Reiss, P. D., Zuurendonk, P. F., & Veech, R. L. (1984) *Anal. Biochem. 140*, 162–171.
Reynolds, O. (1883) *Philos. Trans. R. Soc. London 174*, 935–982.
Sauers, C. K., Jencks, W. P., & Groh, S. (1975) *J. Am. Chem. Soc. 97*, 5546–5549.
Smith, M. H. (1973) *Biophys. J. 13*, 817–821.
Smith, R. M., & Alberty, R. A. (1956) *J. Am. Chem. Soc. 78*, 2376–2380.
Stackhouse, J., Nambiar, K. P., Burbaum, J. J., Stauffer, D. M., & Benner, S. A. (1985) *J. Am. Chem. Soc. 107*, 2757–2763.
Stubbe, J., & Abeles, R. (1980) *Biochemistry 19*, 5505–5512.
Travers, F., Barman, T. E., & Bertrand, R. (1979) *Eur. J. Biochem. 100*, 149–155.
Trentham, D. R., Eccleston, J. F., & Bagshaw, C. R. (1976) *Q. Rev. Biophys. 9*, 217–281.
Veech, R. L., Guynn, R., & Veloso, D. (1972) *Biochem. J. 127*, 387–397.
Wells, T. N. C., & Fersht, A. R. (1986) *Biochemistry 25*, 1881–1886.
Wilkinson, K. D., & Rose, I. A. (1979) *J. Biol. Chem. 254*, 12567–12572.
Williamson, J. R. (1965) *J. Biol. Chem. 240*, 2308–2321.
Williamson, J. R. (1966) *J. Biol. Chem. 241*, 5026–5036.
Williamson, J. R., Scholz, R., Browning, E. T., Thurman, R. G., & Fukami, M. H. (1969) *J. Biol. Chem. 244*, 5044–5054.
Wold, F., & Ballou, C. E. (1957) *J. Biol. Chem. 227*, 301–312.
Wold, F., & Barker, R. (1964) *Biochim. Biophys. Acta 85*, 475–479.
Wong, C.-H., & Whitesides, G. M. (1981) *J. Am. Chem. Soc. 103*, 4890–4899.

Differences in the Effects of Phorbol Esters and Diacylglycerols on Protein Kinase C†

Mohammad D. Bazzi and Gary L. Nelsestuen*
Department of Biochemistry, The University of Minnesota, St. Paul, Minnesota 55108
Received March 15, 1989; Revised Manuscript Received July 12, 1989

ABSTRACT: The binding of protein kinase C (PKC) to membranes and appearance of kinase activity are separable events. Binding is a two-step process consisting of a reversible calcium-dependent interaction followed by an irreversible interaction that can only be dissociated by detergents. The irreversibly bound PKC is constitutively active, and the second step of binding may be a major mechanism of PKC activation [Bazzi & Nelsestuen (1988) *Biochemistry 27*, 7589]. This study examined the activity of other forms of membrane-bound PKC and compared the effects of phorbol esters and diacylglycerols. Like the membrane-binding event, activation of PKC was a two-stage process. Diacylglycerols (DAG) participated in forming an active PKC which was reversibly bound to the membrane. In this case, both activity and membrane binding were terminated by addition of calcium chelators. DAG functioned poorly in generating the constitutively active, irreversible PKC–membrane complex. These propreties differed markedly from phorbol esters which activated PKC in a reversible complex but also promoted constitutive PKC activation by forming the irreversible PKC–membrane complex. The concentration of phorbol esters needed to generate the irreversible PKC–membrane complex was slightly higher than the concentration needed to activate PKC. In addition, high concentrations of phorbol esters (≥50 nM) activated PKC and induced irreversible PKC–membrane binding in the absence of calcium. Despite these striking differences, DAG prevented binding of phorbol esters to high-affinity sites on the PKC–membrane complex. Taken together, the results may suggest that a low-affinity interaction between PKC, phorbol esters, and/or the membrane component was responsible for the irreversible membrane-binding event that produced the constitutively active kinase. These different behaviors of DAG and phorbol esters may be consistent with their different and complex effects in whole cells and tissues.

Phorbol esters are potent tumor-promoting agents that elicit a variety of biological responses (Blumberg et al., 1984; Ashendel, 1985; Blumberg, 1988) which may stem from their effect on potein kinase C (PKC).[1] The activity of this enzyme is sensitive to calcium and diacylglycerol and is believed to be involved in signal transduction by the phosphatidylinositol cycle (Nishizuka, 1986a,b; Kikkawa & Nishizuka, 1986). Phorbol esters and diacylglycerol (DAG) are often thought to exert their function via the common mechanism of PKC activation. The more pronounced effects of phorbol esters may stem from the rate of metabolism. DAG is rapidly metabolized and will activate PKC in a transitory fashion while phorbol esters are degraded very slowly and can activate PKC over a longer time (Nishizuka, 1986a).

Phorbol esters alter the cellular distribution of PKC by producing a stable membrane-associated form that can only be solubilized with detergents (Kraft et al., 1982; Kraft &

† Supported in part by Grant GM 38819 from the National Institutes of Health.

[1] Abbreviations: BSA, bovine serum albumin; DAG, diacylglycerol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol; PC, phosphatidylcholine; PDBu, phorbol 12,13-dibutyrate; PKC, Ca^{2+}- and phospholipid-dependent protein kinase; PMA, phorbol 12-myristate 13-acetate; PS, phosphatidylserine.

0006-2960/89/0428-9317$01.50/0

Anderson, 1983; Gopalakrishna et al., 1986). Tissue fractionation revealed the presence of two forms of membrane-bound PKC, reversible and irreversible complexes (Kikkawa et al., 1982; Shearman, 1987). Reversible and irreversible membrane-bound forms of PKC have also been generated in purified systems. This shows that the in vivo observations may arise entirely from the properties of PKC, without participation of other cellular components. It was further shown that, in a pure system, the activity of membrane-inserted PKC was not influenced by calcium or phorbol esters (Bazzi & Nelsestuen, 1988). This raises the possibility that formation of membrane-inserted PKC is the major or only mechanism of PKC activation.

This study was initiated to compare the effects of DAG and phorbol esters on PKC, and to determine if active PKC could be generated in a reversible membrane-bound complex. The results showed major differences in the ability of the two cofactors to form the irreversible PKC–membrane complex. DAG activated PKC largely in a reversible membrane complex while phorbol esters functioned largely by forming the irreversible complex. These differences and other observed properties may help to explain the different effects of phorbol esters and DAG on pure PKC and on whole cells.

Experimental Procedures

Materials. Histone III-S, Triton X-100, bovine serum albumin (BSA), diolein, and protamine sulfate were purchased from Sigma Chemical Co. Diolein consists of a mixture of isomers containing approximately 15% 1,2-*sn*-dioleoylglycerol (manufacturer's estimate). [γ-^{32}P]ATP (3 Ci/mmol) was purchased from Amersham Corp. 1-Oleoyl-2-acetyl-*sn*-glycerol (OAG) was purchased from Avanti Polar Lipids. Bovine brain phosphatidylserine (PS) and egg yolk phosphatidylcholine (PC) were obtained from either Avanti Polar Lipids or Sigma Chemical Co. Phorbol 12-myristate 13-acetate (PMA), phorbol 12,13-dibutyrate (PDBu), and 4-α-phorbol 12,13-dibutyrate (4-α-PDBu) were purchased from LC Services Corp. ^{45}Ca (44.5 mCi/mg) and [^{3}H]PDBu (15–20 Ci/mmol) were purchased from New England Nuclear. Polycarbonate filters (0.1 μm pore size) were purchased from Nucleopore Corp. Other chemicals and reagents were of the highest grade available.

PKC was purified from bovine brain according to a published procedure (Bazzi & Nelsestuen, 1987). The enzyme activity was assayed by ^{32}P incorporation into histone III-S. The activity measurements were performed in the presence of either phospholipid vesicles as reported by Kikkawa et al. (1982) or Triton–PS mixed micelles as reported by Hannun et al. (1985).

Measuring Irreversibility of PKC–Phospholipid Interaction. Upon insertion into membranes, the activity of PKC becomes Ca^{2+} independent (Bazzi & Nelsestuen, 1988). This property was utilized to measure the formation of the irreversible PKC–membrane complex (membrane-inserted PKC). The formation of the irreversible PKC–membrane complex was confirmed in each case by gel filtration chromatography of the mixture in the presence of EGTA (see below).

Initially, the Ca^{2+}-dependent and -independent activity of PKC was monitored as a function of time. PKC was mixed with phospholipids [vesicles (0.2 mg/mL) or Triton mixed micelles (containing 10 mol % PS) ± phorbol esters or diacylglycerols] in buffer containing (unless otherwise specified) 20 mM Tris (pH 7.5), 10 mM $MgCl_2$, 0.2 mg/mL histone, 0.1 mM $CaCl_2$, and 0.1 mM EGTA. Under these conditions, the free calcium concentration, calculated as described by Storer and Cornish-Bowden (1976), was approximately 0.6 μM. The phosphorylation reaction was initiated by the addition of [^{32}P]ATP (final concentration 15 μM), and product was measured at timed intervals. Aliquots (250 μL each) were withdrawn from the reaction mixture, and phosphorylation was terminated by the addition of 1 mL of 20% TCA. To terminate Ca^{2+}-dependent phosphorylation, a large volume of this incubation mixture was withdrawn and made 2.0 mM in EGTA, and phosphorylation was continually monitored as outlined above.

Formation of the irreversible PKC–phospholipid complex was also measured as a function of the concentrations of various activators in the medium. In this case, PKC was mixed with phospholipid vesicles (0.2 mg/mL) in buffer containing 20 mM Tris (pH 7.5), 10 mM $MgCl_2$, 0.25 mM $CaCl_2$, 0.25 mM EGTA (free calcium concentration was approximately 1.4 μM), and a specified concentration of either PMA, PDBu, or OAG. The mixture was incubated for 30 min at room temperature, and two aliquots were withdrawn. EGTA was added to one aliquot to give a final concentration of 2.0 mM. The mixture was incubated for 20 min to allow complete dissociation. The kinase activity of each aliquot (plus EGTA and plus calcium) was then assessed after addition of 15 μM [^{32}P]ATP and histone (0.2 mg/mL).

Dissociation of Calcium from Membrane-Inserted PKC. PKC was mixed with phospholipid vesicles in buffer containing 20 mM Tris (pH 7.5), 10 mM $MgCl_2$, 50 nM PDBu, and 2 μM ^{45}Ca. After the mixture (250 μL total volume) was incubated for 30 min at room temperature, EGTA was added to a final concentration of 2.0 mM. The sample was incubated for an additional 20 min and was then applied on a Sephacryl S-300 column (1.0 × 40 cm) equilibrated and eluted (1-mL fractions) with buffer containing 20 mM Tris (pH 7.5), 1.0 mM EGTA, 1.0 mM EDTA, 30 mM β-mercaptoethanol, 10% (v/v) glycerol, and 0.5 mg/mL BSA. The kinase activity in each fraction (100-μL aliquots) was measured by using protamine sulfate as the substrate (Takia et al., 1977). The concentration of Ca^{2+} in the elution profile was determined from the radioactivity present in each fraction (25-μL aliquots) and the known specific radioactivity of ^{45}Ca.

PKC–PDBu Binding. The binding of PDBu to PKC was measured under equilibrium conditions by using gel filtration chromatography (Hummel & Dryer, 1962) as described previously (Bazzi & Nelsestuen, 1989). Bio-Gel A 1.5-m (Bio-Rad) columns (1.0 × 40 cm) were equilibrated and eluted with buffer consisting of Tris (20 mM, pH 7.5), glycerol (10% v/v), β-mercaptoethanol (30 mM), Ca^{2+} (0.5 mM), [^{3}H]PDBu (2 nM), and BSA (0.5 mg/mL). PKC was mixed with 0.6 mL of the equilibration buffer and phospholipid vesicles [composed of 25% PS and 75% PC ± 10% or 30% OAG (w/w with respect to total lipid)]. The samples were incubated for 30 min at room temperature and then applied on the gel filtration columns. The concentrations of PDBu (250-μL aliquots) and the kinase activity of each fraction (100-μL aliquots) were measured as described previously (Bazzi & Nelsestuen, 1989). Total yield of enzyme activity from the columns was greater than 80%.

Phospholipid Preparations. Unilammelar vesicles composed of PS/PC/diolein (25:75:0 or 25:65:10) were prepared by several cycles of freeze–thaw followed by sonication in a bath (Laboratory Supplies Inc., Hicksville, NY). The phospholipids were dried from organic solvents under a stream of nitrogen and suspended in aqueous buffer (20 mM Tris, pH 7.5) by agitation. The suspension was frozen in dry ice, thawed at room temperature, and suspended by brief bath sonication. This cycle was repeated at least 5 times. When needed, OAG

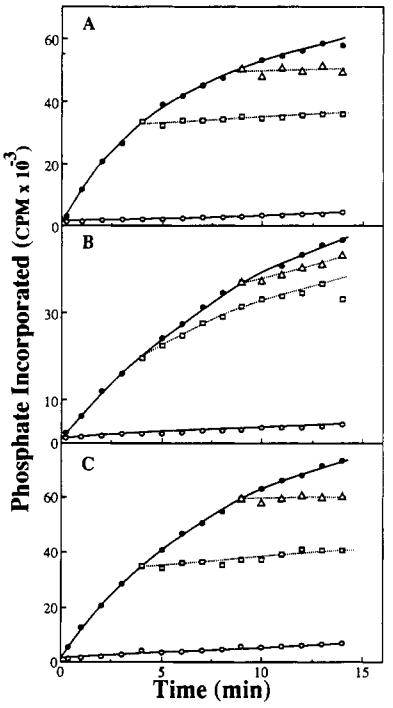

FIGURE 1: Reversible and irreversible activation of PKC by phospholipid vesicles. Activity of PKC was measured by using phospholipid vesicles (0.2 mg/mL) composed of PS/PC/diolein (25:75:0 or 25:65:10) and in the presence of 0.6 μM free Ca^{2+} as described under Experimental Procedures. All panels show the activity of PKC in the presence (●) or the absence (○) of PKC activators. After 4 (□, dashed lines) or 9 min (△, dashed lines), EGTA was added (2 mM final concentration) to aliquots of the assay mixture to inhibit the activity of the reversible PKC–membrane complex. The PKC activator was either 10% (w/w) diolein (panel A), 50 nM PDBu (panel B), or 10% (w/w) OAG (panel C).

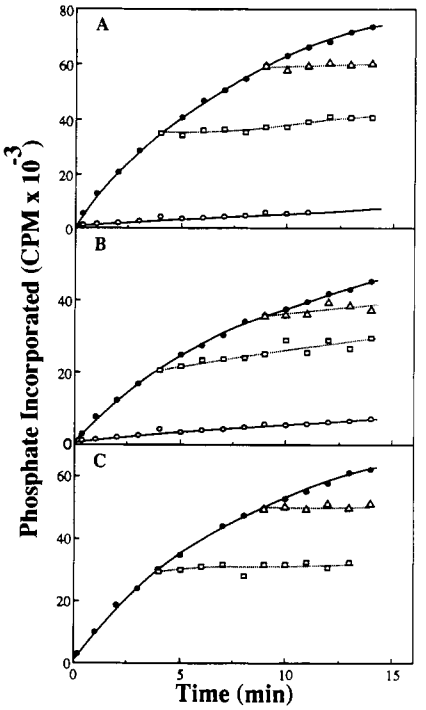

FIGURE 2: Reversible activation of PKC by Triton–PS mixed micelles. The activity of PKC was measured in the presence of 0.15 mM Ca^{2+} by using Triton–PS mixed micelles as the lipid source. The activity was measured with micelles containing 10 mol % PS without (○) or with (●) 2.5 mol % diolein (panel A), 50 nM PDBu (panel B), or 2.5 mol % OAG (panel C). At 4 (□, dashed lines) or 9 min (△, dashed lines), EGTA was added to portions of the assay mixture and the activity of PKC was monitored as a function of time.

was dissolved in ethanol (at either 5 or 50 mg/mL) and delivered to prepared phospholipid vesicles. The concentration of ethanol in the final mixture never exceeded 8% (v/v). Phospholipid concentrations were determined from organic phosphate (Chen et al., 1956) by using a phosphorous to phospholipid weight ratio of 1:25.

Results

Difference between Diacylglycerols and Phorbol Esters in Activating PKC. Previous studies showed two forms of membrane-associated PKC, reversible and irreversible complexes (Gopalakrishna et al., 1986; Bazzi & Nelsestuen, 1988). While the irreversible complex was active in the presence or the absence of both Ca^{2+} and/or phorbol esters (Bazzi & Nelsestuen, 1988), it was not known whether reversible complexes were active.

The results in Figure 1A show the incorporation of ^{32}P into histone as a function of time. At 4 or 9 min, EGTA terminated the kinase activity in the mixture containing DAG (Figure 1A). These results suggested that the majority of active PKC was bound reversibly to the membrane. Gel filtration of a product of this incubation (see below for an example) showed that, indeed, virtually all the PKC was dissociated from the membrane by EGTA.

Activation of PKC by phorbol esters showed different properties (Figure 1B). Like diolein, phorbol esters (50 nM PDBu) activated PKC, but this activation was largely uninhibited by later addition of EGTA. Bocckino and Exton (1986) also reported that calcium was needed to initiate PKC activity but was not needed to maintain the activity in the presence of PMA. Gel filtration experiments (see below) showed that the calcium-independent activity arose from membrane-inserted PKC and was not due to proteolytic degradation of PKC. These results suggested a critical difference between the activation of PKC by diolein and phorbol esters.

The experiments in Figure 1A,B were performed with two preparations of phospholipid vesicles (±10% diolein). To eliminate possible differences in individual phospholipid preparations, OAG was used as the activator since it was added to preformed vesicles. OAG behaved in a manner similar to diolein so that activity was terminated by EGTA (Figure 1C). This showed a major difference between DAG and phorbol esters in that the tumor-promoting agent, PDBu, was more efficient in producing membrane-inserted PKC, a constitutively active kinase.

The reversibility of PKC activation was also examined by using Triton–PS mixed micelles as the lipid source (Figure 2). The activity was monitored in the presence of 10 mol % PS and either 2.5 mol % diolein (Figure 2A), 50 nM PDBu (Figure 2B), or 2.5 mol % OAG (Figure 2C). As in the case of phospholipid vesicles, activation by diolein (Figure 2A) or OAG (Figure 2C) was dependent on the presence of Ca^{2+}. However, unlike the case with phospholipid vesicles, activation by phorbol esters was also dependent on the continuous presence of Ca^{2+} (Figure 2B). These results showed that some activation properties of PKC were dependent on whether the phospholipids were provided in the form of vesicles or micelles. It is possible that the dynamic nature of micelles prevented formation of irreversible protein–phospholipid complexes.

Dissociation of Calcium from Membrane-Inserted PKC. It has been proposed that the irreversible PKC–membrane complex arises from tight calcium binding. To test this possibility, PKC was incubated with phospholipid vesicles in the presence Ca^{2+} and phorbol esters at room temperature to induce maximum insertion of PKC into vesicles. The sample

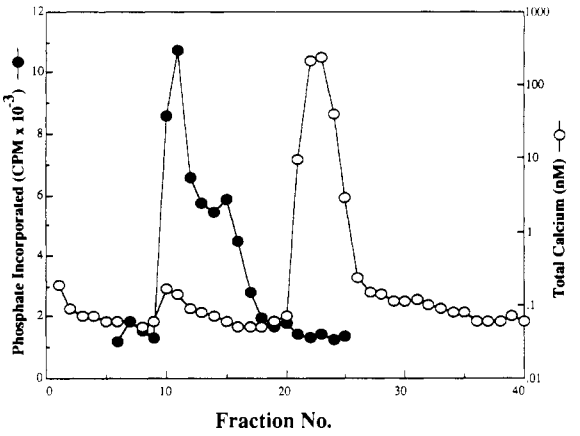

FIGURE 3: Dissociation of calcium from membrane-inserted PKC. PKC (32 pmol) was mixed with 62.5 μg of phospholipid vesicles composed of PS/PC (50:50) in 0.5 mL of buffer containing 20 mM Tris (pH 7.5), 10% glycerol, 10 mM $MgCl_2$, 0.5 mg/mL BSA, 30 mM β-mercaptoethanol, 50 nM PDBu, and 2 μM ^{45}Ca. The sample was incubated for 30 min at room temperature to allow insertion, made 2 mM EGTA, incubated for an additional 20 min to allow dissociation, and then applied on a Sephacryl S-300 column (1 × 40 cm) equilibrated with a buffer containing 20 mM Tris, pH 7.5, 0.5 mg/mL BSA, 1.0 mM EGTA, 1.0 mM EDTA, 30 mM β-mercaptoethanol, and 10% glycerol. The kinase activity (●) and ^{45}Ca concentration (○) associated with each fraction are shown.

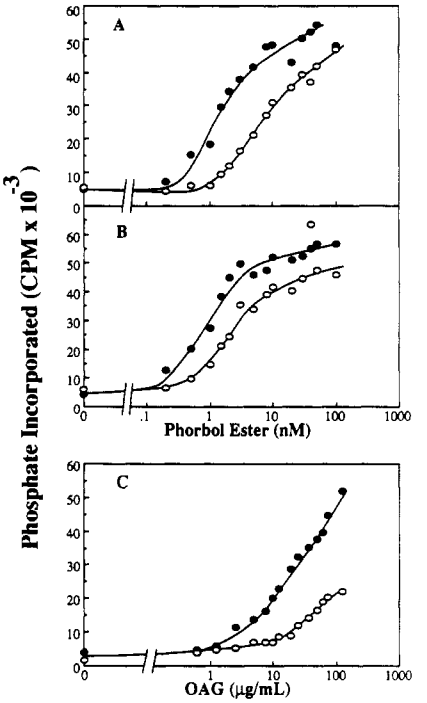

FIGURE 4: Dependence of PKC–membrane insertion on phorbol ester concentration. PKC was incubated with phospholipid vesicles in the presence of 1.4 μM free Ca^{2+} and a specified concentration of either PDBu (panel A), PMA (panel B), or OAG (panel C). The vesicles were composed of PS/PC (25:75), and all assays contained 0.2 mg/mL concentrations of these vesicles. After a 30-min incubation, two aliquots were withdrawn and EGTA was added to one aliquot to give a final concentration of 2.0 mM. The kinase activities of both aliquots [plus calcium (●) and plus EGTA (○)] are shown.

was dissociated by the addition of EGTA, and the components were separated by gel filtration. The elution profile (Figure 3) indicated that the majority of PKC became inserted into membrane under these conditions and eluted at the exclusion volume of the column. A smaller fraction of the PKC eluted at the position of the free kinase (M_r = 80000). A very small amount of ^{45}Ca eluted with membrane-inserted PKC. The ^{45}Ca/PKC ratio in these fractions was slightly variable in different experiments but was always less than 0.1 (the data shown in Figure 3 corresponded to 0.02 mol of ^{45}Ca/mol of PKC). Experiments that measured background (PKC was omitted) showed a similar elution profile for Ca^{2+} (data not shown). Consequently, the mol ratios of Ca^{2+}/PKC obtained in Figure 3 represented an upper limit. These results indicated that the stability of the irreversible PKC membrane complex was not dependent on residual Ca^{2+} in the complex.

Dependence of PKC–Membrane Insertion on the Concentration of Activators. The ability of phorbol esters to activate and induce insertion of PKC into membranes was examined as a function of phorbol ester concentration. At the Ca^{2+} concentration used in Figure 4 (1.6 μM free calcium), PDBu was essential for PKC activation and insertion into membranes. These graphs plot total rather than free PDBu concentrations and cannot be used to estimate equilibrium binding parameters. Nevertheless, the results (Figure 4A) showed a difference in the relative amounts of PDBu needed to produce activity and membrane insertion. These titrations suggested the presence of a reversible, calcium-dependent PKC–membrane–PDBu complex that was active.

PMA also activated PKC and was even more efficient than PDBu in causing irreversible PKC–membrane binding (Figure 4B). For example, the same level of calcium-independent activity (30000 cpm, for example) required 2 nM PMA versus 8 nM PDBu. This was consistent with the known efficacy of PMA and PDBu in producing biological responses (Blumberg et al., 1984).

Activation and insertion of PKC into membranes were also examined with OAG. In agreement with the results in Figure 1, OAG activated PKC in the presence of calcium but induced little insertion into membranes (Figure 4C). Low levels of calcium-independent activity were obtained with amounts of OAG that approached the amount of phospholipid. The latter may therefore be due to nonspecific events such as structural alteration of bilayers by OAG.

Ca^{2+}-independent kinase activity can be generated by insertion of PKC into the phospholipid bilayer (Bazzi & Nelsestuen, 1988). However, a proteolytic fragment of PKC, PKM (M_r = 55000), also exhibits Ca^{2+}-independent kinase activity (Kishimoto et al., 1983; Huang & Huang, 1986). Reaction mixtures similar to those shown in Figures 1–4 (above) were subjected to gel filtration chromatography to assess whether the Ca^{2+}-independent activity was generated from membrane-inserted or proteolytically cleaved PKC. After PKC was incubated with phospholipid under various conditions, mixtures were made 2.0 mM in EGTA and chromatographed on columns equilibrated with buffer containing EGTA. A typical result is shown in Figure 5. When PKC was incubated in the presence of 50 nM PDBu or PMA, the majority of the PKC became inserted into membranes and eluted with the phospholipid vesicles at the exclusion volume of the column. As expected, incubation of PKC with phospholipid vesicles containing OAG (10% w/w with respect to phospholipids) resulted in little membrane-inserted PKC. Since both Ca^{2+} (Figure 3) and PDBu (Bazzi & Nelsestuen, 1989) dissociate from membrane-inserted PKC, the differences between DAG and phorbol esters did not arise from the presence of residual cofactors. Results of these experiments showed that Ca^{2+}-independent activity observed in the presence of phorbol esters (Figures 1–4) was due to the formation of membrane-inserted PKC.

To test the specificity of the PKC–membrane insertion event, titrations were carried out with PDBu and/or 4-α-PDBu (Figure 6). Two titrations were performed. In both cases,

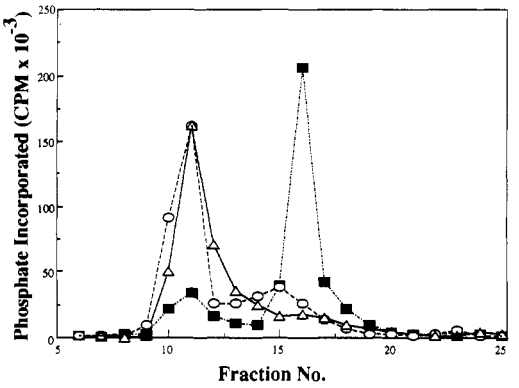

FIGURE 5: Reversible and irreversible association of PKC with phospholipid vesicles. PKC was incubated with phospholipid vesicles composed of PS/PC (25:75) in 20 mM Tris buffer (pH 7.5) containing 10 mM $MgCl_2$, 0.25 mM EGTA, 0.25 mM $CaCl_2$, and either 50 nM PMA (△, solid line), 50 nM PDBu (○, dashed line), or 10% OAG (w/w with respect to phospholipid) (■, dashed–dotted line). The sample was incubated at room temperature for 30 min, made 2.0 mM with EGTA, and then applied on a Sephacryl S-300 column (1 × 40 cm) equilibrated and eluted with a buffer containing 20 mM Tris (pH 7.5), 0.5 mg/mL BSA, 1.0 mM EGTA, 1.0 mM EDTA, 30 mM β-mercaptoethanol, and 10% glycerol. The activity associated with each fraction (1 mL) was measured by using protamine sulfate as the substrate.

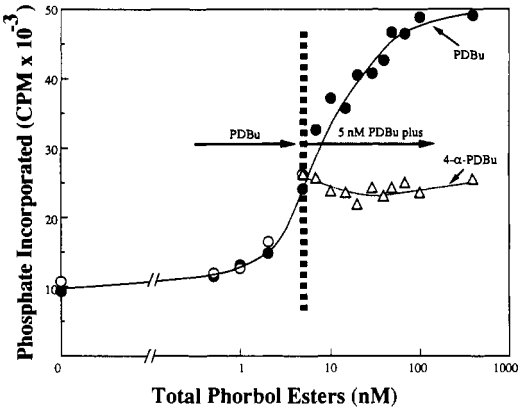

FIGURE 6: Specificity of PKC insertion into membranes. The formation of membrane-inserted PKC was monitored as function of total phorbol ester concentration (4-β- plus 4-α-phorbol esters). In one case (●), the titration was carried out entirely with PDBu. In the second case (○), the titration used PDBu up to 5 nM followed by 4-α-PDBu (△) to give the indicated concentration of total phorbol esters. In both titrations, PKC was incubated (230 μL total volume) for 30 min with phospholipid vesicles (0.2 mg/mL) in a buffer containing 20 mM Tris (pH 7.5), 10 mM $MgCl_2$, 0.15 mM Ca^{2+}, 0.2 mg/mL histone, and the indicated concentration of total phorbol esters. The mixture was made 2.0 mM with EGTA and incubated for an additional 20 min, and the kinase activity was measured as described under Experimental Procedures.

PDBu was added up to 5 nM which was sufficient to activate the enzyme and produce partial insertion of PKC into the membrane (Figures 6 and 4A). Further additions consisted of either PDBu or 4-α-PDBu. The results (Figure 6) showed that 4-α-PDBu failed to enhanced PKC-membrane insertion. Thus, activation and insertion of PKC into membranes were both stereospecific events.

A surprising observation was that calcium was not essential for forming membrane-inserted PKC. The results in Figure 7A showed that calcium-dependent binding of PKC to the membrane reduced the concentration of PDBu needed to cause irreversible PKC–membrane association. However, in the absence of calcium, high concentrations of phorbol esters (≥50 nM PDBu) activated PKC by forming the irreversible membrane-bound complex (Figure 7A). This activation required

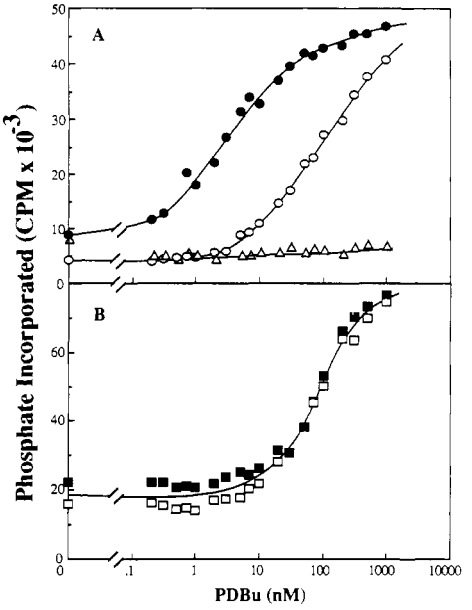

FIGURE 7: Ca^{2+}-independent insertion of PKC into membranes. Panel A shows comparison of Ca^{2+}-dependent and -independent insertion. PKC was incubated at the given concentration of PDBu with phospholipid (0.2 mg/mL of PS/PC, 25:75) in the presence of either 0.15 mM Ca^{2+} (●) or 3.0 mM EGTA (○). Incubations without phospholipid (+EGTA) are shown for reference (△). After a 30-min incubation, EGTA or Ca^{2+} was added to give the same final concentrations in all assays. After an additional 10 min, the Ca^{2+}-independent activity was measured. Panel B shows formation of Ca^{2+}-independent activity in membranes containing DAG. PKC was incubated with phospholipid vesicles (0.2 mg/mL) for 30 min in a buffer containing 20 mM Tris (pH 7.5), 10 mM $MgCl_2$, 0.2 mg/mL histone, 3.0 mM EGTA, and the concentration of PDBu that is given. The phospholipid vesicles (25% PS) contained 5% OAG (■) or 10% diolein (□), and the remaining lipid was PC. To assure complete chelation of contaminating Ca^{2+}, all reagents were made 3 mM in EGTA before addition of PKC. The kinase activity was measured as described under Experimental Procedures.

phospholipids (Figure 7A) and required preincubation of PKC with phospholipid before addition of substrate. The latter aggregated the phospholipid and made it less accessible to the kinase. Gel filtration experiments such as those shown in Figure 5 showed virtually complete association of PKC with the vesicles at high PDBu concentrations (data not shown).

It was possible that DAG may have altered the vesicles so that formation of the irreversible PKC–membrane complex was not possible. Alternatively, DAG might destabilize the complex once it was formed. The results in Figure 7B showed that this was not the case. PDBu promoted formation of irreversible PKC complexes with vesicles containing high concentrations of DAG. Furthermore, PDBu was able to form the irreversible PKC–membrane complex even in the presence of high levels of both DAG and EGTA.

Previous reports indicate that DAG and phorbol esters compete for the same site on PKC (Sharkey et al., 1984). However, the differences between these cofactors observed in this study suggested the need to reexamine this property with the materials used in this study. In agreement with the earlier result, binding of phorbol esters to PKC was inhibited by high levels of OAG (Figure 8). Thus, OAG appeared to bind to the high-affinity PDBu binding site on the PKC–membrane complex.

Discussion

Both phorbol esters and DAG are known to activate PKC, and their similarities have suggested analogous, if not identical, functions. For example, both compounds reduce the Ca^{2+} concentration required for in vitro activation of PKC, and in

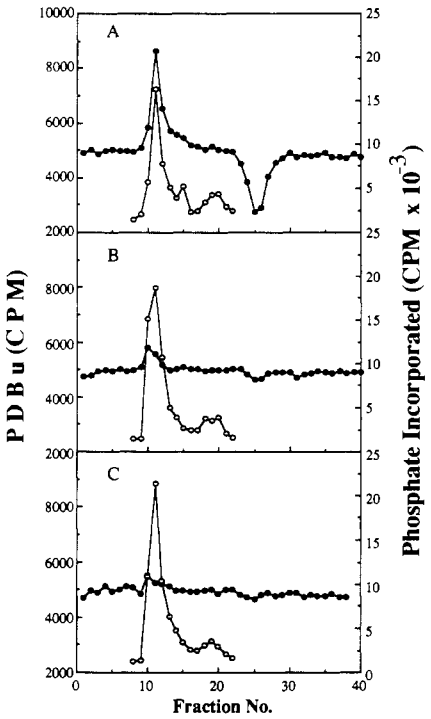

FIGURE 8: Inhibition of PKC–PDBu binding by OAG. Protein kinase C was mixed with 0.6 mL of the equilibration buffer and phospholipid vesicles composed of PS/PC (25:75) as described under Experimental Procedures. OAG was added to the phospholipid vesicles to give a ratio of 0% (w/w with respect to phospholipid) (panel A), 10% (panel B), or 30% (panel C). The mixture was incubated at 4 °C for 30 min and applied on a Bio-Gel A 1.5-m column (1.0 × 40 cm) equilibrated and eluted with buffer containing 20 mM Tris (pH 7.5), 10% glycerol, 30 mM β-mercaptoethanol, 0.5 mM Ca^{2+}, 2.0 nM [^{3}H]PDBu, and 0.5 mg/mL BSA. All panels show the PDBu (●) and the kinase activities (○) associated with each fraction.

many cases, both induce similar biological responses (Kikkawa & Nishizuka, 1986). The tumor-promoting activity of phorbol esters may arise from their slow metabolism, which allows PKC activation for long time periods (Nishizuka, 1986a). The results obtained in this study showed a more basic difference between these materials; phorbol esters induced sustained in vitro activation of PKC by forming an irreversible PKC–membrane complex, while DAG acted primarily through the reversible PKC–membrane complex.

The larger difference between DAG and phorbol esters may actually pose a new problem: DAG had low efficacy in generating membrane-inserted PKC. However, evidence from tissue fractionations suggests that formation of the irreversible PKC–membrane complex is a physiological event. Substantial portions of cellular PKC, especially in brain, are irreversibly associated with the membrane and can only be released by treatment with detergents (Kikkawa et al., 1982; Shearman et al., 1987; Yoshida et al., 1988). Thus, insertion of PKC into membranes is not associated exclusively with tumor promotion and phorbol esters. While DAG did not induce efficient insertion of PKC into membranes under the in vitro conditions used in these experiments, other factors may enhance its efficacy in vivo. Possible contributors might include events that occur in conjunction with cell signaling such as changes in transmembrane potentials, membrane curvature, or membrane fusion. Alternatively, it is possible that the effect of DAG is small but accumulative so that multiple signals are needed to cause insertion of an appreciable amount of PKC into the membrane. In the latter case, the small effects of DAG observed in this study may be adequate to function as a basis for long-term cell potentiation or memory through the PKC enzyme (Bazzi & Nelsestuen, 1988).

Some properties of the phorbol ester–PKC interaction suggested multiple interactions. Activation and insertion of PKC into the membrane arose from specific interactions but required different concentrations of phorbol esters (Figure 4). In addition, high concentrations (about 50 nM) of phorbol esters caused irreversible association of PKC with membranes in the absence of calcium (Figure 7). It should be emphasized that 50 nM PDBu was adequate to activate PKC but was still very low compared to the concentration of phospholipid (~250 μM). Furthermore, both activation and insertion of PKC into the membrane represented stereospecific interactions, and 4-α-PDBu was ineffective (Figure 6). Thus, both of the phorbol ester induced events involved highly specific interactions rather than general disruption of membrane structure. In general, insertion events required higher concentrations of phorbol esters than activation. These properties may be consistent with the observation that different concentrations of phorbol esters elicit different biological responses (Blumberg, 1988) and with the report of calcium-independent, phorbol ester induced formation of the chelator-resistant PKC–membrane complex in cell membranes (Gopalakrishna et al., 1986).

Explanation of the phorbol ester effects may require two phorbol ester binding events consisting of a high-affinity interaction which is calcium- and membrane-dependent and a low-affinity interaction which is calcium-independent. Reversible activation would result from the high-affinity PKC–phorbol ester interaction. This would be the association that is easily observed in binding measurements [Bazzi and Nelsestuen (1989) and references cited therein]. A low-affinity interaction might be responsible for PKC–membrane insertion. The existence of a second interaction of PKC and/or membranes with phorbol esters represents speculation based exclusively on unexplained PKC behavior. Previous studies failed to detect more than one binding site for PDBu on the PKC–membrane complex (Kikkawa et al., 1983; Tanaka et al., 1986; Bazzi & Nelsestuen, 1989). In addition, no phorbol ester binding to soluble PKC was detected in the absence of calcium (Sando & Young, 1983; Dougherty & Niedel, 1986; Huang & Huang, 1986; Bazzi & Nelsestuen, 1988). Calcium-independent binding of phorbol esters to PKC occurs with membrane-inserted PKC (Bazzi & Nelsestuen, 1989) and also to soluble PKC in the presence of polycationic materials (Bazzi & Nelsestuen, 1989; Thompson et al., 1988). These conditions did not appear relevant to the titrations in Figures 4 and 7. Nevertheless, the second phorbol ester induced event is an irreversible process so that product formation is not representative of total binding. Filling of an extremely small portion of the sites, followed by efficient protein–membrane insertion, would lead to accumulation of PKC in the membrane. In fact, binding affinity could be so low that direct evidence for such an interaction could be difficult to obtain.

The preparation of PKC used in this study probably contained a mixture of PKC isozymes (Huang et al., 1986; Jaken & Kiley, 1987), and differences between the PKC isozymes have been observed (Huang et al., 1988; Nishizuka, 1988). While quantitative differences between the isozymes with respect to the propreties documented here may exist, this study and previous studies (Bazzi & Nelsestuen, 1989) showed that nearly all of the PKC molecules became inserted into membranes (Figure 4) and the reported behavior should apply qualitatively to all of the isozymes present in these preparations.

It has been proposed that the tight association of PKC with membranes results from increased affinity for Ca^{2+} (Bell,

1986). However, this study showed that added calcium dissociated from the irreversible PKC–membrane complex (Figure 3). In addition, irreversible PKC–membrane binding occurred in the presence of high concentrations of EGTA (Figure 7). Previous studies (Bazzi & Nelsestuen, 1989) showed that phorbol esters also dissociated from the irreversible PKC–membrane complex. Thus, the irreversible membrane-bound form of PKC must involved interactions such as insertion of protein components into the hydrocarbon region of the bilayer. While such interactions may also occur between PKC and phospholipids dispersed in Triton micelles, the dynamic nature of micelles may render such interactions unstable. Activation of PKC by lipids in detergent micelles required the continual presence of calcium (Figure 2), and this system may not be useful for study of the irreversible stage of PKC–membrane binding.

The current study showed complex in vitro behavior of PKC which may be consistent with the in vivo behavior of the relevant second messengers, tumor promotors, and PKC. Further studies will be needed to determine whether other factors participate in the similar events that occur in vivo. Especially important are questions of how PKC becomes irreversibly associated with cell membranes under normal physiological conditions; the second messenger, DAG, had low efficacy in the in vitro system used here.

References

Ashendel, C. L. (1985) *Biochim. Biophys. Acta 822*, 219.

Bazzi, M. D., & Nelsestuen, G. L. (1987) *Biochemistry 26*, 115.

Bazzi, M. D., & Nelsestuen, G. L. (1988) *Biochemistry 27*, 7589.

Bazzi, M. D., & Nelsestuen, G. L. (1989) *Biochemistry 28*, 3577.

Bell, R. M. (1986) *Cell 45*, 631.

Blumberg, P. M. (1988) *Cancer Res. 48*, 1.

Blumberg, P. M., Jaken, S., Konig, B., Sharkey, N. A., Leach, K. L., Jeng, A. Y., & Yeh, E. (1984) *Biochem. Pharmacol. 33*, 933.

Bocckino, S., & Exton, J. H. (1986) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 45*, Abstract 1865.

Chen, P. S., Toribara, T. Y., & Warner, H. (1956) *Anal. Chem. 28*, 1756.

Dougherty, R. W., & Niedel, J. E. (1986) *J. Biol. Chem. 261*, 4097.

Gopalakrishna, R., Barsky, S. H., Thomas, T. P., & Anderson, W. B. (1986) *J. Biol. Chem. 261*, 16438.

Hannun, Y. A., Loomis, C. R., & Bell, R. M. (1985) *J. Biol. Chem. 260*, 10039.

Huang, K.-P., & Huang, F. L. (1986) *Biochem. Biophys. Res. Commun. 139*, 320.

Huang, K.-P., Nakabayashi, H., & Huang, F. L. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8535.

Huang, K.-P., Huang, F. L., Nakabayashi, H., & Yoshida, Y. (1988) *J. Biol. Chem. 263*, 14839.

Hummel, J. P., & Dreyer, W. J. (1962) *Biochim. Biophys. Acta 63*, 530.

Jaken, S., & Kiley, S. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4418.

Kikkawa, U., & Nishizuka, Y. (1986) *Annu. Rev. Cell Biol. 2*, 149.

Kikkawa, U., Takai, Y., Minakuchi, R., Inohara, S., & Nishizuka, Y. (1982) *J. Biol. Chem. 257*, 13341.

Kikkawa, U., Takai, Y., Tanaka, Y., Miyake, R., & Nishizuka, Y. (1983) *J. Biol. Chem. 258*, 11442.

Kishimoto, A., Kajikawa, N., Shiota, M., & Nishizuka, Y. (1983) *J. Biol. Chem. 258*, 1156.

Kraft, A. S., & Anderson, W. B. (1983) *Nature (London) 301*, 621.

Kraft, A. S., Anderson, W. B., Cooper, H. L., & Sando, J. J. (1982) *J. Biol. Chem. 257*, 13193.

Nishizuka, Y. (1988) *Nature (London) 334*, 661.

Nishizuka, Y. (1986a) *JNCI, J. Natl. Cancer Inst. 76*, 363.

Nishizuka, Y. (1986b) *Science (Washington, D.C.) 233*, 305.

Sando, J. J., & Young, M. C. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 2642.

Sharkey, N. A., Leach, K. L., & Blumberg, P. M. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 607.

Shearman, M. S., Naor, Z., Kikkawa, U., & Nishizuka, Y. (1987) *Biochem. Biophys. Res. Commun. 147*, 911.

Storer, A. C., & Cornish-Bowden, A. (1976) *Biochem. J. 159*, 1.

Takai, Y., Kishimoto, A., Inoue, M., & Nishizuka, Y. (1977) *J. Biol. Chem. 252*, 7603.

Tanaka, Y., Miyake, R., Kikkawaw, U., & Nishizuka, Y. (1986) *J. Biochem. 99*, 257.

Thompson, N. T., Bonser, R. W., Hodson, H. F., & Garland, L. G. (1988) *Biochem. J. 255*, 417.

Yoshida, Y., Huang, F. L., Nakabayashi, H., & Huang, K.-P. (1988) *J. Biol. Chem. 263*, 9868.