that activation of cellular protein kinase C is involved in the process of differentiation of leukemia cells into macrophages (Fig. 5).

Several properties of the diacyglycerols are worthy of mention. Diacylglycerols with saturated acyl side chains were as effective as diacylglycerols with unsaturated side chains in kinase activation and in inhibition of phorbol diester binding with the *in vitro* assays. $DiC_{18:1}$, diC_8, and diC_{10} were equipotent. However, acyl length was important in determining interaction with the regulatory site on the kinase. DiC_6 and diC_4 were both less active, suggesting that diacylglycerols with side chains of >6 carbons are optimal for *in vitro* kinase activation. Although $diC_{18:1}$ and diC_{10} interacted with the regulatory site *in vitro*, neither was an effective inhibitor of the phorbol diester receptor of intact cells, indicating that side chains of 10 carbons or greater prevented interaction of these diacylglycerols with the important cellular regulatory site. DiC_8 appeared to offer optimal properties of cell permeability and kinase activation. OAG, the only other cell-permeable diacylglycerol previously used (reviewed in ref. 11), was a good *in vitro* kinase activator, but it did not have access to all cellular [^{3}H]PDB sites; hence, it may not be ideal for assessment of diacylglycerol biological effects. Finally, the hydroxyl in position 3 is critical for activity because 1,2-dioctanoylpropanediol (deoxy-diC_8) was totally inactive *in vitro* and *in vivo*. Cell permeable diacylglycerols such as OAG and diC_8 should be handled with caution, because they mimic many of the biological effects of phorbol diesters, which are known tumor promoters. Whether these diacylglycerols will also be active in two-stage carcinogenesis remains to be determined.

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