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Research Article

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Possible Mechanism of Phorbol Diester-induced Maturation of Human Promyelocytic Leukemia Cells

Activation of Protein Kinase C

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Abstract. The phorbol diesters are the most potent inducers of differentiation of the promyelocytic leukemia cell line, HL-60. Soluble phorbol diester receptors from HL-60 cells were obtained from the cytosolic fraction and from the particulate fraction by either divalent ion chelation or detergent extraction. The partially purified soluble phorbol diester receptors required exogenous Ca^{2+} and phospholipid for maximal binding and displayed a dissociation constant (K_D) of 8.1 nM for [^{3}H]phorbol 12,13-dibutyrate. Phorbol diester analogues inhibited [^{3}H]phorbol 12,13-dibutyrate binding in a stereospecific manner consistent with their biologic potency. The soluble phorbol diester receptors prepared by all three methods copurified in a constant ratio with the Ca^{2+}/phospholipid-dependent protein kinase C through ammonium sulfate precipitation, DEAE ion exchange, and gel filtration chromatography. Partially purified protein kinase C was directly activated by the phorbol diesters even in the absence of exogenous Ca^{2+}. The ability of a series of phorbol analogues to activate the kinase correlated with their known activity as inducers of cell differentiation. In addition, phorbol diester stimulation altered the phosphate acceptor substrate profile of protein kinase C, at least in part, by alteration of the Michaelis constant (K_m). These data suggest that protein kinase C is the phorbol diester receptor and that phorbol diester-induced macrophage maturation of HL-60 cells may be mediated by activation of intracellular protein kinase C.

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Introduction

The phorbol diesters are a group of agents capable of tumor promotion when administered topically following subthreshold doses of a carcinogen (1). In addition to tumor promotion, a wide range of functional and biochemical changes have been ascribed to the phorbol diesters in numerous experimental systems (2). Recently, the phorbol diesters have been shown to induce differentiation of HL-60 cells to macrophagelike cells (3–5). Phorbol 12-myristate 13-acetate (PMA)[1] treatment brought about rapid cessation of DNA synthesis and cell proliferation. The cells became adherent and phagocytic, lost azurophilic granules, demonstrated increased levels of nicotinamide-adenine dinucleotidase, nonspecific esterase, and acid phosphatase, became cytocidal and cytostatic for tumor targets, and synthesized a surface antigen reactive with anti-monocyte serum (6–9). Other effects of PMA on HL-60 cells have included increased polyamine levels (10, 11), altered phosphatidylcholine and triglyceride metabolism (12, 13), decreased synthesis of high-molecular-weight glycopeptides (14) and generation of potent procoagulant activity (15). The phorbol diesters exert these effects via stereospecific receptors (16–18).

The nature of the phorbol diester receptor and the possible mechanism by which the effects of the phorbol diesters were transduced across membranes had remained unknown. However, recently Castagna et al. (19) have shown that PMA produced a direct activation of the Ca^{2+} and phospholipid-dependent protein kinase, termed protein kinase C. This is a novel protein kinase, originally described in brain (20), and widely distributed in mammalian tissues (21, 22) including various types of human leukemic cells (23). It has been partially purified from platelets (24) and peripheral lymphocytes (25) as well as highly purified from bovine heart (26), rat brain (27), and pig spleen (28). The kinase is cyclic nucleotide and calmodulin insensitive but phos-

1. *Abbreviations used in this paper:* CHAPS, 3-[(3-cholamido-propyl)-dimethylammonia]-1-propane sulfonate; PDBu, phorbol 12,13-dibutyrate; 4αPDD, 4α-phorbol 12,13-didecanoate; PDD, phorbol 12,13-didecanoate; PMA, phorbol 12-myristate, 13-acetate; PMSF, phenylmethylsulfonyl fluoride.

pholipid and Ca^{2+} dependent and further stimulated by diacylglycerol. It may undergo limited proteolysis by calcium-dependent neutral protease (29). Using chelator extracts of the particulate fraction of rat brain, we recently tested the hypothesis that protein kinase C may indeed be the phorbol diester receptor (30). We found that stereospecific, saturable phorbol diester receptors copurified with protein kinase C activity through several steps. In addition, we confirmed the observation of PMA activation of protein kinase C. After initial submission of this paper, others have now demonstrated specific phorbol diester receptors in mouse brain cytosol that copurified with a Ca^{2+}/phospholipid-dependent kinase activity through gel filtration (31). Indeed, Ashendel et al. (32, 33), using detergent extracts of the particulate fraction of mouse brain, have shown copurification to apparent homogeneity of a specific phorbol diester receptor and a Ca^{2+}/phospholipid-dependent protein kinase. Operationally, three pools of phorbol diester receptors have been identified; a cytosolic pool which remains soluble even in the presence of Ca^{2+}, a cytosolic pool that binds to membranes in the presence of Ca^{2+} and can be solubilized by chelator extraction of the Ca^{2+} membranes, and a membrane pool that resists chelator extraction and requires detergent treatment for solubilization.

We have now extended these observations to the human leukemia cell line HL-60, in which the phorbol diesters exert well characterized biological effects. Utilizing all three of the aforementioned fractions, the cytosolic fraction, the chelator extract, and the detergent extract of the particulate fraction, we found copurification of phorbol diester receptors and protein kinase C activity through several steps. The two activities copurified in a constant ratio. Inhibition of [^{3}H]phorbol 12,13-dibutyrate ([^{3}H]PDBu) binding to the soluble receptor, as well as stimulation of the kinase activity, by a series of phorbol analogues demonstrated potency and specificity paralleling their known biological activities. Phorbol activation of protein kinase C, in the absence of Ca^{2+}, altered the phosphate acceptor substrate profile, at least in part, by an alteration of the apparent Michaelis constant (K_m) for histones. Thus the phorbol diesters may mediate their transmembrane effects by activation of protein kinase C, with resultant alterations of cellular phosphoproteins.

Methods

Reagents. Solutions of calf thymus histone type III-S, calf thymus histone type V-S (H1), casein, salmon protamine-free base, ovalbumin from chicken egg (Sigma Chemical Co., St. Louis, MO), and myelin basic protein (a gift from Dr. Eugene Day, Duke University) were made up in deionized water to 1 mg/ml immediately before use. Diolein and phosphatidylserine (Sigma Chemical Co.) were stored dessicated at 0°C in chloroform. Just before use, the chloroform was evaporated and the lipids resuspended by sonication in deionized water to 500 μg/ml. The stock solution of [^{3}H]PDBu (New England Nuclear, Boston, MA) was diluted to 1,000 nM in 20 mM Tris–HCl, pH 7.5, and 0.01% Pentax bovine serum albumin (Miles, Laboratories, Inc., Research Products Div., Elkhart, IN). PMA, PDBu, phorbol-12,13-didecanoate (PDD), and 4αphorbol-12,13-didecanoate (4αPDD) (PL Biochemicals, Inc., Milwaukee, WI) were dissolved in dimethyl sulfoxide at 10 mg/ml and stored at 0°C. Just before use they were diluted with deionized water. [γ^{32}P]ATP (ICN Radiochemicals, Inc., Covina, CA) was diluted to 10 μM ATP with 2–4 × 10^5 cpm [γ^{32}P]ATP/nmol.

Solubilization and partial purification of the phorbol receptor and protein kinase C. HL-60 cells (a gift from R. C. Gallo, National Institutes of Health) were grown in 3-liter spinner flasks in RPMI 1640 with 2 mM glutamine, 10 mM Hepes, penicillin (50 U/ml), streptomycin (50 μg/ml), and 10% heat-inactivated fetal bovine serum (Gibco Laboratories, Grand Island, NY). At a density of 1.0–1.5 × 10^6 cells/ml the cells were harvested by centrifugation and washed two times with Dulbecco's phosphate-buffered saline without divalent ions. The cells were resuspended in 5 vol of a lysis buffer consisting of 20 mM Tris–HCl, pH 7.5, 50 mM 2-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride (PMSF), and 0.01% leupeptin (Sigma Chemical Co.) and 1 mM $CaCl_2$ or 10 mM EGTA and 2 mM EDTA. Cells were broken by sonication at maximal output (Biosonik, Bronwil Scientific, Rochester, NY) for 2 min, then centrifuged at 100,000 *g* for 2 h. With cells lysed in the presence of chelators, the resultant supernatant and pellet were termed the chelator cytosol and chelator pellet, respectively. With cells lysed in the presence of $CaCl_2$, the resultant pellet was termed the Ca^{2+} pellet.

The chelator cytosol was brought up to 10% sucrose (wt/vol) and added to an excess of DEAE cellulose (Whatman Chemical Separation, Inc., Clifton, NJ) equilibrated in 20 mM Tris–HCl, pH 7.5, 10 mM EGTA, 2 mM EDTA, 50 mM 2-mercaptoethanol, and 10% sucrose. The mixture was added to a 4 × 50-cm column and washed with one column volume of the equilibration buffer then washed with 2 vol of 20 mM Tris–HCl, pH 7.5, 0.1 mM EGTA, 0.1 mM EDTA, 50 mM 2-mercaptoethanol, and 10% sucrose. Proteins were eluted with a 200-ml linear gradient of 0.0–0.3 M NaCl in the second wash buffer. Active fractions were pooled, concentrated by ultrafiltration, and applied to a 1 × 45-cm Sephadex G-150 (Pharmacia Fine Chemicals, Piscataway, NJ) column equilibrated in the same buffer. Blue dextran (Pharmacia Fine Chemicals), bovine serum albumin (Sigma Chemical Co.), and lactalbumin (Sigma Chemical Co.) were run separately as markers.

The chelator pellet was resuspended by several strokes of a glass-teflon tissue homogenizer in 20 mM Tris–HCl, pH 7.5, 2 mM PMSF, 10 mM EGTA, 2 mM EDTA, 50 mM 2-mercaptoethanol, 0.01% leupeptin, and 20 mg/ml of 3-[(3-cholamido-propyl)dimethylammonia]-1-propane sulfonate (CHAPS) and stirred at 4°C for 15 min. The mixture was then centrifuged at 100,000 *g* for 2 h. The resultant supernatant, termed detergent extract of pellet, was then carried through DEAE ion exchange as described above.

The Ca^{2+} pellet was resuspended by several strokes of a glass-teflon tissue homogenizer in 20 mM Tris–HCl, pH 7.5, 5 mM EGTA, 2 mM EDTA, 2 mM PMSF, and 0.01% leupeptin and stirred at 4°C for 1 h. The mixture was centrifuged at 100,000 *g* for 2 h. To the supernatant was added ammonium sulfate up to 21% (wt/vol), the precipitate was pelleted, and the resultant supernatant was brought up to 45% ammonium sulfate. The 21–45% ammonium sulfate pellet was collected by centrifugation and resuspended in 20 mM Tris–HCl, pH 7.5, 2 mM EGTA, 2 mM EDTA, 50 mM 2-mercaptoethanol, and 10% sucrose. After dialysis against this same buffer, the 21–45% ammonium sulfate pellet was applied to an excess of DEAE cellulose equilibrated with the same buffer. After a two column volume wash with this buffer, a 60-ml linear gradient of 0.0–0.6 M NaCl in equilibration buffer was applied. Active fractions were pooled and used for further studies.

Phorbol receptor assay. Whole cell phorbol receptor assay was done according to the method described by Goodwin and Weinberg (34). The soluble phorbol receptor assay was done as previously described (30). Briefly, 50 μl of receptor preparation was added to a 150 μl mixture

containing 20 nM [^{3}H]PDBu in 20 mM Tris–HCl, pH 7.5, 1.0 mM $CaCl_2$, and 20 μg/ml phosphatidylserine. After incubation at 22°C for 15 min, receptor bound and free [^{3}H]PDBu were separated by filtration through 1.8-ml AcA 44 columns (LKB). Radioactivity was measured in 15 ml of Ultrafluor in a Beckman LS6800 counter (Beckman Instruments Inc., Fullerton, CA).

Protein kinase C assay. Protein kinase C was assayed by measuring the incorporation of ^{32}P from [γ^{32}P]ATP into specified phosphate acceptor substrates. The reaction mixture (250 μl) consisted of 10 μM ATP (2–4 × 10^5 cpm [γ^{32}P]ATP/nmol), 20 μg/ml phosphatidylserine, 2 μg/ml diolein, either 1 mM EGTA or $CaCl_2$ as designated, 10 mM $MgCl_2$, and 50 μl of enzyme preparation. The reaction was started by the addition of enzyme source, incubated at 30°C for 10 min, and stopped by the addition of 500 μg of bovine albumin and 1 ml of 4°C 25% trichloroacetic acid (TCA). Precipitated proteins were collected and washed with 10 ml of 4°C 25% TCA on Millipore HA filters (0.45 μm, Millipore Continental Water Systems, Bedford, MA). Dried filters were suspended in 10 ml Ultrafluor and ^{32}P counted in a Beckman LS6800 counter.

Results

Extraction and partial purification of the phorbol diester receptor and protein kinase C. Based on observations that ~60% of rat brain phorbol diester receptors and protein kinase C were membrane-bound following tissue homogenization in the presence of Ca^{2+} (27, 30), we initially homogenized HL-60 cells in 1 mM $CaCl_2$, separated the cytosol and crude particulate fractions by centrifugation, and then assayed for both phorbol diester receptors and protein kinase C activity. By Scatchard (35) analysis (data not shown), the starting HL-60 cells demonstrated a K_D = 21 nM for [^{3}H]PDBu and 120,000 receptors/cell, in agreement with previous determinations (17, 18). As seen in Table I, 93% of the total cellular receptors was recovered in the crude homogenate. The supernatant obtained after EGTA/EDTA extraction of the 100,000 *g* pellet was fractionated with ammonium sulfate; 12% of the total phorbol diester receptors was recovered in the 21–45% ammonium sulfate pellet. In four separate preparations, a yield of 10–13% was obtained at this step.

In an attempt to characterize all pools of the phorbol diester receptor and protein kinase C, we also prepared cellular homogenates in the presence of chelators. The presence of chelators is necessary to characterize the cytosolic component in order to avoid Ca^{2+}-mediated proteolysis (27). About 75% of the original whole cell phorbol diester receptors can be accounted for in the chelator cytosol and the chelator pellet combined. Hence, three pools of soluble phorbol diester receptors, cytosolic receptors prepared in the presence of chelators, membrane receptors extracted with detergent, and receptors extracted with chelators from Ca^{2+}-prepared membranes, can be examined. These fractions represent at least 75% of total phorbol diester receptors (Table I); the remainder of the receptor activity is either lost during the initial sonication and centrifugation (7–20%) or remains associated with the particulate after chelator or detergent extraction (5–15%).

We also attempted to follow the distribution of protein kinase C during the homogenization and extraction sequences. How-

Table I. Recovery of Phorbol Diester Receptors and Protein Kinase C

Fraction	Yield phorbol binding	Specific activity [^{3}H]PDBu binding	Phorbol binding / Protein kinase C	
	%	*pmol/mg protein*		
Whole cells	100			
Cellular homogenate	93			
Chelator cytosol	55	4.5		
Chelator pellet	20	2.9		
Chelator extract Ca^{2+} pellet	12			
DEAE pool of chelator cytosol	40	55.1	Ca^{2+} PMA	0.0284 0.0657
DEAE pool of detergent extract	11	11.1	Ca^{2+} PMA	0.0292 0.0928
DEAE pool of chelator extract	4		Ca^{2+} PMA	0.0305 0.0924
G-150 pool of DEAE-purified chelator cytosol	12	95.4	Ca^{2+} PMA	0.0300 0.0908

After measurement of whole cell phorbol diester receptors, the cells were resuspended in buffers containing 20 mM Tris–HCl, pH 7.5, 50 mM 2-mercaptoethanol, 2 mM PMSF, 0.01% leupeptin and either 1 mM $CaCl_2$ or 10 mM EGTA and 2 mM EDTA and lysed by sonication (cellular homogenate). The homogenates were centrifuged at 100,000 *g* for 2 h and the fractions termed chelator cytosol, chelator pellet, and Ca^{2+} pellet obtained. The Ca^{2+} cytosol was discarded. The chelator cytosol was applied to DEAE cellulose and eluted with a linear (0–0.3 M) NaCl gradient. Active fractions were pooled, their total [^{3}H]PDBu binding activity determined and compared with whole cell total binding activity (percent yield). [^{3}H]PDBu binding of the pooled fractions (counts per minute per milliliter) was compared with protein kinase activity of the same pooled fractions stimulated by either 500 μM $CaCl_2$ and 20 μg/ml phosphatidylserine or 16 nM PMA and 20 μg/ml phosphatidylserine (counts per minute per milliliter) and expressed as a ratio of phorbol binding/protein kinase activity. Part of the pooled fractions from the DEAE column of the chelator cytosol was subjected to Sephadex G-150 gel sizing chromatography. Active fractions were pooled and percent recovery and phorbol binding/protein kinase ratio determined as above. The chelator pellet was suspended by tissue homogenization in the original lysis buffer, an aliquot taken for [^{3}H]PDBu binding and CHAPS added to a final concentration of 20 mg/ml and stirred for 15 min at 4°C. The detergent extraction solution was centrifuged and the supernatant applied to a DEAE cellulose column and eluted with a linear (0–0.3 M) NaCl gradient. Active fractions were pooled, percent recovery [^{3}H]PDBu binding and phorbol binding/protein kinase ratios determined as above. The Ca^{2+} pellet was resuspended by glass-teflon homogenization in 20 mM Tris–HCl, pH 7.5, 5 mM EGTA, 2 mM EDTA, 2 mM PMSF, and 0.01% leupeptin and incubated for 1 h at 4°C. A 21–45%-ammonium sulfate fraction was prepared and subjected to DEAE cellulose chromatography. Active fractions were pooled and percent recovery [^{3}H]PDBu binding and phorbol binding/protein kinase ratios determined as above. Protein was determined according to the method of Bradford (61) and picomoles [^{3}H]PDBu per milligram protein determined and reported as specific activity.

ever, Ca^{2+}- and lipid-stimulated kinase activity was evident only after ammonium sulfate or DEAE ion exchange steps. An endogenous inhibitor or specific phosphatase masks the activity during the early steps. Therefore, parallel calculations of protein kinase C and phorbol diester receptor yields were not possible.

Three points are worthy of mention. Treatment of the cells with 5 mM diisopropylfluorophosphate before sonication did not increase the yield of kinase or receptor (31). Second, when cells were washed with Ca^{2+}-containing buffers before homogenization, subsequent extraction of the crude particulate fraction with chelators was less efficient (n = 4). Therefore, cells were always washed without divalent ions before cell disruption in the lysis buffer. Finally, the chelator extraction mixture of 5 mM EGTA and 2 mM EDTA was found to be superior to 20 mM EGTA alone and to chelators plus 500 mM NaCl.

Next, the cytosolic, the chelator-extracted, and the detergent-extracted fractions were purified by DEAE ion exchange chromatography. Fractions were assayed for specific phorbol diester receptor activity and for Ca^{2+}/phospholipid-dependent and independent protein kinase activity. In addition, the active cytosolic and detergent extracted column fractions were assayed for PMA-stimulated protein kinase activity, while the active chelator extracted column fractions were pooled and used for further phorbol activated kinase studies (see below). As illustrated in Fig. 1*A–C*, the phorbol diester receptor activity and the Ca^{2+}/phospholipid-stimulated and PMA-stimulated kinase activities all coeluted as a single peak (n = 3; n = 6; n = 3, respectively). Greater than 50% of the original phorbol diester receptors could be accounted for in the combined active DEAE fractions (Table I).

At this stage of purification the kinase exhibited an absolute dependence on added phospholipid and Ca^{2+} or PMA. The phorbol diester receptor shared the lipid requirement and was also stimulated by exogenous Ca^{2+}. The active fractions from the cytosolic DEAE column were applied to a Sephadex G-150 sizing chromatography column as shown in Fig. 2. Again, the phorbol diester receptor and the Ca^{2+}/phospholipid- and PMA-stimulated kinase activities comigrated as a single peak, just ahead of bovine serum albumin. Similar results were seen when the chelator-extracted pool was subjected to sizing chromatography (data not shown).

Since it was not possible to compare yields of phorbol receptor and protein kinase C, the ratio of the two activities during the later stages of purification was determined. The active fractions from the three ion exchange columns and from the sizing column were each pooled, dialyzed against a common buffer, and assayed for specific phorbol diester receptor activity and phorbol and Ca^{2+}/phospholipid-dependent kinase activity. As shown in Table I, the ratios of phorbol binding to protein kinase activities was very similar, independent of the method of solubilization or purification.

Characterization of the partially purified soluble phorbol diester receptor. Fig. 3 depicts the saturation curve for binding of [^{3}H]PDBu to aliquots of the pooled DEAE fractions of chelator extracted membranes. Saturation was evident above 25 nM with 50% of the sites occupied at 7.5 nM [^{3}H]PDBu. Scatchard analysis (35) showed a strictly linear relationship with a derived K_D = 8.1 nM.

As stated above, Ca^{2+} and phospholipid were required in order to demonstrate high-affinity binding of [^{3}H]PDBu to the soluble receptor. To further characterize this requirement, Ca^{2+} and phosphatidylserine dose-response curves were done as shown in Fig. 4. In the presence of 20 μg/ml phosphatidylserine, [^{3}H]PDBu binding was maximal at 10^{-4} M $CaCl_2$ (Fig. 4 *A*). In the presence of EGTA, about one-third maximal binding was seen, while $CaCl_2$ concentrations above 10^{-3} M inhibited binding to the unstimulated level. The absolute phospholipid requirement is depicted in Fig. 4 *B*. The curve has the shape of a typical saturation curve, with half-maximal stimulation at 20 μg/ml and saturation >100 μg/ml.

Phorbol activation of partially purified protein kinase C. Because of the observations that the phorbol diesters could directly activate rat brain protein kinase C (19, 30), similar experiments were undertaken using the enzyme partially purified from HL-60 cells. Using the pooled fractions from chelator extracted DEAE chromatography, the effects of phosphatidylserine, diolein, and/or PMA on kinase activity, as a function of Ca^{2+} concentration, were determined (Fig. 5). In the presence of EGTA, neither diolein nor phosphatidylserine, alone or together, activated protein kinase C. However, in the presence of 10^{-6} M Ca^{2+}, the addition of phosphatidylserine dramatically increased the kinase activity. Increasing the Ca^{2+} concentration brought about a dose-dependent increase in enzyme activity with maximum activity at 10^{-4} M Ca^{2+}. The further addition of the unsaturated diacylglycerol, diolein, was associated with only a modest (n = 3) enhancement of kinase activity at less than maximum Ca^{2+} concentrations. Likewise, PMA alone, at 16 nM, did not alter the enzyme. However, the addition of PMA plus phosphatidylserine, even in the presence of EGTA, greatly enhanced the protein kinase activity to 60% of the maximum Ca^{2+}/phospholipid-stimulated activity. The further addition of only 10^{-6} M Ca^{2+} was associated with maximum enzyme activity. The stimulation of kinase activity by the combination of PMA at 16 nM plus optimal concentrations of Ca^{2+}, phosphatidylserine and diolein was identical to the stimulated activity seen with either inducer alone (i.e., the two methods of activation were not additive).

Knowing the optimal conditions for enzyme activation, phosphate acceptor substrate specificity was examined. Because of initial differences seen in the phosphate acceptor substrate specificity of Ca^{2+}/phospholipid-stimulated and PMA-stimulated kinase activities, studies were done to clarify this difference. As seen in Fig. 6 *A*, Histone H1, histone type IIIS, and myelin basic protein were all excellent phosphate acceptors for the Ca^{2+}-stimulated kinase (solid lines). The histones were also good substrates for PMA-stimulated enzyme (dashed lines) and the initial differences seen at the usual substrate concentrations of 200 μg/ml, could be explained by a change in K_m (Fig. 6 *B*). However, the differences seen using myelin basic protein may be more complex and may involve more than just an alteration in K_m. We could not achieve concentrations of myelin basic protein in the assay, which were high enough to determine an

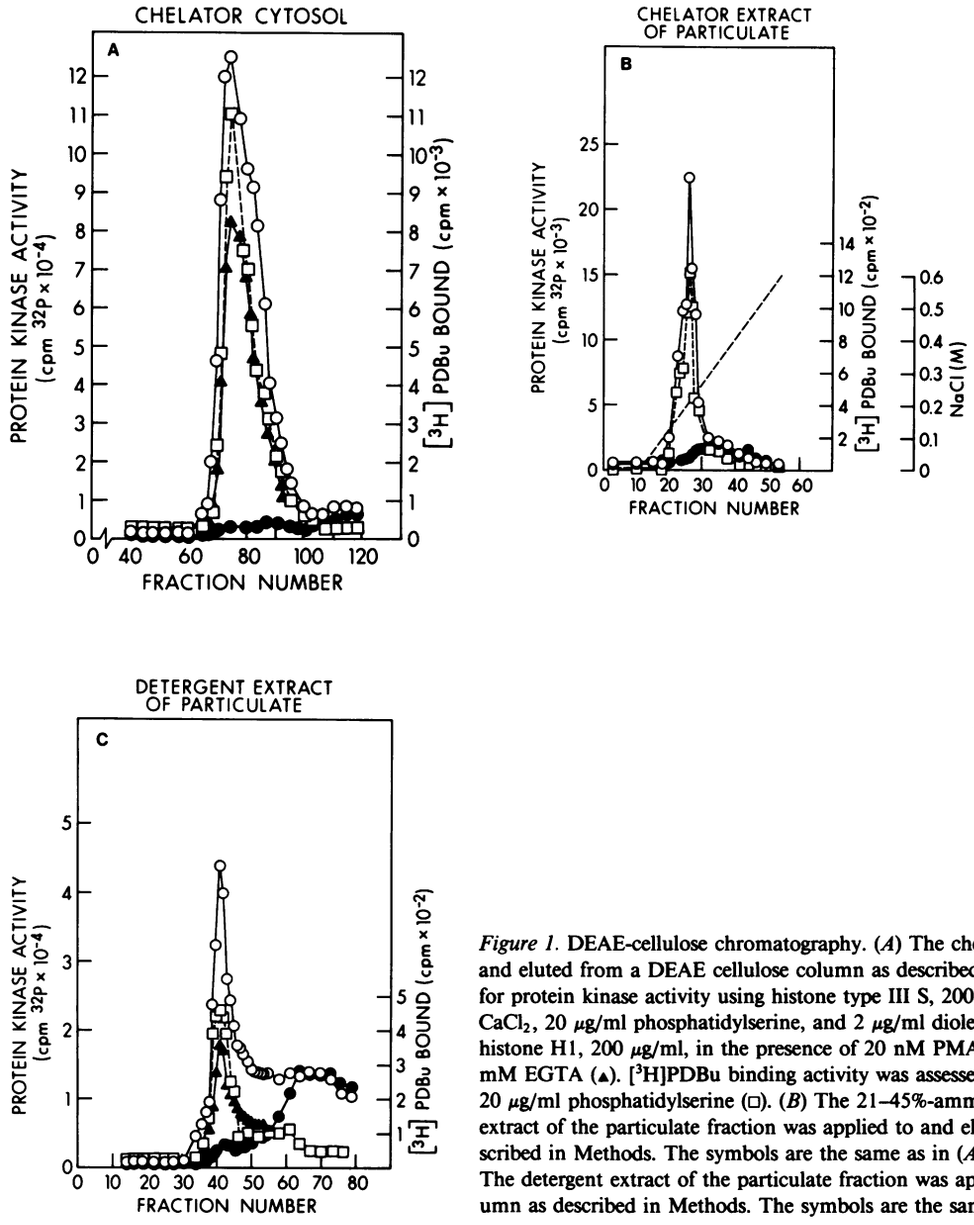

Figure 1. DEAE-cellulose chromatography. (*A*) The chelator cytosol was prepared, applied to and eluted from a DEAE cellulose column as described in Methods. Fractions were assayed for protein kinase activity using histone type III S, 200 μg/ml, in the presence of 500 μM $CaCl_2$, 20 μg/ml phosphatidylserine, and 2 μg/ml diolein (○) or 1 mM EGTA (●), or using histone H1, 200 μg/ml, in the presence of 20 nM PMA, 20 μg/ml phosphatidylserine, and 1 mM EGTA (▲). [³H]PDBu binding activity was assessed in the presence of 1 mM $CaCl_2$ and 20 μg/ml phosphatidylserine (□). (*B*) The 21–45%-ammonium sulfate fraction of the chelator extract of the particulate fraction was applied to and eluted from a DEAE column as described in Methods. The symbols are the same as in (*A*) except for NaCl gradient (-----). (*C*) The detergent extract of the particulate fraction was applied to and eluted from a DEAE column as described in Methods. The symbols are the same as in (*A*).

accurate K_m with the PMA-stimulated kinase. In similar studies, protamine, casein, and ovalbumin were modest-to-poor acceptor substrates in all instances (data not shown).

Phorbol diester stereospecificity of the soluble phorbol diester receptor and of protein kinase C activation. The specificity of the soluble phorbol diester receptor was examined. As illustrated in Fig. 7, the inhibition of [³H]PDBu binding by various phorbol analogues was assessed over a range of concentrations. Binding inhibition mirrored known biologic potency. PMA, the most potent phorbol diester analogue, completely inhibited binding of [³H]PDBu at 20 nM with 50% inhibition at 3 nM. PDD and PDBu are less potent as inducers of HL-60 maturation. Near maximal binding inhibition for both was seen at 250 nM with 50% inhibition at 21 nM and 7 nM, respectively. The biologically inactive phorbol diester, 4αPDD, did not inhibit [³H]PDBu binding over the concentration range of 50 to 500 nM.

Finally, the dose-response and stereospecificity of the activation of protein kinase C by phorbol diester analogues was examined. The results are illustrated in Fig. 8. Since it was known that phorbol stimulation altered the K_m for the histone

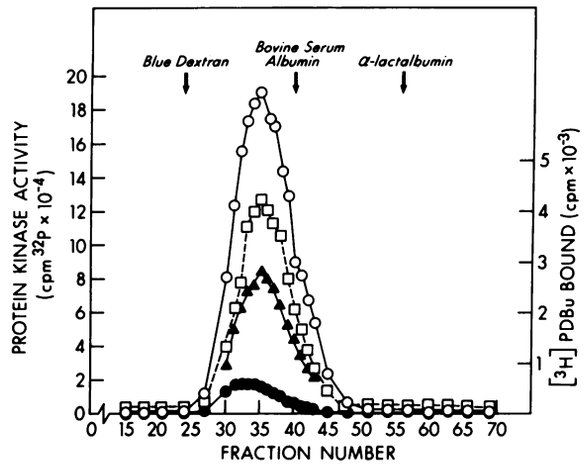

Figure 2. Sephadex G-150 chromatography. Active fractions from the chelator cytosol DEAE column were subjected to Sephadex G-150 chromatography as described in Methods. Fractions were assayed for protein kinase activity using histone type III-S, 200 μg/ml, in the presence of 500 μM $CaCl_2$, 20 μg/ml phosphatidylserine, and 2 μg/ml diolein (○) or 1 mM EGTA (●) or using histone H1, 200 μg/ml, in the presence of 20 nM PMA, 20 μg/ml phosphatidylserine, and 1 mM EGTA (▲). [^{3}H]PDBu-binding activity was assessed in the presence of 1 mM $CaCl_2$ and 20 μg/ml phosphatidylserine (□). The vertical arrows denote the elution positions of the standard markers.

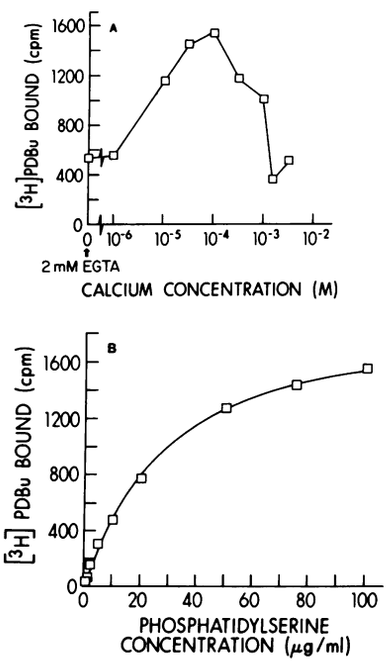

Figure 4. (*A*) Binding of [^{3}H]PDBu as a function of $CaCl_2$ concentration. 50-μl aliquots of pooled DEAE fractions were assayed for [^{3}H]PDBu binding activity in the presence of 20 μg/ml phosphatidylserine and 20 nM [^{3}H]PDBu and various concentrations of $CaCl_2$ or 2 mM EGTA. Data points are averages of duplicate measurements. Simultaneous nonsaturable binding, assessed in the presence of a 100-fold excess (5 μM) of nonradioactive PDBu were consistently <100 cpm ^{3}H. (*B*) Binding of [^{3}H]PDBu as a function of phosphatidylserine concentration. 50-μl aliquots of pooled DEAE fractions were assayed for [^{3}H]PDBu binding in the presence of 1 mM $CaCl_2$ and 20 nM [^{3}H]PDBu and various concentrations of phosphatidylserine. Points are averages of duplicates and nonsaturable binding was as in (*A*). These experiments are representative of two such experiments.

phosphate acceptor substrates, two acceptors were used, histone type III-S and histone H-1. Using type III-S as substrate (Fig. 8 *A*), PMA, in the presence of EGTA and phosphatidylserine, was the most potent activator of protein kinase C. Maximum enzyme activity occurred at 50 nM and 50% activation at 8 nM PMA. However, PDBu was only slightly less potent with equal stimulation at 100 nM and 50% activation at 16 nM. PDD was a less potent enzyme activator with maximum kinase activity at 250 nM and 50% activity at 30 nM. Activation by all three analogues resulted in the same maximal kinase activity. The biologically inactive analogue 4α-PDD was ineffective as an activator of protein kinase C even at 500 nM. Changing the phosphate acceptor substrate to histone H1 did not alter the rank order potency of the phorbol diester analogues (Fig. 8 *B*). Under these conditions, maximal kinase activation occurred at 100 nM PMA with an ED_{50} = 11 nM PMA. PDBu- and PDD-stimulated kinase activity showed ED_{50} values of 24 nM PDBu and 70 nM PDD. Again, the same maximal kinase activity was achieved with each of the phorbol analogues. 4α-PDD was ineffective as an enzyme activator. Hence, as regards phorbol

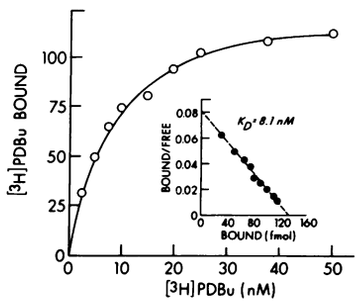

Figure 3. Binding of [^{3}H]PDBu as a function of increasing concentration. Data points are averages of duplicate measurements of 50 μl from pooled DEAE fractions. Scatchard analysis is shown in the insert. This is representative of three such experiments with derived K_D = 7.0, 8.1, 10.0 nM [^{3}H]PDBu.

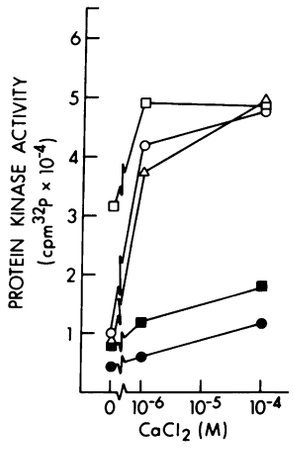

Figure 5. Activation of protein kinase C by PMA and lipids as a function of $CaCl_2$ concentration. Chelator-free pooled DEAE fractions from chelator extract were assayed for protein kinase activity using histone type III-S, 200 μg/ml, in the presence of 1 mM EGTA or added $CaCl_2$ at 1–100 μM. Incubation mixtures contained: 2 μg/ml diolein (●); 16 nM PMA (■); 20 μg/ml phosphatidylserine (△); 2 μg/ml diolein and 20 μg/ml phosphatidylserine (○); 16 nM PMA and 20 μg/ml phosphatidylserine (□). Data points are averages of duplicate measurements and this data is representative of three experiments.

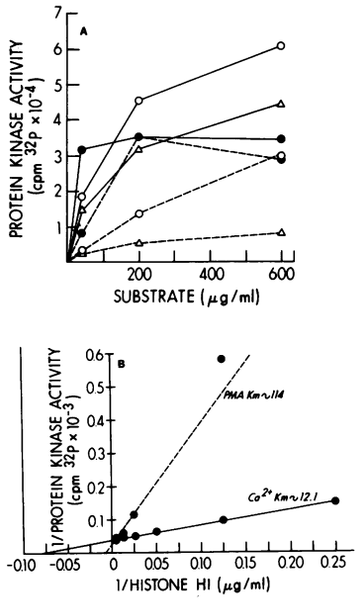

Figure 6. Activity of protein kinase C stimulated by PMA or $CaCl_2$. (*A*) Activity as a function of phosphate acceptor substrate concentration. Pooled chelator cytosol DEAE fractions were assayed for kinase activity stimulated by either 500 μM $CaCl_2$, 20 μg/ml, phosphatidylserine, and 2 μg/ml diolein (solid lines) or 16 nM PMA, 20 μg/ml phosphatidylserine and 1 mM EGTA (dashed lines) in addition to 0–600 μg/ml of the following substrates: histone type III S (○), histone H1 (●), or myelin basic protein (△). (*B*) Line-Weaver-Burk plot of the reciprocal of the protein kinase activity stimulated by either 500 μM $CaCl_2$, 20 μg/ml phosphatidylserine, and 2 μg/ml diolein (solid line) or 16 nM PMA, 20 μg/ml phosphatidylserine, and 1 mM EGTA (dashed line) as a function of the reciprocal of various concentrations of histone H1 substrate. Data points were obtained by averaging duplicate counts from two separate experiments.

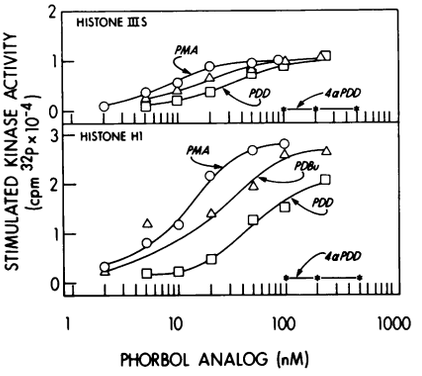

Figure 8. (*A*) Activation of protein kinase C by phorbol analogues utilizing histone III-S as substrate. 50-μl samples from pooled DEAE fractions were assayed for protein kinase activity in the presence of 1 mM EGTA, 20 μg/ml phosphatidylserine, and 200 μg/ml of histone III-S in addition to one of the following: PMA at 2–100 nM (○); PDD at 5–250 nM (□); PDBu at 5–250 nM (△); 4αPDD at 100–500 nM (*). Data points are averages of duplicate values. (*B*) Activation of protein kinase C by phorbol analogues utilizing H1 as substrate. 50-μl of the same source as in (*A*) were assayed for kinase activity in the presence of 1 mM EGTA, 20 μg/ml phosphatidylserine, and 200 μg/ml of histone H1 in addition to one of the following: PMA at 2–100 nM (○); PDD at 5–250 nM (□); PDBu at 2–250 nM (△); 4αPDD at 100–500 nM (*). Data points are averages of duplicate values from a single experiment and are representative of data from three experiments.

diester activation of protein kinase C, enzyme activation potency reflects the known biologic potency of the phorbol analogues.

Discussion

The HL-60 cell line was established from a patient with acute promyelocytic leukemia and has proven to be an experimentally accessible model for the study of myeloid differentiation (37, 38). The phorbol diester tumor promoter, PMA, has been the most potent and efficacious inducer of macrophage maturation of this cell line (3–5). The macrophage nature of the differentiated cells has been repeatedly confirmed (4,6–8). The abilities of a series of phorbol diester analogues to induce macrophage maturation followed a rank order of potency and specificity consistent with their known abilities to promote tumors (5).

Other cell lines have also been found to be responsive to the differentiating effects of the phorbol diesters. The myeloblastic leukemia cell lines KG-1 and ML-3 and the monocytic leukemia cell line THP-1 were also induced to macrophage maturity with PMA treatment (7, 39). This success with differentiation of continuous cell lines was extended to fresh explants of human leukemia cells. Cells from patients with acute and chronic myelocytic or myelomonocytic leukemias have been treated in primary culture with the phorbol diesters. The treated cells universally acquired differentiated macrophage characteristics (40–42).

Phorbol diester-induced leukemic cell differentiation exhibited the potency and stereospecificity characteristic of a receptor-mediated response. Driedger and Blumberg (43) and Delclos et al. (44) developed [^{3}H]PDBu as a specific ligand for the phorbol diester receptor and this ligand was used to demonstrate specific receptors in a variety of normal and transformed cells and tissues, including the HL-60 cell line (16, 45, 46). Solanki et al. (17) found 80,000 phorbol diester receptors per cell with a K_D = 50 nM in both phorbol responsive and unresponsive HL-60 cells. Cooper et al. (18) found 190,000 receptors per HL-60 cell with

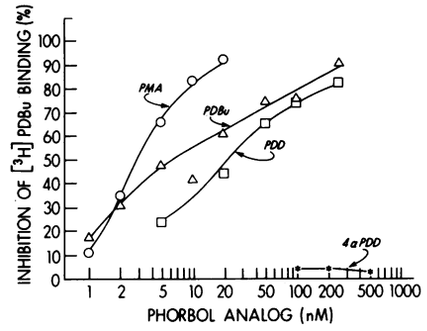

Figure 7. Inhibition of [^{3}H]PDBu binding by various phorbol diester analogues. 50-μl aliquots from pooled DEAE fractions were incubated with 20 nM [^{3}H]PDBu, 1 mM $CaCl_2$, and 20 μg/ml phosphatidylserine in addition to one of the following phorbol analogues: PMA at 1–20 nM (○); PDD at 5–250 nM (□); PDBu at 5–250 nM (△); 4αPDD at 100–500 nM (*). Data points are averages of duplicate values from two separate experiments.

a K_D = 23 nM. The phorbol diesters also appear to exert their effects in fresh leukemic explants via specific receptors (46a).

However, the biochemical nature of the phorbol diester receptors had remained unknown until recently. Castagna et al. (19) demonstrated direct activation of rat brain Ca^{2+}/phospholipid-dependent protein kinase C by the active phorbol analogues. We confirmed this observation and also showed that soluble phorbol diester receptors copurified with protein kinase C from rat brain (30) lending further support to the identity of protein kinase C as the phorbol diester receptor. The very recent reports (32, 33) demonstrating that both activities from mouse brain apparently reside in a single molecule, further strengthen this argument.

In this paper we have extended these observations to the HL-60 cell line in which the phorbol diesters elicit a known biological response. The initial whole cell phorbol receptor measurements of 120,000 receptors/cell with a K_D = 21 nM were similar to those previously reported (17, 18). Following cell lysis and fractionation by centrifugation, the sum of the cytosolic and membrane-associated receptors nearly equaled the total quantity of receptors measured in the intact cells. This suggests that both the cytosolic and membrane-associated receptors are measured in the whole cell binding assay. But if approximately one-third of the total receptors are cytosolic in the presence of Ca^{2+}, and two-thirds are cytosolic in the presence of divalent ion chelators (27, 30), how can they be measured in the whole cell receptor assay? Recently Kraft et al. (47) and Kraft and Anderson (48), using a thymoma cell line and yolk sac cells, demonstrated vastly decreased cytosolic protein kinase C activity and increased membrane-associated activity after whole cell treatment with PMA. Indeed, using this thymoma cell line, EL4, Sando and Young (49) have very recently identified a cytosolic, high-affinity phorbol diester receptor. Thus, phorbol diester exposure appears to bring about translocation of cytosolic protein kinase C/phorbol diester receptor to the membrane, so that most of the kinase/receptor is membrane-bound and hence accessible to the radioligand.

We attempted to study all forms of the phorbol diester receptor that could be solubilized. The phorbol diester receptor and protein kinase C activities copurified through ammonium sulfate precipitation, DEAE ion exchange, and Sephadex G-150 sizing chromatography from all pools studied. In fact, in all studies done to date, specific phorbol diester receptors have always been associated with the protein kinase activity. Through these steps, the phorbol diester receptor developed a requirement for both exogeneous Ca^{2+} and phospholipid. Identical requirements have been well documented for protein kinase C (26, 27). In addition, similar requirements have recently been demonstrated for a soluble phorbol diester receptor found in several tissue types (31, 32, 49, 50). In this study, optimal receptor binding and maximum kinase activity occurred at the identical Ca^{2+} concentration of 10^{-4} M. In addition, the relative mobilities of both activities from HL-60 cells on sizing chromatography are in accord with that found previously for protein kinase C from other sources (26, 27).

The probable identity of protein kinase C and the phorbol receptor was further supported by the studies of kinase activation by the phorbol analogues. PMA, at biologically relevant concentrations, directly activated protein kinase C, even in the absence of Ca^{2+}. This activation was dose dependent and stereospecific, with activation potency correlated with known biologic potency for tumor promotion and induction of cell differentiation. In addition, phorbol activation of protein kinase C brought about a change in the phosphate acceptor substrate profile of the enzyme by altering the K_m for the histone substrates, although the differences noted using myelin basic protein as substrate may be more complex. It would seem that the phorbol diesters exert their effects on the HL-60 cell by initially binding to stereospecific, high-affinity sites on protein kinase C. Resultant activation of this enzyme may be reflected in vivo by increased membrane-associated kinase activity with subsequent changes in cellular phosphoproteins. Indeed, we have characterized several phosphoprotein changes in HL-60 cells after treatment with PMA and other active phorbol analogues (Vandenbark, G. R., and J. E. Niedel, manuscript in preparation).

The linkage of the phorbol diester receptor with protein kinase C has interesting implications. Recently much evidence has accumulated demonstrating a causal role for protein phosphorylation in oncogenesis induced by retroviruses (51–54). Modulation of normal growth by epidermal growth factor and platelet-derived growth factor may also involve activation of membrane-associated protein kinases (55–57). Tumor promotion and cellular differentiation now also appear to be tightly associated with activation of a protein kinase. Thus, we are now attempting to characterize the endogenous phosphate acceptor substrates for phorbol diester-activated protein kinase C in HL-60 cells.

In summary, in the HL-60 cell line we have: (*a*) demonstrated soluble phorbol diester-binding protein from three cellular pools accounting for at least 75% of the total whole cell phorbol diester receptors, and shown them to have the affinity, saturability, and stereospecificity expected of the phorbol diester receptor; (*b*) shown that the phorbol diester receptor and Ca^{2+}/phospholipid-dependent protein kinase C cosolubilize and copurify in a constant ratio; (*c*) demonstrated direct activation of protein kinase C by the phorbol diesters at biologically active concentrations and with a specificity paralleling the known biologic potency of the various analogues. With these data and the rapid cellular phosphoprotein changes seen in PMA-treated HL-60 cells (Vandenbark, G. R., and J. E. Niedel, manuscript in preparation), we propose that phorbol binding to and activation of protein kinase C may be causal in the induction of macrophage maturation of myeloid leukemic cells by the phorbol diesters. Biologically relevant (i.e., lymphokines, monokines) inducers of cellular differentiation may activate protein kinase as well (58–60).

Note added in proof. Feuerstein and Cooper (1983. *J. Biol. Chem.* 258:10786–10793) have demonstrated rapid changes in HL-60 phosphoproteins induced by PMA.

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