Activation of Distinct Protein Kinase C Isozymes by Phorbol Esters: Correlation with Induction of Interleukin 1β Gene Expression

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ABSTRACT: Treatment of human promyelocytic leukemia cells U937 with phorbol 12-myristate 13-acetate (TPA) induces them to differentiate into monocytic cells [Harris, P., & Ralph, P. (1985) *J. Leukocyte Biol. 37*, 407–422]. Here we investigated the effects of TPA on interleukin 1 gene expression and the possible role of protein kinase C (PKC) in this process. Addition of TPA to serum-starved U937 cells induced the expression of the interleukin 1β (IL-1β) gene. This effect was apparent as early as 2 h and peaked at 24 h in the presence of 5×10^{-8} M TPA. Higher concentrations of TPA, which partially or totally depleted protein kinase C levels in the cells (10^{-9}–2×10^{-5} M), had an inhibitory effect on IL-1β mRNA expression. Cell-permeable 1,2-dioctanoyl-*sn*-glycerol (diC_8), a diacylglycerol that activates PKC in intact cells and cell-free systems, did not mimic the effect of TPA on the IL-1β mRNA induction. To determine the protein kinase C isozymes present in the control and TPA- (5×10^{-8} M) treated U937 cells, we prepared antipeptide antibodies that specifically recognize the α, β, and γ isoforms of protein kinase C in rat brain cytosol and U937 cell extracts. In "control" U937 cells, 30% of PKC α was particulate, and PKC β was cytosolic, while there was no detectable PKC γ. Upon TPA treatment, there was a time-dependent translocation (maximum 1 h) of PKC α to a particulate compartment, followed by its gradual disappearance (70% by 3 h, not detectable by 6 h), with no concomitant rise in the cytosolic form. PKC β remained cytosolic during TPA treatment, while PKC γ appeared at 6 h and continued to increase in abundance by 24 h, mostly in particulate form. Exposure of $^{32}PO_4$-labeled cells to TPA (5×10^{-8} M) for 30 min enhanced the phosphorylation of several major substrates; 5 of 10 were in the same postmitochondrial fraction into which PKC α translocated. Exposure of U937 cells to diC_8 (5×10^{-5}–10^{-4} M) failed to induce PKC α translocation. Although diC_8 induced the phosphorylation of five substrates, these were cytosolic and were distinct from the substrates phosphorylated in the presence of TPA. D-Sphingosine of H-7, PKC antagonists, prevented the accumulation of IL-1β transcripts and TPA-induced phosphorylation of endogenous substrates in a dose-dependent manner. Our data suggest a potential role for PKC α mediated phosphorylation of substrates in the initial events leading to TPA-induced differentiation of a promonocytic cell to a monocyte/macrophage.

TPA[1] is a member of the family of plant diterpenes that elicits a multitude of responses in a variety of cells leading to an altered pattern of growth or differentiation (Vandenbark & Niedel, 1984). Addition of TPA to U937 cells induces these cells to differentiate into a monocyte/macrophage [for review, see Harris and Ralph (1985)]. This process has been associated with the induction of several cellular protooncogenes including c-*fos* (Mitchell et al., 1985), c-*fms* and c-*sis* (Pantazis et al., 1986), changes in morphological appearance, and adherence to substratum (Mitchell et al., 1985). Addition of TPA has also been shown to induce the production and secretion of interleukin 1 by the U937 human promonocytic cell line or the murine macrophage cell line P388D1 (Mizel et al., 1978) as measured by the proliferative response of murine thymocytes.

The cellular effects of TPA are mediated by the activation of the calcium- and phospholipid-dependent protein kinase C [for reviews, see Kikkawa and Nishizuka (1984) and Niedel and Blackshear (1986)]. Physiologically, protein kinase C (PKC) is activated by a rise in endogenous diacylglycerol generated in response to receptor-mediated stimulation of phospholipase C (Nishizuka, 1984). Recent molecular cloning analysis has shown that PKC is a family of multiple isoforms having closely related, but different, structures (Parker et al., 1986; Coussens et al., 1986; Knopf et al., 1986; Ohno et al., 1987, 1988; Ono et al., 1988). Thus far, the sequences of eight isoforms (α, βI, βII, γ, δ, ε, ξ, and η) have been deduced from the cDNAs obtained from brain libraries. In the brain, three major distinct types of PKC can be resolved upon chromatography: α, a mixture of βI, βII, and γ (Ono et al., 1987; Jaken & Kiley, 1987). The PKC isoforms thus far identified appear to show tissue-specific expression and a slightly different mode of activation and kinetic properties (Ono et al., 1987; Jaken & Kiley, 1987; Huang et al., 1987). The most recently identified isozyme, n-PKC, shows a more dramatic difference in that it does not require calcium for activity (Ohno et al., 1988).

In the present investigation, we analyzed the effect of phorbol esters and cell-permeable 1,2-diacyl-*sn*-glycerols on the regulation of IL-1 gene expression in the U937 cell line. In order to determine whether PKC mediates this effect, we have analyzed the phosphorylation patterns in situ as well as the abundance and distribution of PKC α, PKC β, and PKC

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[1] Abbreviations: TPA, 12-*O*-tetradecanoylphorbol 13-acetate; PDBu, phorbol 12,13-dibutyrate; 4α-PDD, 4α-phorbol 12,13-didecanoate; diC_8, 1,2-dioctanoyl-*sn*-glycerol; OAG, 1-oleoyl-2-acetylglycerol; H-7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; PBS, phosphate-buffered saline; SDS/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; DMEM, Dubecco's modified Eagle's medium; EDTA, (ethylenedinitrilo)tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; NP40, nonidet P-40; DMSO, dimethyl sulfoxide.

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γ after TPA or diC_8 treatment. Here we report that treatment of U937 cells with TPA, but not diC_8, promotes the differential translocation of PKC α to a particulate compartment of the cell and has no effect on PKC β. This process is associated with the specific phosphorylation of substrate proteins and the induction of the IL-1 gene. The selective activation of PKC α after TPA treatment appears to be one of the initial events in the TPA-induced monocytic differentiation.

Experimental Procedures

Cells and Reagents. U937 cells were grown in RPMI 1640 medium (Gibco, Grand Island, NY), supplemented with 10% heat-inactivated fetal calf serum, 50 units/mL penicillin, 50 μg/mL streptomycin, and 2 mM glutamine. (2–3) × 10^6 cells were washed and incubated with RPMI 1640 and 0.1% BSA (fraction V, Sigma) in the presence of the agents described in the legends to the figures. Human IL-1α and IL-1β cDNA clones were obtained from Immunex (Seattle, WA). Pure rat brain PKC α and β and partially purified rat brain PKC were purchased from Sphinx Biotechnologies (Durham, NC). $^{32}PO_4$ was from Du Pont/NEN. [^{3}H]PDBu was obtained from Amersham. TPA, PDBu, and 4α-PDD were from Sigma. H-7 was purchased from Seikagaku-America, Inc., Miami, FL. D-Sphingosine (Sigma) was prepared as a stock solution of ethanol and BSA as described (Lambeth et al., 1988) to avoid cell toxicity.

Cytoplasmic Dot Hybridization. Serum-starved U937 cells were induced and treated as described in the legends to the figures. Samples of total RNA for cytoplasmic dot blots (or slot blots) were prepared exactly as described (White & Bancroft, 1982). Prehybridization and hybridization were performed in 50% formamide, 5 × SSC, 5 × PIPES, 5 × Denhardt, 0.1% SDS, 100 μg/mL salmon sperm DNA, and yeast tRNA (boiled and chilled before use). cDNA probes were labeled with [^{32}P]αCTP by nick-translation using a nick translation kit from BRL (Bethesda, MD). Bound radioactivity was separated from unincorporated by a spun column procedure as described in detail by Maniatis (1982).

Blots were washed in 2 × SSC/0.2% SDS for 2 h at 42 °C with five changes of wash buffer. The air-dried blots were exposed to Kodak XAR film with intensifying screens at −70 °C for 2 days. The extent of hybridization was quantitated by densitometric tracing of the autoradiographs using a Zeineh soft laser scanning densitometer (Model SLR, Biomed Instruments, Inc., Fullerton, CA) and the Zeineh Videophoresis II software program for the Apple II computer. To correct for differences in the amount of RNAs loaded per slot, results were normalized to signals generated by probing with β-actin cDNA (Gunning et al., 1983).

$^{32}PO_4$ Incorporation. Prior to labeling, cultures were rinsed with serum-free, phosphate-free HEPES-buffered (25 mM, pH 7.5) DMEM. One milliliter of the same medium containing 0.1 mCi/mL $^{32}PO_4$ was added for 4 h at 37 °C. At the end of the labeling period, cells were washed and agents were added for indicated times. To stop the reaction, cells were washed with ice-cold PBS and lysed on ice in a buffer containing 10 mM Tris-HCl, pH 7.4, 1% NP40, 0.3% SDS, 500 mM NaCl, 1 mM EDTA, 5% β-mercaptoethanol, 0.1 mM PMSF, and 0.01% leupeptin. Samples were boiled for 5 min and processed for two-dimensional SDS/polyacrylamide gel electrophoresis by the method of O'Farrell (1975). First dimension electrofocusing gels contained 5% BDH ampholines pH 4–8 (Gallard-Schlessinger, Inc., San Francisco, CA), and second dimension gels were 8% SDS/polyacrylamide. Gels were dried and exposed to Kodak SB film at room temperature for 3–4 days. The same number of TCA-precipitable counts (usually 1 × 10^6 cpm) were loaded per sample. For determination of phosphoproteins in cytosolic and particulate fractions, cells in suspension (control and short-term TPA treated) or attached to substratum (24-h TPA) were washed with ice-cold PBS and quickly frozen in liquid N_2. Subsequently, the frozen cells were thawed and processed exactly as described before (Strulovici et al., 1987).

Preparation of Peptide Antigens and Antipeptide Antibodies. The peptides were synthesized on *p*-methylbenzhydrylamine resin using N^{α}-*tert*-butoxycarbonyl- and benzyl-based side-chain protection for the amino acids [except Arg-Tos, Cys(pMeOBzl)]. The polypeptides were deprotected and removed from the resin as the C-terminal amides by treatment with redistilled (CoF_3), anhydrous liquid HF (−10 °F for 30 min, then 0 °F for 30 min). The crude peptides were purified by preparative reversed-phase high-performance liquid chromatography on a 2.5 × 100 cm column (Altex) of C-18 packing (Vydac 218TP, 20 μm) using CH_3CN/0.1% CF_3CO_2H eluents. The percent CH_3CN used varied with the peptide (α, 13%; β, 20%; γ, 15%; V_5, 23%). Purity of greater than 90% was achieved, and all peptides had acceptable amino acid analyses.

The peptides were coupled to keyhole limpet hemocyanin with *N*-maleimidobenzoic acid *N*-hydroxysuccinimide ester (MBS) (Lerner et al., 1981). The immunogen was prepared by diluting 500 μg of peptide to 1 mL with water and mixing with an equal volume of Freund's complete adjuvant. Each keyhole limpet hemocyanin–peptide conjugate was injected subcutaneously into three male New Zealand White rabbits. Rabbits were given booster injections of 250 μg of peptide per rabbit in Freund's incomplete adjuvant every 3 weeks and were bled 10 days after each boost. Sera were screened for antipeptide antibodies by an enzyme-linked immunosorbent assay as described before (Massoglia et al., 1981).

Western Blot. Cytosolic and particulate extracts of U937 cells were prepared by lysing the cells in a hypotonic buffer as described, with the difference that, after obtaining the cytosolic fraction, the high-speed pellet was extracted in a buffer containing 10 mM Tris, pH 7.4, 1% NP40, 100 mM NaCl, 1 mg/mL BSA, 1 mM EDTA, 2 μg/mL aprotinin, and 0.01% leupeptin. The particulate extracts were centrifuged at 100000*g* for 10 min at 4 °C, and the supernatant was used. After addition of SDS sample buffer (25 mM Tris-HCl, pH 6.8, 10% SDS, 10% glycerol, 5% β-mercaptoethanol), cytosolic and particulate extracts were boiled for 5 min and subjected to polyacrylamide slab gel electrophoresis according to Laemmli (1970). The transfer to nitrocellulose paper was performed as described (Burnette, 1981). Nonspecific sites were blocked by incubating the nitrocellulose paper with 5% instant milk (Carnation) and 0.5% Tween 20 for 1 h at 41 °C. Following 2-h incubation with the various dilutions of antisera and two washes (Tris-buffered saline, 0.5% Tween 20, 5 min each), the membrane was incubated with ^{125}I-protein A (Amersham) (0.1 μCi/mL). After three more washes of 10 min each, the nitrocellulose paper was air-dried and exposed to Kodak XA5 film with intensifying screens at −70 °C for 2 days. PKC was confirmed as the major antigen reacting with the crude sera.

Results

Regulation of IL-1 mRNA Expression by Phorbol Esters. We analyzed IL-1 mRNA levels in serum-starved U937 cells by cytoplasmic dot hybridization using IL-1α- and IL-1β-specific cDNA probes. A time course of TPA-induced IL-1β gene expression is shown in Figure 1. The effect of 5 × 10^{-8} M TPA was detectable as early as 2 h after treatment and

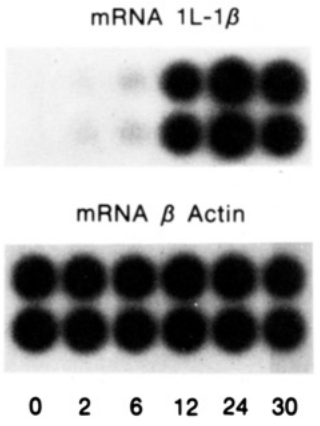

FIGURE 1: Time course of TPA-induced IL-1β mRNA expression. Serum-starved U937 cells were treated with TPA (5×10^{-8} M) for the times indicated. The blots were hybridized consecutively with the IL-1β and β-actin cDNA probes. A representative experiment is shown. The experiment was repeated four times with comparable results.

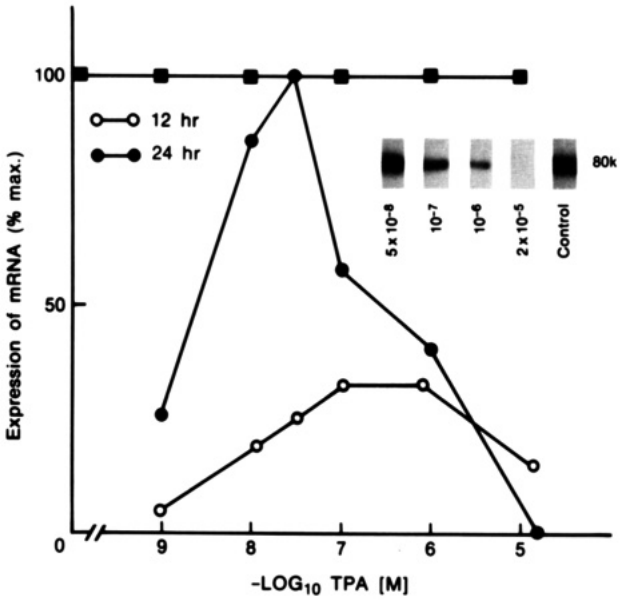

FIGURE 2: Dose dependence of TPA-induced IL-1β mRNA expression at 12- and 24-h treatment. Quantitation of the degree of IL-1β or β-actin (filled squares) mRNA expression was performed by densitometric scanning of autographs (see Experimental Procedures). Results are expressed as percentages of the IL-1β mRNA expressed in the presence of TPA 5×10^{-8} M at 24 h. The result is representative for four independent experiments. Inset: Western blot analysis of total protein kinase C. Extracts (150 μg) from control and TPA-treated cells (24 h) were electrophoresed on a 10% SDS/PAGE (as described under Experimental Procedures). The result is typical of two separate, independent experiments.

increased up to 24 h. IL-1α mRNA was not detectable. When the U937 cells were treated with various concentrations of TPA for 12 h, there was a concentration-dependent increase in the accumulation of IL-1β transcripts ($EC_{50} = 2 \times 10^{-8}$) (Figure 2). The concentration–response relationship at 24 h was biphasic, with both an inductive and an inhibitory component. The fact that prolonged treatment with higher concentrations of TPA induced a decrease in response suggested to us that the protein kinase C in these cells might have been down-regulated or depleted (Rozengurt et al., 1983). In order to determine the PKC levels in the TPA-treated U937 cells, we made use of a polyclonal antibody to PKC prepared against purified brain PKC (Ballester & Rosen, 1985). The inset to Figure 2 shows a Western blot of cell lysates before ("control") and after treatment with various concentrations of TPA for

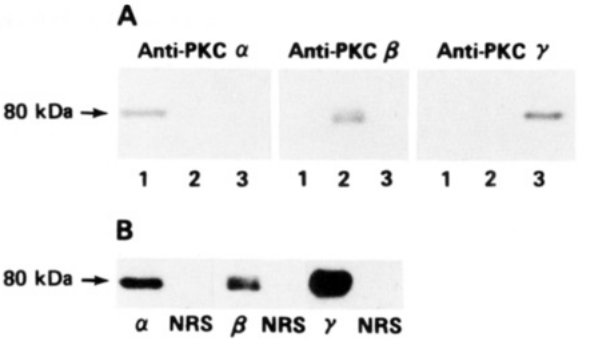

FIGURE 3: Specificity of the anti-PKC antisera. (A) Immunoreactivity of antisera to PKC α, β, or γ with pure rat brain PKC α or β and partially purified PKC γ. PKC α or β (50 ng) and partially purified PKC γ (0.1 μg) were subjected to SDS/PAGE, transferred to nitrocellulose paper, and immunoblotted with the antisera (see Experimental Procedures). (B) Immunoblot of cytosolic partially purified rat brain PKC with antisera to PKC α, β, or γ. PKC (0.5 μg) was subjected to SDS/PAGE. The experiment was repeated twice with similar results.

24 h. The antiserum, which measures total PKC-immunoreactive protein, recognized a protein band that migrated at 80 kDa, the apparent molecular weight of PKC. There was a marked decrease in the levels of immunoreactive enzyme at 10^{-7} and 10^{-6} M TPA, while at 2×10^{-5} M there was no longer detectable PKC. This finding was also validated by measuring [^{3}H]PDBu binding sites in the intact cells exposed TPA for 24 h (46% reduction in binding sites at 10^{-7} M TPA, 65% at 10^{-6} M, and complete down-regulation at 2×10^{-5} M TPA, respectively) (not shown). This result was reproduced in two separate experiments ($p \ll 0.05$). Thus, the decrease in immunoreactive PKC correlated with the decrease in IL-1β mRNA accumulation at the same concentrations of TPA (Figure 2).

Antipeptide Antibodies That Recognize PKC α, β, and γ. In order to characterize the PKC isozyme(s) present in control and induced U937 cells, we developed specific antibodies that recognize the PKC α, β, and γ isozymes.

The synthetic peptides containing sequences unique to bovine PKC α, PKC β, and PKC γ were the following: for PKC α, ISPSEDRRQPSNNL, corresponding to residues 318–331; for PKC β, EKTTNTISKFDNNG (residues 319–332); for PKC γ, VRTGPSSSPIPSPSPT (residues 316–332). All three were derived from the variable sequence, V_3, which connects the catalytic and regulatory domains (Coussens et al., 1986). We also selected an additional sequence for PKC γ (PISPTPVPVM) which corresponds to residues 688–697, derived from a variable sequence found at the carboxyl terminus (V_5) which is 10 residues longer than the aligned PKC α and β sequences (Coussens et al., 1986).

The specificity of antisera raised against the peptide immunogens was tested. Equal amounts of BSA-coupled peptides (10 ng/lane) were loaded on nitrocellulose paper (dot blots), followed by the addition of each one of the antisera (1:200 dilution). Immunoreactivity was measured by determining ^{125}I-protein A bound to the peptide–antibody complex. Each antipeptide antiserum recognized its own antigen and failed to cross-react with the heterologous peptide or with preimmune serum (not shown). The specificity of the antisera was further tested by using purified rat brain PKC α and β as antigens. Instead of PKC γ (which we did not have available) we used a purified brain PKC preparation enriched in the γ isoform. As shown in Figure 3A, each antiserum recognized only its own antigen. When the immunoreactivity of the antipeptide antibodies was tested against a purified rat brain PKC prep-

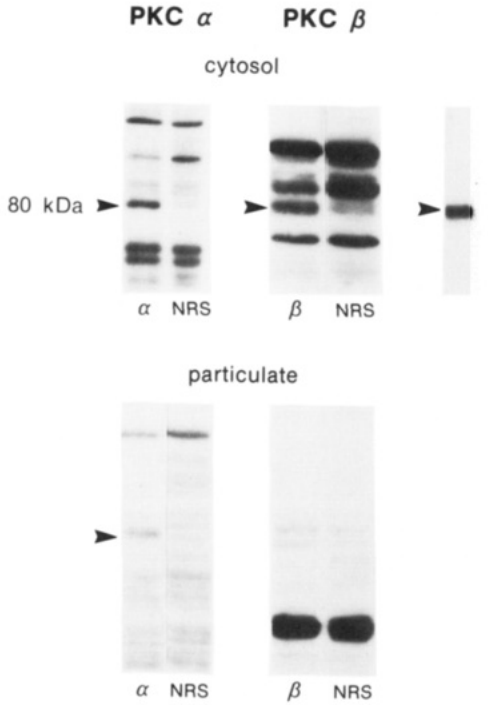

FIGURE 4: Immunoblot of cytosol and particulate extracts from control U937 cells with antisera to PKC α or β. Membrane and cytosolic fractions were prepared and subjected to SDS/PAGE (150 μg of protein/lane). Purified brain PKC β (0.1 μg) was immunoblotted with anti-PKC β antiserum (upper far right lane).

aration containing a mixture of the isozymes, the anti PKC γ antipeptide antisera (V_3 and V_5) reacted more strongly than the antisera against PKC α or β ($\gamma > \beta > \alpha$) (Figure 3B), proving on one hand the specificity of the antisera, and on the other hand confirming the prevalence of PKC γ over β or α isozymes in brain (Huang et al., 1987). PKC β appeared as a doublet, presumably due to the heterogeneity of brain PKC β (a mixture of the two subspecies, βI and βII).

Identification of PKC Isozymes in Cytosolic and Particulate Extracts from Control and TPA-Treated U937 Cells. We first made use of these specific antibodies to determine the abundance of each PKC isozyme in control U937 cells. Cytosolic and particulate extracts of U937 cells were immunoblotted with each antiserum or preimmune rabbit serum. Figure 4 shows a Western blot of cytosolic and particulate extracts from control U937 cells. PKC α was ~30% in particulate form and ~70% cytosolic; PKC β, the more abundant species among the PKC isozymes, was detected only in the cytosol. PKC γ isozyme was undetectable in both compartments.

It was of interest to test the effect of TPA (5×10^{-8} M) on the abundance and distribution of the PKC isozymes α, β, and γ. This concentration of TPA apparently does not cause a decrease in immunoreactive PKC levels (Figure 2, inset) as assessed by immunoblotting with an antiserum that recognizes all three isozymes. Figure 5 shows an autoradiograph of immunoblotted PKC α from U937 cells incubated for various times with 5×10^{-8} M TPA. Between 30 min and 1 h, there was a sizable decrease in cytosolic PKC α and a corresponding increment in the particulate form. However, the particulate PKC α started to disappear at 3 h and became undetectable by 6 h after 5×10^{-8} M TPA treatment. During early TPA treatment (30 min), PKC β remained cytosolic (Figure 6, upper lanes 1), and PKC γ was still not detectable (Figure 6, lower lanes 1 and 2). PKC γ became detectable at 6 h and kept increasing in abundance during the 24-h observation time—mostly in particulate form (Figure 7), when PKC α

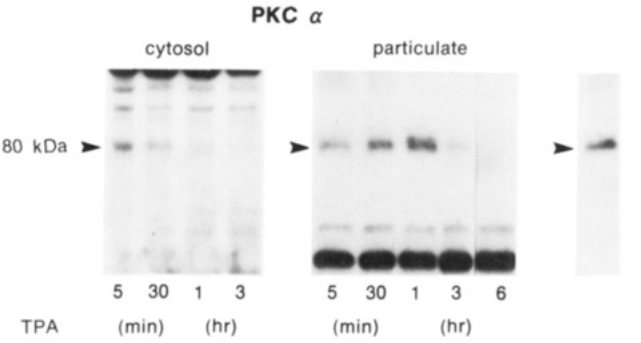

FIGURE 5: Immunoblot of cytosol and particulate extracts from TPA-treated U937 cells with antiserum to PKC α. Cells were treated with TPA (5×10^{-8} M) for different periods of time. Membrane and cytosolic fractions were prepared as described under Experimental Procedures. Equal amounts of protein were subjected to SDS/PAGE (100–150 μg/lane). Antiserum to PKC α was used at 1:200 dilution ($n = 2$). The lane to the far right of the picture is a purified preparation of brain PKC α (0.1 μg of protein) immunoblotted with antiserum to PKC α.

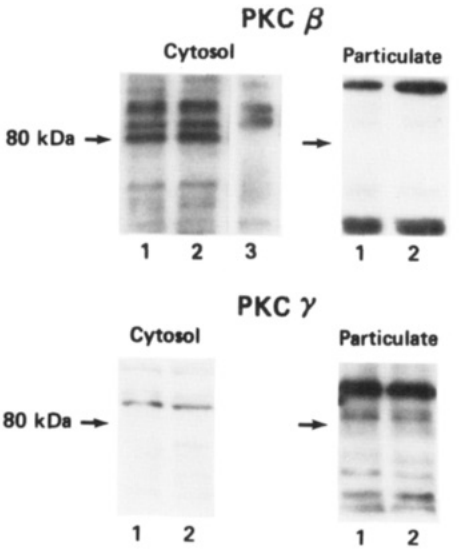

FIGURE 6: Immunoblot of cytosol and particulate extract from TPA-treated U937 cells with antiserum to PKC β and γ. PKC β cytosol: lane 1 = 30 min; lane 2 = 24-h TPA, immunoblotted with anti-PKC β antibody (1:200); lane 3 = 24-h TPA, immunoblotted with preimmune serum. PKC β particulate: lane 1 = 24-h TPA, immunoblotted with antiserum to PKC β; lane 2 = 24-h TPA, immunoblotted with preimmune serum. PKC γ cytosol and particulate: lane 1 = control; lane 2 = 30-min TPA, both immunoblotted with antiserum to PKC γ. Equal amounts of protein were subjected to SDS/PAGE and transferred to nitrocellulose paper as described ($n = 2$).

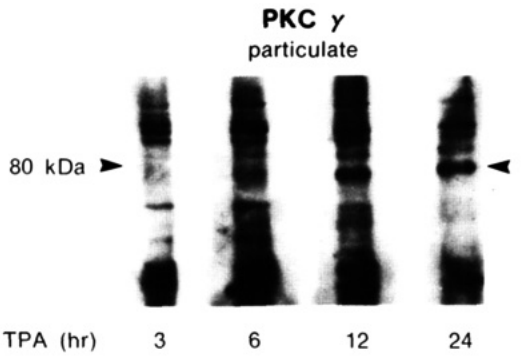

FIGURE 7: Immunoblot of particulate extract from TPA-treated U937 cells with antiserum to PKC γ. See legend to Figure 5 and Experimental Procedures for details. Anti-PKC γ antiserum was used at a 1:200 dilution ($n = 2$).

Table I: Effect of PKC Antagonists on IL-1β mRNA Expression[a]

treatment and concn of agonist (M)	antagonist and its concn (M)	expression of IL-1β mRNA (% maximum)
no treatment		ND[b]
TPA, 5×10^{-8}	none	100 ± 8
TPA, 5×10^{-8}	H-7, 5×10^{-6}	47 ± 6
TPA, 5×10^{-8}	H-7, 5×10^{-5}	10 ± 5
TPA, 5×10^{-8}	D-sphingosine, 5×10^{-7}	91 ± 21
TPA, 5×10^{-8}	D-sphingosine, 5×10^{-6}	75 ± 9.3
TPA, 5×10^{-8}	D-sphingosine, 5×10^{-5}	22 ± 1.4
TPA, 5×10^{-8}	D-sphingosine, 10^{-4}	ND[b]

[a] Serum-starved U937 cells were preincubated with H-7 or D-sphingosine for 1 h before the addition of TPA for 24 h. Quantitation of degree of IL-1β mRNA expression was performed by densitometric scanning of autoradiographs (±SE; $n = 3$). [b] Not detectable by scan densitometry.

reappeared (not shown) and β increased also in abundance in the cytosol (Figure 6, lane 2). Treatment of cells with the cell-permeable synthetic diacylglycerol diC_8 did not induce translocation of PKC α, nor did it increase the abundance of all PKC isozymes tested during the 24-h treatment (not shown).

Specificity of Response. We investigated in greater detail the role of protein kinase C in the IL-1β response, by using PKC inhibitors.

Recently, long-chain bases (sphinganine and sphingosine) have been reported as potent inhibitors of PKC in broken cell preparations and intact cells (Merill et al., 1986; Hannun & Bell, 1987; Lambeth et al., 1988). Addition of D-sphingosine to U937 cells prevented TPA from inducing IL-1β mRNA expression in a dose-dependent manner (Table I). H-7 (Hidaka et al., 1984) had a similar effect (Table I).

PDBu, which binds to and activates PKC to a lesser extent than TPA, induced the IL-1β mRNA to a lesser extent than TPA, while 4α-PDD, an inactive phorbol analogue, was without effect. When the U937 cells were incubated with these phorbol analogues for 24 h (not shown), the rank order of potency for stimulating accumulation of IL-1β transcripts was identical with their order or potency for protein kinase C activation: TPA > PDBu ≫ 4α-PDD (Dunn & Blumberg, 1983). DiC_8 or OAG (not shown) did not mimic the effect of phorbol esters, under the same regimen (repeated additions) used to induce differentiation of HL-60 cells into cells with morphologic characteristics of macrophages (Ebeling et al., 1985).

Phorbol Ester Stimulated Protein Phosphorylation. Since it was established that phorbol esters bind to and activate PKC, a proposed mechanism of action for these compounds has been the phosphorylation of specific protein substrates by the kinase (Castagna et al., 1982; Nishizuka, 1984; Niedel & Blackshear, 1986). Under the specific conditions we chose for the two-dimensional analysis of the phosphoproteins, TPA (5×10^{-8} M) added for 30 min specifically stimulated the phosphorylation of 10 polypeptides (Figure 8A,B) in $^{32}PO_4$-labeled U937 cells. PDBu (10^{-7} M) induced the same pattern of phosphorylation (not shown). Analysis of cytosolic and particulate substrates phosphorylated in the intact cells in the presence of TPA is shown in Table II. Five out of ten substrates were found in the 100000*g* pellet. pp 73/6.3 was found in both the cytosolic and particulate fractions. To ascertain if D-sphingosine prevented the effect of TPA on IL-1β gene expression by inhibiting PKC activity, we tested the effect of D-sphingosine on the TPA-induced phosphorylation of endogenous substrates in situ. Preincubation of ^{32}P-labeled cells with D-sphingosine for 1 h before TPA treatment prevented

Table II: Effects of TPA or DiC_8 on the Phosphorylation Pattern of U937 Cells after Cell Fractionation[a]

TPA		diC_8	
C	P	C	P
94/5.62	86/5.7	100/6.16	73.6.3
63/6.09	85/5.6	73/6.3	
61/5.6	74/5.6	55/6	
59/5.6	73/6.3	26/6.3	
45/5.25	36/5.2		

[a] Intact $^{32}PO_4$-labeled U937 cells were treated with TPA (5×10^{-8} M) or diC_8 (5×10^{-5} M) for 30 min. Separation of cytosolic and particulate fractions as well as two-dimensional SDS/PAGE and autoradiography were performed as described under Experimental Procedures. The phosphoproteins are designated by MW/pI, according to their migration on a two-dimensional SDS/PAGE system. Data shown are the patterns of phosphorylation in the cytosolic and particulate fractions of U937 cells fractionated after in situ phosphorylation. Autoradiograms were analyzed visually in relation to a reference protein (which remained unchanged during the various treatments). There were no other phosphoproteins that increased their phosphate content as assessed by visual inspection. These data represent the results from three separate experiments.

to a large extent the ability of TPA to induce phosphorylation of the majority of protein substrates (Figure 8D). The agent was not toxic to the U937 cells as assessed by trypan blue exclusion (95% viability).

Since diC_8 was unable to mimic the effect of TPA on the regulation of IL-1β mRNA and on the translocation of PKC α, it was of interest to test whether treatment of the U937 cells with this agent would induce the same phosphorylation pattern as TPA. Our data show (Figure 8C and Table II) that treatment with diC_8 (5×10^{-5} M) for 30 min enhanced the phosphorylation of five substrates, only one of which (pp 73/6.3) was both cytosolic and particulate. DiC_8 at 10^{-6} M was not as effective (only pp 73 was noted), while at 10^{-4} M, the agent was quite toxic to the cells, although further enhancement of the same phosphorylation pattern could be observed (not shown). By analyzing the patterns of phosphorylation induced by TPA vs diC_8, several additional differences were noticed: pp 73/6.3 was the only common substrate, while pp 100/6/16, 55/6, and 26/6.3 were phosphorylated only during diC_8 treatment. Lower concentrations of TPA or PDBu induced a lesser response (not shown), but the pattern of phosphorylation did not resemble that induced by diC_8. Presence of TPA (5×10^{-8} M) for 24 h induced the phosphorylation of additional substrates, while several other substrates became dephosphorylated (Figure 8E).

Discussion

This investigation has focused on the effect of phorbol esters on the regulation of the IL-1 gene as a marker of differentiation of the U937 cell from a promonocyte to a cell with a macrophage-like function.

TPA causes an increase in the level of IL-1β mRNA expression, possibly by activation of IL-1β gene transcription. Several lines of evidence indicate that this effect is mediated by protein kinase C. Treatment with PKC antagonists blocks the ability of these agents to stimulate both the phosphorylation of endogenous substrates and the accumulation of IL-1β transcripts. Down-regulation of the enzyme resulted in diminished accumulation of IL-1β transcripts, thus demonstrating that the ability of TPA to induce the IL-1β gene depends directly on the level of protein kinase C present in the cell.

Recently, it has been shown (Kiss et al., 1988) that treatment of HL-60 cells with 10^{-9} M TPA induces translocation of PKC to the nucleus after 1 day, as assessed by immuno-

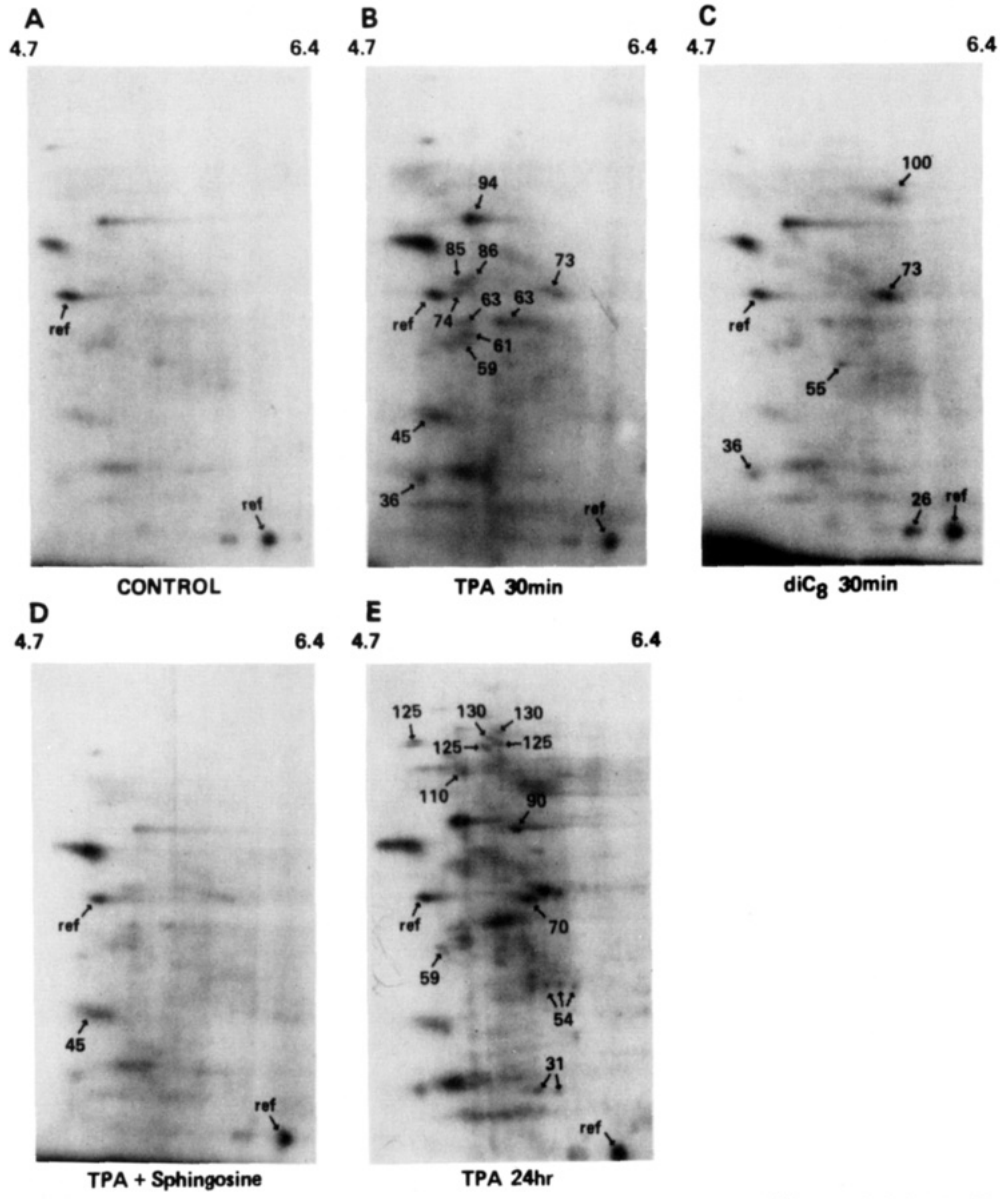

FIGURE 8: Two-dimensional SDS/PAGE analysis of phosphorylated proteins in intact U937 cells. ^{32}P-Labeled U937 cells were left untreated (control) or treated with TPA (5×10^{-8} M) or diC$_8$ (5×10^{-5} M) for 30 min and analyzed by two-dimensional gel electrophoresis. To assess the effect of D-sphingosine (5×10^{-5} M), the U937 cells were pretreated with this agent for 1 h prior to 30-min TPA treatment. In order to analyze the phosphorylation pattern induced by TPA in more differentiated U937 cells, TPA (5×10^{-8} M) was added to the cultures for 20 h. The cells, which became adherent to substratum, were rinsed in phosphate-free DMEM, labeled with $^{32}PO_4$ for 4 h, and then washed, and TPA (5×10^{-8} M) was added for 30 min. The reaction was stopped and the cells were processed as described under Experimental Procedures. Experiments were repeated three times with identical results. A representative experiment is shown.

chemical methods using an anti-PKC antibody. The authors, however, do not discriminate between the PKC isozymes, and correlate this translocation of PKC with cell-free phosphorylation of substrates in purified nuclei.

Numerous reports have documented that active phorbol esters provoke a rapid association of PKC with the cellular particulate fraction [for review, see Niedel and Blackshear (1986)]. However, the molecular mechanism and physiological significance of PKC translocation is unclear.

Our data show the following: (a) In U937 cells activated with TPA, there is preferential translocation of PKC α versus PKC β at early time points. (b) This translocation is associated with in situ phosphorylation of specific substrates in the same postmitochondrial fraction into which the isozyme translocates. This suggests that early events induced by TPA could be correlated directly with the activation of PKC α and the phosphorylation of specific substrates. Early responses such as the appearance of c-fos mRNA which peaks at 45 min (Mitchel et al., 1985) and the early phase of the IL-1β mRNA induction (2–4 h, Figure 1) might be mediated via activation of PKC α. The particulate substrates that become phosphorylated during this early phase might be implicated in this process. However, later stages in the TPA-induced differentiation process could be mediated via other PKC isozymes, since PKC α, β, and γ become more abundant by 24 h. Isozyme-selective PKC inhibitors would be needed to resolve these questions.

The data showing that PKC α preferentially becomes translocated and subsequenly down-regulated as assessed by the anti-PKC α antiserum shows that caution must be exercised in interpreting PKC "down-regulation" data using an antibody that recognizes all PKC isozymes (Figure 2, inset): due to different distribution patterns and synthesis of the isozymes, subtle differences among isozymes can be entirely missed. In addition, the caveat exists that some isoforms of PKC interact differently with the membrane (some stronger

than others), in which case their activation (translocation) will not be detected as readily. However, there is no simple way, as yet, to address the issue of whether all PKC isoforms interact with the cell membrane in identical manner.

Recently, it has been demonstrated (Makowske et al., 1988) that, in the HL-60 cell line, PKC α, β, and γ isozymes increase in abundance during cell differentiation. All three isozymes have been shown to increase at least 2-fold by 48-h treatment with 1.2% DMSO. However, by 96 h there was further increase in abundance of the α and β isoforms, without further increase in the abundance of the γ isozyme. When HL-60 cells were treated with 1 μM retinoic acid, all three isozymes incrased 3-fold in abundance by 96 h. In a closely related cell line—U937—we find that TPA treatment induces an increase in the abundance of PKC α, β, and γ by 24-h observation time. However, the kinetics and extent of effect seem to be different (more enzyme, particularly PKC γ, shorter time). This could be due to different cell line (HL-60 vs U937) and inducer (TPA vs DMSO or retinoic acid) and/or different mechanism(s) of regulation (transcriptional expression of mRNA, protein synthesis, etc.).

Although PKC is the cellular receptor for TPA, it is not yet clear whether all the cellular effects of TPA involve PKC. The ability of diC_8 or OAG, which can activate PKC, to induce differentiation of HL-60 cells has been the subject of controversy. Ebeling et al. (1985) demonstrated that diC_8 induced monocytic morphology and adherence of HL-60 cells. Using OAG, several groups (Kreutter et al., 1985; Morin et al., 1987) were unable to reproduce these results, leading to the hypothesis that phorbol esters may activate distinct pathways in addition to PKC. Data from several laboratories have also shown that OAG, when added to HL-60 cells, can induce phosphorylation of 9 of 14 proteins whose phosphorylation increases in response to TPA (Morin et al., 1987). A recent report using OAG- and TPA-treated U937 cells shows that only TPA but not OAG stimulated differentiation of these cells to a monocyte-like cell (Ways et al., 1987), although OAG increased particulate PKC activity and stimulated the phosphorylation of the same three substrates as TPA did. However, Salehi et al. (1988) demonstrated that diC_8, like TPA, decreased the transcription of c-myc in HL-60 cells, in a PKC-dependent manner. The authors suggest that persistent activation of PKC is necessary to elicit chemically induced cell differentiation.

Our data show that the synthetic diacylglycerols do not mimic TPA in inducing IL-1β gene expression in U937 cells. However, in contrast to Ways et al., we have noticed large differences between the ability of TPA or diC_8 to stimulate the phosphorylation of endogenous substrates (Figure 8 and Table II). In addition, we were able to identify a specific PKC isozyme which becomes preferentially translocated during TPA treatment, but not with diC_8 treatment. Our findings suggest that activation of distinct PKC isozymes provides a potential explanation for the difference in the ability of TPA and diC_8 to induce a biological response. Although this is an attractive possibility, there are caveats to drawing parallels between the effects of TPA and diC_8. Among them, TPA induces irreversible translocation of PKC followed by its degradation, while diacylglycerols produced via PI turnover or added exogenously induce reversible translocation (Anderson et al., 1985; Issandou et al., 1988). Perhaps persistent activation of PKC (Salehi et al., 1988), in this case the α form, is indeed required.

Reservations have been expressed about the nonspecific action of PKC inhibitors such as sphingosine (Gazzi & Nelsestuen, 1987; Jefferson & Schulman, 1988) or H-7 (Hidaka et al., 1984). However, the use of more lines of evidence in this report (PKC inhibitors, other PKC agonists, inactive analogue of phorbols, PKC down-regulation) validates the involvement of PKC in the TPA response. Nevertheless, the involvement of other kinases in this process cannot be excluded.

The pathway responsible for transducing the signal generated by the activation of PKC to the transcriptional machinery is not known. Recently, several phorbol ester inducible genes have been found to contain a consensus DNA sequence recognized by TPA-modulated trans-acting factors (Lee et al., 1987; Imagawa et al., 1987; Angel et al., 1987). TPA, presumably via PKC activation, might induce a posttranslational modification (phosphorylation) of such factors and increase their activity or, alternatively, might inactivate an inhibitory protein whose role is to regulate the activity of such trans-acting factors via a phosphorylation reaction (Angel et al., 1987).

In summary, we have documented that TPA induces IL-1β gene expression in U937 cells, most likely due to activation of PKC. In addition, we have demonstrated differential translocation of PKC α and β isoforms by TPA but not diC_8 and the differential phosphorylation of proteins by these agents. The mechanisms responsible for selective translocation of PKC α vs PKC β, and the mechanism and role of isotype switching, remain to be explored. Similarly, additional work will be necessary to determine the subcellular localization of the activated PKC(s) and its substrates as well as the link between these events and gene regulation.

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Registry No. TPA, 16561-29-8; PKC, 9026-43-1; PDBu, 37558-16-0; ISPSEDRRQPSNNL, 119110-32-6; EKTTNTISKFDNNG, 119110-33-7; VRTGPSSSPIPSPSPT, 119110-34-8; PISPTPVPVM, 119110-35-9.

REFERENCES

Anderson, W. B., Estival, A., Tapiovaara, H., & Gopalakrishna, R. (1985) *Adv. Cyclic Nucleotide Protein Phosphorylation Res. 19*, 287–306.

Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R. J., Rahmsdorf, H. J., Jonat, C., Herrlich, P., & Karin, M. (1987) *Cell 49*, 729–739.

Balavoine, J.-F., de Rochemonteix, B., Williamson, K., Seckinger, P., Cruchaud, A., & Dayer, J.-M. (1986) *J. Clin. Invest. 78*, 1120–1124.

Ballester, R., & Rosen, O. M. (1985) *J. Biol. Chem. 260*, 15194–15199.

Burnette, W. N. (1981) *Anal. Biochem. 112*, 195–203.

Cambier, J. C., Newell, M. K., Justement, L. B., McGuire, J. C., Leach, K. L., & Chen, Z. Z. (1987) *Nature 327*, 629–632.

Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U., & Nishizuka, Y. (1982) *J. Biol. Chem. 257*, 7847–7851.

Coussens, L., Parker, P. J., Rhee, L., Yang-Feng, T. L., Chen, E., Waterfield, M. D., Francke, U., & Ulrich, A. (1986) *Science 233*, 859–866.

Dinarello, C. A. (1984) *Rev. Infect. Dis. 6*, 51–89.
Driedger, L. E., & Blumberg, P. M. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 567–571.
Dunn, J. A., & Blumberg, P. M. (1983) *Cancer Res. 43*, 4632–4637.
Ebeling, J. G., Vandenbark, G. R., Kuhn, L. J., Ganong, B. R., Bell, R. M., & Niedel, J. E. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 815–819.
Gunning, P., Ponte, P., Okayama, H., Engel, J., Blau, H. M., & Kedes, L. (1983) *Mol. Cell. Biol. 3*, 787–795.
Hannun, Y. A., & Bell, R. M. (1987) *Science 235*, 670–674.
Harris, P., & Ralph, P. (1985) *J. Leukocyte Biol. 37*, 407–422.
Hidaka, H., Inagaki, M., Kawamoto, S., & Sasaki, Y. (1984) *Biochemistry 23*, 5036–5041.
Huang, F. L., Yoshida, Y., Nakabayashi, H., & Huang, K.-P. (1987) *J. Biol. Chem. 262*, 15714–15720.
Imagawa, M., Chiu, R., & Karin, M. (1987) *Cell 51*, 251–260.
Issandou, M., Bayard, F., & Darbon, J.-M. (1988) *Cancer Res. 48*, 6943–6950.
Jaken, S., & Kiley, S. C. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4418–4422.
Kikkawa, U., & Nishizuka, Y. (1986) *Annu. Rev. Cell Biol. 2*, 149–178.
Kiss, Z., Deli, E., & Kuo, J. F. (1988) *FEBS Lett. 231*, 41–46.
Knopf, J. L., Lee, M. H., Sultzman, L. A., Kritz, R.-W., Loomis, C. R., Hewick, R. M., & Bell, R. M. (1986) *Cell 46*, 491–502.
Kreutter, D., Caldwell, A. B., & Morin, M. J. (1985) *J. Biol. Chem. 260*, 5979–5984.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Lambeth, J. D., Burnham, D. N., & Tyagi, S. R. (1988) *J. Biol. Chem. 263*, 3818–3822.
Lapetina, E. G., Reep, B., Ganong, B. R., & Bell, R. M. (1984) *J. Biol. Chem. 260*, 1358–1361.
Lee, W., Haslinger, A., Karin, M., & Tjian, R. (1987) *Nature (London) 325*, 368–371.
Lerner, R., Green, N., Alexander, H., Liu, F.-T., Sutcliffe, J., & Shinock, T. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3404–3407.
Makowske, M., Ballester, R., Cayre, Y., & Rosen, O. M. (1988) *J. Biol. Chem. 263*, 3402–3410.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) in *Molecular Cloning—A Laboratory Manual*, pp 466 and 467, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Massoglia, S. L., Kenney, J. S., & Gospodarowicz, D. J. (1981) *J. Cell. Physiol. 132*, 531–537.
Merrill, A. H., Sereni, A. M., Stevens, V. L., Hannun, Y. A., Bell, R. M., & Kinkade, M., Jr. (1986) *J. Biol. Chem. 261*, 12610–12615.
Mitchell, R. L., Zokas, L., Schreiber, R. D., & Verma, I. M. (1985) *Cell 40*, 209–217.
Mizel, S. B., Rosenstreich, D. L., & Oppenheim, J. J. (1978) *Cell. Immunol. 40*, 230–235.
Morin, M. J., Kreutter, D., Rasmussen, H., & Sartorelli, A. C. (1987) *J. Biol. Chem. 262*, 11758–11763.
Niedel, J. E., & Blackshear, P. J. (1986) in *Phosphoinositides and Receptor Mechanisms* (Putney, J. W., Ed.) pp 47–88, Alan R. Liss, New York.
Niedel, Y. E., Kuhn, L. J., & Vandenbark (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 36–40.
Nishizuka, Y. (1984) *Science 225*, 1365–1370.
O'Farrell, P. H. (1975) *J. Biol. Chem. 250*, 4007–4021.
Ohno, S., Kawasaki, M., Imajoh, S., & Suzuki, K. (1987) *Nature (London) 325*, 161–166.
Ohno, S., Akita, Y., Konno, Y., Imajoh, S., & Suzuki, K. (1988) *Cell 53*, 731–741.
Ono, Y., Kikkawa, U., Ogita, K., Fujii, T., Kurokawa, T., Asaoka, Y., Sekiguchi, K., Ase, K., Igarashi, K., & Nishizuka, Y. (1987) *Science 236*, 1116–1120.
Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Igarashi, K., & Nishizuka, Y. (1988) *J. Biol. Chem. 263*, 6927–6932.
Pantazis, P., Sariban, E., Kufe, D., & Antoniades, H. N. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 6455–6459.
Parker, P. J., Coussens, L., Totty, N., Rhee, L., Young, S., Chen, E., Stabel, S., Waterfield, M. D., & Ulrich, A. (1986) *Science 233*, 853–859.
Pelosin, J.-M., Vilgrain, I., & Chambaz, E. M. (1987) *Biochem. Biophys. Res. Commun. 147*, 382–391.
Pujol, J.-P., & Loyau, G. (1987) *Life Sci. 41*, 1187–1198.
Rozengurt, E., Rodriguez-Pena, M., & Smith, K. A. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 7244–7248.
Salehi, Z., Taylor, J. D., & Niedel, J. E. (1988) *J. Biol. Chem. 263*, 1898–1903.
Sen, R., & Baltimore, D. (1986) *Cell 47*, 921–928.
Strulovici, B., Tahilramani, R., & Nestor, J. J., Jr. (1987) *Biochemistry 26*, 6005–6011.
Vandenbark, G. R., & Niedel, J. E. (1984) *J. Natl. Cancer Inst. 73*, 1013–1019.
Ways, D. K., Dodd, R. C., & Earp, H. S. (1987) *Cancer Res. 47*, 3344–3350.
White, B. A., & Bancroft, F. C. (1982) *J. Biol. Chem. 257*, 8569–8572.