Mechanism of Activation of Protein Kinase C: Roles of Diolein and Phosphatidylserine†

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ABSTRACT: We studied the roles of lipid concentration, phosphatidylserine (PS), and diolein (DG) contents, as well as Ca^{2+} concentration, on the partitioning of protein kinase C (PKC) between aqueous and membrane environments as well as the relationship of this partitioning to the activation of the enzyme. Physiological concentrations of 1 mol % DG increased the apparent binding constant of PKC to the 3:1 PC/PS membrane 500 times. This increase was proportional to the mol % DG. Over 50% PKC was bound to that membrane at micromolar concentrations of Ca^{2+} and physiologically relevant total concentration of lipid only when 1 mol % DG was included. PKC bound either to PS alone or to PS and DG was enzymatically competent; however, the rate of phosphorylation was doubled in the presence of 1 mol % diolein. The dependence of PKC binding on the mol % PS was highly sigmoidal. The Hill coefficient was in the range of 4–6, with the higher values found at the lower lipid concentrations. These results suggest that the observed apparent cooperativity is due, at least in part, to the change in dimensionality when PKC binds to the membrane.

Protein kinase C (PKC)[1] is a family of structurally related serine/threonine kinases (Nishizuka, 1988; Kikkawa et al., 1989; Parker et al., 1989) implicated in the regulation of a variety of cellular responses (Nishizuka, 1988, 1989). Two distinct subgroups of this kinase family differ in their Ca^{2+} requirement for activation (Nishizuka, 1988; Stabel & Parker, 1991). Anionic lipids (Takai et al., 1979; Sekiguchi et al., 1988; Huang et al., 1988), in particular L-phosphatidylserine (PS) (Lee & Bell, 1989; Burns et al., 1990), are necessary to activate PKC. To obtain fully functional PKC, binding of either diacylglycerol (DAG) or phorbol ester to the enzyme is also required (Kaibuchi et al., 1981; Hannun et al., 1985; Burns et al., 1990), with one possible exception (Ways et al., 1992). Although the molecular mechanism of PKC activation by DAG remains elusive, there seems to be an agreement at the phenomenological level. Four recent papers proposing the molecular mechanism of activation of PKC by DAG all suggested similar models (Stabel & Parker, 1991; Burns & Bell, 1992; Orr & Newton, 1992b; Zidovetzki & Lester, 1992) which originated in that proposed by Hannun and Bell (1986). According to the proposed scheme, a rise in calcium concentration to a micromolar level causes the binding of Ca^{2+}-dependent isoforms of PKC to membranes containing PS or other anionic lipids. The enzyme, however, remains inactive. The release of DAG from either phosphatidylinositol bisphosphate (PIP_2) (Leach et al., 1991) or phosphatidylcholine (PC) (Diaz-Laviada et al., 1990) allows its binding to PKC and simultaneous activation of the enzyme. PKC may be also activated by phorbol esters which compete with DAG for the same binding site (Sharkey et al., 1984). Two cysteine-rich fragments of PKC, present in the highly conserved region C1 in almost all isoforms of this kinase family (Kikkawa et al., 1989; Parker et al., 1989; Stabel & Parker, 1991), each bind the phorbol ester in a manner similar to that of the intact enzyme although with the lower affinity (Burns & Bell, 1991).

Although different modes of PKC activation were reported (Walker & Sando, 1988; Zalewski et al., 1990; Lee & Bell, 1991; Lester et al., 1991; Khan et al., 1992), the basic mode of activation of Ca^{2+}-dependent PKC involves its binding in the presence of calcium ions to a membrane containing anionic lipids and subsequent binding of DAG or phorbol ester to the enzyme (Bell & Burns, 1991; Zidovetzki & Lester, 1992). Surprisingly, only a few studies on PKC binding to the membrane can be found in the literature (Wolf et al., 1985; Bazzi & Nelsestuen, 1987a; Rodriguez-Paris et al., 1989; Brumfeld & Lester, 1990; Orr & Newton, 1992a,b). The most extensive study was presented in a series of papers by Bazzi & Nelsesteuen (1987a, 1989, 1990, 1991a,b, 1992). They found that PKC bound to membranes containing PS with high affinity so that as little as 5 mol % PS was sufficient to cause virtually complete PKC binding to the membrane. The binding of PKC to the membrane did not depend on the presence of DAG (Bazzi & Nelsestuen, 1987a). The non-physiological levels of calcium required to bind a significant fraction of the enzyme to the membrane (Bazzi & Nelsestuen, 1987a, 1990) were explained in terms of a "sequential binding model" (Bazzi & Nelsestuen, 1991b). The presence of phosphatidylethanolamine significantly reduced the Ca^{2+} concentration required for PKC binding to the membrane (Bazzi & Nelsestuen, 1992). However, PKC binding to Triton X-100 mixed micelles (Lee & Bell, 1989; Orr & Newton, 1992b) or multilamellar vesicles (Bolen & Sando, 1993) depended on DAG.

To further study the mechanism of activation of PKC, we decided to use a novel binding assay recently developed to study the binding of phospholipase C to the membrane of large unilamellar vesicles (LUVs) (Rebecchi et al., 1993). This assay utilizes sucrose-loaded LUVs (SLV) which can be easily sedimented by centrifugation to separate the free from the membrane-bound enzyme. In the present study, we employed LUVs formed from a mixture of PS, PC, and DAG.

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[1] Abbreviations: ATP, adenosine triphosphate; BSA, bovine serum albumin; CL, cardiolipin; DAG, diacylglycerol; dansyl(DNS)-PE, *N*-(5-(dimethylamino)naphthalene-1-sulfonyl)-L-α-phosphatidylethanolamine; DG, 1,2-dioleoyl-*sn*-glycerol; EGTA, ethylene glycol bis(β-aminoethyl ether-*N*,*N*,*N*′,*N*′-tetraacetic acid; LUV(s), large unilamellar vesicle(s); PKC, protein kinase C; PA, phosphatidic acid; PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIP_2, phosphatidylinositol bisphosphate; PS, phosphatidylserine; PO, 1-palmitoyl-2-oleoyl form of the aforementioned phospholipids; SLV, sucrose-loaded vesicle.

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LUVs constitute a well-characterized, stable membrane system suitable for the study of PKC activation (Boni & Rando, 1985).

As has been pointed out several times (Bazzi & Nelsestuen, 1987a; Trudell et al., 1989), most of the studies on PKC activation were conducted at low salt concentration. A salt concentration close to the physiological level significantly decreases the activity of PKC (Bazzi & Nelsestuen, 1987a; Hannun & Bell, 1989; Trudell et al., 1989). We investigated both the binding of PKC to the membrane and the activity of the enzyme at a KCl concentration close to that encountered in a cell.

The dependence of PKC activation on the mol % PS in a micelle or membrane is highly sigmoidal. Hill coefficients in the range from 4 to 11 have been obtained from this dependence (Hannun et al., 1985; Newton & Koshland, 1989, 1990; Burns et al., 1990). This has been interpreted as resulting from the cooperative binding of PS molecules to the enzyme (Newton & Koshland, 1989). Orr and Newton (1992a,b) recently reported that the PS dependence of PKC binding to Triton X-100 mixed micelles exhibits identically strong cooperativity and that the binding and activation are interdependent. Interestingly, both the binding (Orr & Newton, 1992a,b) and the autophosphorylation of PKC (Newton & Koshland, 1990), in the presence of small unilamellar vesicles, display only a moderate level of cooperativity, giving Hill coefficients smaller than those found in the micellar system. The investigation of PKC binding to the membrane of LUVs at different lipid/protein ratios, allows new light to be shed on this phenomenon.

This study on the binding of PKC to membranes was conducted on a mixture of the enzyme isoforms purified from rat brain. However, the principle rat brain isoforms of the enzyme expressed in a baculovirus system exhibit similar regulation by PS, DAG, and Ca^{2+} (Burns et al., 1990). Thus, although there may be quantitative differences among these principle isoforms, their general characteristics are well described by the mixture (Jaken & Kiley, 1987; Huang et al., 1988; Orr & Newton, 1992a,b).

EXPERIMENTAL PROCEDURES

Materials. *N*-(5-(Dimethylamino)naphthalene-1-sulfonyl)-dipalmitoyl-L-α-phosphatidylethanolamine was purchased from Molecular Probes, Eugene, OR. All other lipids were from Avanti Polar Lipids, Pelham, AL. Histone III-S, protamine sulfate, bovine serum albumin fraction V, ATP sodium salt, and EGTA were from Sigma Chemical Co., St. Louis, MO. The peptide, acetyl-Phe-Lys-Lys-Ser-Phe-Lys-Leu-amide, was a generous gift of Dr. R. E. Williams, NRC, Canada. Tris was the ultrapure grade from BRL, Gaithersburg, MD. [γ-^{32}P]ATP and [9,10-^{3}H(n)]dipalmitoylphosphatidylcholine were purchased from NEN, Montréal, Québec. All other chemicals were at least analytical grade.

PKC Purification. Rat brain PKC was purified by a modification of the procedure described by Huang et al. (1986). The phenyl-Sepharose chromatography was followed by filtration through a Diaflo (Amicon, Danvers, MA) 100-kDa-cutoff filter. The filter was then washed recurrently with small volumes of the buffer containing 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 10% glycerol, and 20 mM Tris, pH 7.5. The filtrates containing high PKC activity were pooled together and concentrated over a Diaflo 30-kDa-cutoff filter. Subsequent steps followed the published procedure (Huang et al., 1986). Purified PKC displayed a single band on silver-stained electrophoresis gels. The specific activity of the enzyme in a micellar assay was 2 μmol of phosphate transferred onto histone per minute per milligram of PKC. The phospholipid-independent activity constituted 4–5% of the total kinase activity and was subtracted from both the activity and binding results.

PKC Binding. The sucrose-loaded vesicles (SLV) method was adopted, with minor modifications, from the procedure of Rebecchi et al. (1993). Mixtures of lipids in chloroform were dried under a stream of nitrogen, subsequently evacuated under high vacuum for 2 h, and suspended in solution A containing 170 mM sucrose, 0 or 5 mM $MgCl_2$, buffered with 20 mM Tris–HCl to pH 7.0. Aliquots of 0.5 mL of 10 mM lipid suspension were subjected to 5 freeze–thaw cycles, followed by extrusion through two stacked 0.1-μm polycarbonate filters (Costar, Cambridge, MA) in a microextruder Liposofast (MM Developments, Ottawa, Ontario). The suspension of LUVs in solution A was mixed in the ratio 1:5 with solution B containing 100 mM KCl, or 5 mM $MgCl_2$, buffered with 20 mM Tris–HCl to pH 7.0. After centrifugation at 100000g for 30 min at 25 °C (Beckman TL-100 centrifuge, TLA-100.2 rotor), the supernatant was removed and the lipid pellet was resuspended in solution B. The lipid concentration was monitored by liquid scintillation counting of [^{3}H]PC which was routinely included in trace amounts in all lipid mixtures. These SLV were then suspended in a standard buffer used for the PKC binding assay: 100 mM KCl, 5 mM $MgCl_2$, 0.2 mM $CaCl_2$, 3 mg/mL BSA, buffered with 20 mM Tris to pH 7.0. The addition of BSA was necessary to prevent losses of PKC activity due to multiple transfers of the enzyme to different vials. The 750-μL suspension of LUVs at the desired concentration and 180 ng of PKC were centrifuged at 100000g for 30 min at 25 °C. Both the top 550 μL and the bottom 200 μL were each diluted to a 750-μL total volume with the standard buffer. The activity of PKC toward either protamine sulfate or histone III-S was determined using the activity assay described below. The sum of these activities was equal to the activity of PKC in the centrifuged but unseparated tube and about 10% lower than the activity of an identical sample before centrifugation. A similar decrease of activity was found in a sample stored for the same period of time at ambient temperature. The relative amount of lipid in each fraction was evaluated from the liquid scintillation counting of ^{3}H from PC. The absolute concentration of phospholipid was checked by phosphate analysis. The fraction of membrane-bound enzyme was assumed to be equal to the fraction of kinase activity associated with the SLV. The vesicles-associated kinase activity, A_v, was calculated according to the following formula:

$$A_v = \frac{\beta A_b + (\beta - 1)A_t}{\alpha + \beta - 1}$$

where A_b is kinase activity of the bottom 200 μL of suspension, A_t is kinase activity of the top 550 μL of suspension; α is the fraction of sedimented vesicles; and β is the fraction of kinase activity found in the bottom 200 μL of suspension in the absence of SLV. The parameter β accounted for the nonspecific sedimentation of PKC, which did not exceed 5% of the total kinase activity. The distribution of the enzyme after centrifugation was identical in the samples containing either no lipid or 500 μM egg PC in the form of SLV. More details are given in the Appendix.

Fluorescence energy transfer was another method used to measure PKC binding to the membrane of either small (sonicated) or large unilamellar vesicles (Bazzi & Nelsestuen, 1987a). The excitation and the emission wavelengths were 280 and 496 nm, respectively. The experiments were carried

out in the same buffer as in the SLV method but BSA was omitted. BSA significantly increased the intensity of emission at 496 nm and hence diminished the relative magnitude of emission caused by the energy transfer from the tryptophan residues on PKC. The midpoints of the binding isotherms measured in the presence of BSA were shifted by about 0.1 unit toward higher PKC/lipid ratio.

Ultrafiltration was the third technique used to study the binding of PKC to vesicles. A suspension of 1 μg/mL PKC in the standard buffer was filtered under pressure not exceeding 1 bar, through a 300-kDa-cutoff filter, in the absence of lipid or in the presence of 50 μM lipid formed as LUVs of desired composition. This ultrafiltration membrane was virtually impermeable to LUVs extruded through two stacked 0.1-μm polycarbonate filters as checked by the absence of [^{3}H]PC in the filtrate. The kinase activity toward protamine sulfate or histone was measured in the sample before filtration, as well as in the filtrate and retenate (each constitute a half of the initial volume of the sample). All samples were 10-fold diluted prior to the activity measurements. The results were corrected for the excess of PKC remaining in the retentate (30% of the free enzyme), measured in the absence of lipid or presence of 50 μM POPC in the form of LUVs.

PKC Activity Assay. The activity assay with either histone III-S or protamine sulfate as the substrates was a minor modification of that by Kaibuchi et al. (1981). The 250-μL reaction solution contained 100 mM KCl, 5 mM $MgCl_2$, 0.2 mM $CaCl_2$ or 0.5 mM EGTA, 20 mM Tris (pH 7.0), 3 mg/mL BSA, 0.2 mg/mL histone or protamine sulfate, 0–2.5 mM lipid in the form of LUVs, 20 μM [γ^{32}P]ATP (0.2 μCi/mL), and 240 ng/mL PKC (or 150 μL of centrifuged sample). The reaction was initiated by the addition of ATP, carried on for 10 min at 30 °C, and terminated by the addition of 2 mL of ice-cold 25% TCA. The samples were briefly vortexed, placed on ice for 15 min, and then filtered through GF/C Whatman filters. The filters were subsequently washed four times with 2 mL of 25% TCA, dried, and counted using efficiency-corrected Cherenkov counting. In some assays LUVs were substituted by 0.3% Triton X-100 mixed micelles (Hannun et al., 1985). In those assays KCl was eliminated from the reaction mixture and 0.5 mM DTT was added. PKC activity toward acetyl-FKKSFKL-amide was assayed using conditions identical to those for histone. The peptide concentration was 90 μM. Other details followed the published procedure (Chakravarthy et al., 1991).

The linearity of the time dependence of the reaction was checked by measuring the extent of phosphorylation after 3, 6, and 10 min. When the specific activities calculated for various reaction times differed by more than 5%, the amount of enzyme employed in the assay was appropriately reduced.

RESULTS

Effect of POPS and Diolein on PKC Binding. Figure 1 shows the binding of PKC to LUVs with different mol % POPS for two different total lipid concentrations, 500 and 50 μM, respectively. Two effects are immediately apparent. First, an inclusion of 1 mol % DG dramatically increases the affinity of PKC toward the membranes containing sufficient mol % POPS. Second, the dependence of PKC binding to the LUVs on the mol % PS is highly sigmoidal.

A striking increase of PKC affinity toward the membrane in the presence of 1 mol % DG (filled symbols) is clearly visible for both total lipid concentrations. For 15 mol % POPS and 500 μM total lipid the addition of 1 mol % DG increased the percentage of bound PKC from negligible to over 70%

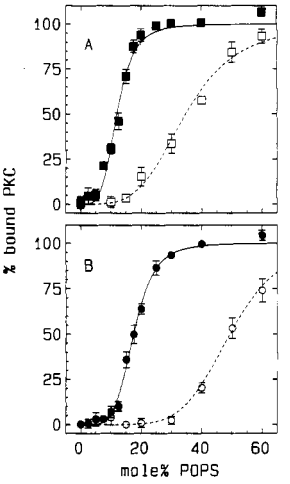

FIGURE 1: Effect of PS and DG on PKC binding to the membrane of LUVs. The binding was assessed by the SLV method. A total of 160 ng of enzyme was suspended in a solution containing 100 mM KCl, 5 mM $MgCl_2$, 0.2 mM $CaCl_2$, 3 mg/mL BSA, and 500 (A, squares) or 50 μM (B, circles) total lipid in a form of SLV, buffered by 20 mM Tris-HCl to pH 7.0. LUVs contained the indicated mol % POPS, none (open symbols) or 1 mol % DG (filled symbols), and POPC as a remainder. The presence of PKC after centrifugation in both the bottom 200 and top 550 μL was assayed with the kinase activity toward protamine sulfate. Percent of bound PKC was calculated as the ratio of the kinase activity bound to vesicles to its sum with the activity of the supernatant, as described in more detail in Experimental Procedures. Points represent an average of at least two independent experiments, each in triplicate. Bars indicate SD, shown if larger than the symbol size. Curves are a graphic representation of the best least-squares fit of the nonlinear Hill equation to the experimental data. The Hill coefficients are (A) 4.0 ± 0.3 (solid line) and 4.2 ± 0.1 (dashed line), (B) 5.1 ± 0.1 (solid line) and 6.0 ± 0.2 (dashed line).

(Figure 1A). The increase in the PKC affinity toward the membrane was equally profound at 50 μM total lipid (Figure 1B). This enhancement amounted to at least 2 orders of magnitude and hence could not be calculated directly from the curves shown in Figure 1. For the same mol % PS, inclusion of 1 mol % DG changed the fraction of bound enzyme from very little to almost 1. Therefore, we compared the total lipid concentrations of vesicles, with the same mol % POPS, which was required to bind similar fractions of PKC. The 2.5 mM 3:1 POPC/POPS (LUVs) bound about 60% of the enzyme. The addition of 1 mol % DG to LUVs of the same lipid composition (25 mol % POPS) resulted in the binding of 60% PKC to the membrane at only 5 μM lipid. Hence, for the membrane of this particular lipid composition 1 mol % DG increased the binding affinity of PKC 500 times.

We used two other techniques to assess the extent of PKC binding to lipid vesicles containing POPS. Since the alternative binding assays required an enzyme concentration 2–3 orders of magnitude higher and they were less accurate than the SLV method, we used them only for six particular compositions of lipid vesicles.

The energy transfer from tryptophan residues in PKC to a dansyl fluorophore attached to the headgroup of phosphatidylethanolamine indicates physical association of the enzyme with the membrane (Bazzi & Nelsestuen, 1987a). Small unilammellar vesicles (SUVs) composed of 10 mol % dansyl-PE, 10 or 50 mol % POPS, and POPC as the remaining lipid were titrated with PKC. Figure 2 shows that 1 mol % DG (closed symbols) significantly increased the extent of the enzyme–vesicle association in agreement with results obtained by the SLV method (Figure 1). The magnitude of DG-induced enhancement in PKC binding to SUVs measured by the

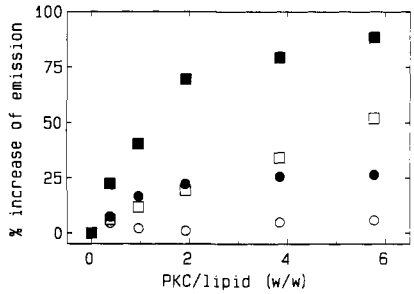

FIGURE 2: Effect of PS on DG and PKC binding to SUVs. Small unilamellar vesicles (1.5 μg total lipid) composed of 10 (circles) or 50 (squares) mol % POPS, 0 (open symbols) or 1 (closed symbols) mol % DG, 10 mol % dansyl-PE, and POPC as the remaining lipid were suspended in 200 μL of solution consisting of 100 mM KCl, 5 mM $MgCl_2$, and 0.2 mM $CaCl_2$ buffered with 20 mM Tris-HCl to pH 7.0. The increase in the fluorescence emission was measured relative to the emission of the dansyl fluorophore in the absence of PKC.

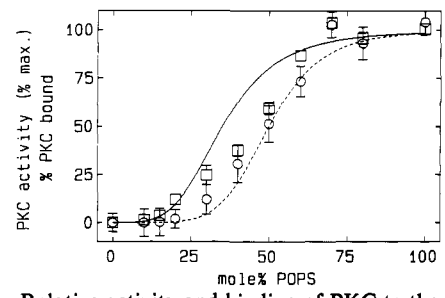

FIGURE 3: Relative activity and binding of PKC to the membrane of LUVs shown to be parallel. Total lipid concentration was 500 (squares and solid line) or 50 μM (circles and dashed line). LUVs were composed of the indicated mol % POPS with POPC as a remainder. The activity of PKC toward 200 μg/mL histone III-S was assayed in the same buffer as binding (Figure 1) with an addition of 20 μM [γ-^{32}P]ATP, 0.1 μCi/mL. The details of the activity assay are given in Experimental Procedures. Points represent mean ±SD (shown if larger than the point size) for two independent experiments, each in triplicate. The curves representing % bound PKC are the same as in Figure 1.

fluorescence energy transfer is difficult to assess. The lack of PKC binding to SUV containing 10 mol % PS (open circles) and the apparent saturation of vesicles composed of the same mol % PS but with 1 mol % DG (closed circles) are consistent with at least a 2 orders of magnitude increase in the PKC affinity toward the membrane induced by DG. Addition of EGTA (1 mM final concentration) restored the fluorescence emission to the level recorded in the absence of PKC, if the vesicles contained 10 mol % PS. In agreement with an earlier report (Bazzi & Nelsestuen, 1987a), for SUVs containing 50 mol % PS, addition of EGTA diminished the increase of the fluorescence emission to about 30% of the maximal value. When SUVs were replaced by LUVs, the apparent saturation of the PKC–membrane association was achieved at a lower PKC/lipid ratio and the maximal increase in the fluorescence emission was 27% lower than that observed for SUVs (data not shown). These observations can be explained by a larger fraction of the phospholipids being exposed on the outer leaflet of the SUV membrane than in the case of the LUV.

Another method used for comparison was filtration of PKC through 300-kDa-cutoff Diaflo ultrafiltration membranes (Amicon, MI) under a pressure of 1 bar. The enzyme was suspended in the standard buffer in the presence of 50 μM total lipid in a form of LUVs. The results obtained for LUVs of two different compositions remained in excellent quantitative agreement with those obtained by the SLV method (data not shown).

Independently of the presence of DG, the binding curves were shifted toward a higher mol % POPS when the total lipid concentration decreased from 500 to 50 μM (Figure 1A,B). For example, in the absence of DG, 50% of the PKC was bound to 500 μM phospholipid at less than 40 mol % POPS (Figure 1A, open squares) whereas 50 μM lipid required about 50 mol % POPS to bind the same fraction of PKC (Figure 1B, open circles).[2] The Hill coefficients[3] describing the sigmoidal dependence of PKC binding on the mol % POPS in the membrane ranged from 4 to 6 depending on the total concentration of lipid. Interestingly, a 10-fold decrease in total lipid concentration increased the Hill coefficient from 4.2 to 6 or from 4.0 to 5.1 with 0 or with 1 mol % DG, respectively. A possible explanation of this phenomenon is discussed below.

Activation and Binding of PKC to Membranes Are Interdependent. Most of the recent models of PKC activation assume that the enzyme may be bound to PS in the membrane but still not be active unless DAG binds to the specific binding site (Burns & Bell, 1992; Stabel & Parker, 1991; Zidovetzki & Lester, 1992; Orr & Newton, 1992a). Figure 3 shows that the PKC activity and binding dependencies on the mol % PS in the membrane of LUVs are very similar in the absence of DG. The relative activities of the enzyme toward histone (symbols) were calculated with respect to the saturation level.

In an extensive study on the kinetic properties of rat brain PKC, Hannun and Bell (1989) showed that DAG increases the catalytic rate constant of the enzyme. Our data are consistent with this finding. The addition of 1 mol % (Figure 4, filled symbols) doubled the maximal observed rate of phosphorylation, at 70 mol % POPS (saturating PS content for all cases, see Figure 3), for two different substrates: histone, a protein substrate widely used in in vitro assays, and a short peptide with high specificity toward PKC-catalyzed phosphorylation (Chakravarthy et al., 1991; Williams et al., 1992), derived from the phosphorylation site sequence of the physiological myristoylated–alanine rich-C kinase substrate (MARCKS) (Graff et al., 1991).

Comparison of the appropriate binding curves from Figure 1 (open and filled squares) with the respective activation curves (Figure 4, same symbols) shows that the activity of PKC is further stimulated by a mol % of PS above that which is required to completely bind PKC to the membrane. We attempted to correlate the efficiency of substrate phosphorylation with its binding to LUVs. However, the interaction of the peptide with LUVs was weak. The intensity of right-angle light scattering at λ = 650 nm was not affected by the presence of 90 μM peptide, indicating a lack of extensive aggregation of LUVs. A direct measurement of peptide

[2] At a given mol % PS, the PKC bound/free ratio decreased by a factor of 6–10, instead of the expected 10, when the total lipid concentration was diminished 10-fold. One of the possible explanations is the limitations of the experimental technique. The fraction of bound enzyme was measured with an accuracy of about 5% of the total enzyme concentration. A 10-fold difference in the total lipid concentration under the best possible experimental conditions would result in more than 75% and less than 25% of the enzyme binding to LUVs at the higher and lower lipid concentrations, respectively. A small, 5% systematic error increasing the fraction of bound enzyme at the lower total lipid concentration would have changed the ratio of the apparent affinities from 9 to 7.

[3] Note that the Hill coefficient is commonly used to assess the degree of cooperativity in a fractional saturation curve. Binding of a macromolecule with multiple binding sites to a membrane containing a monovalent ligand, e.g., a monovalent anionic lipid, may be, however, described by an equation very similar to that for the fractional saturation (Mosior & McLaughlin, 1992a).

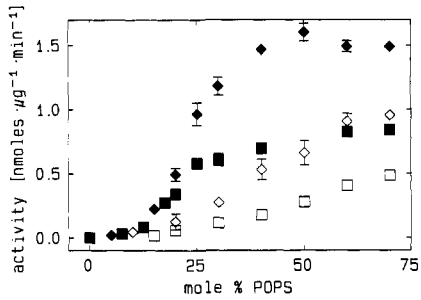

FIGURE 4: Absolute activity of PKC toward histone and acyl-FKKSFKL-amide. The substrate concentrations were 200 μg/mL for histone (squares) or 90 μM for the peptide (diamonds). LUVs were composed of the indicated mol % POPS, 0 (open symbols) or 1 mol % DG (filled symbols), with POPC as a remainder. Total lipid concentration was 500 μM. The activity was measured in the same buffer as the binding (Figure 1) with an addition of 20 μM [γ-^{32}P]-ATP, 0.1 μCi/mL. Other details of the activity assays are given in Experimental Procedures. Points represent the mean ± SD (shown if larger than the point size) for two independent experiments, each in triplicate.

binding to LUVs by a filtration assay (Kim et al., 1991) showed that no more than 10% of the peptide was bound to 3:2 POPS/POPC vesicles at the total lipid concentration of 500 μM. The presence of 90 μM peptide in the binding assay by the SLV method decreased the PKC binding to the membrane by no more than 5% (data not shown). As was reported earlier (Bazzi & Nelsestuen, 1987b,c), histone however, caused precipitation of the lipid from solution. After a brief, 1 min, centrifugation at 2000*g*, 90% of the lipid was collected at the bottom of the centrifuge tube, even when LUVs did not contain sucrose. The amount of lipid precipitated by histone was identical for vesicles containing either 30 or 60 mol % POPS. In contrast, the fraction of kinase activity left in the supernatant was about 40% and 10%, respectively. These values indicated slightly greater binding of PKC to the lipid in the presence of histone than in its absence. This somewhat surprising result cannot be easily interpreted because of the extensive aggregation of the lipid.

Although the extent of PKC activation is not always identical to the extent of binding, our results nevertheless suggest that these two phenomena are closely associated, at least for the membranes containing either PS or PS and DG.

PKC Affinity toward the Membrane Is Linearly Related to Mol % Diolein. We investigated the dependence of PKC binding to the lipid bilayer on the mol % DG. For two different mol % POPS in the membrane, a 5-fold increase in mol % DG resulted in a proportional increase of the bound/free ratio of the enzyme. For LUVs containing 10 mol % POPS, the PKC bound/free ratio increased from 0.45 ± 0.05 to 2.3 ± 0.3 when the mol % DG was raised from 1% to 5%. When the membrane contained 17.5 mol % POPS, the increase of DG from 0.2 to 1 mol % resulted in a change of the PKC bound/free ratio from 1.2 ± 0.08 to 4.8 ± 1. Data represent the mean ± SD for two independent experiments, each in triplicate. This finding is consistent with a 1:1 stoichiometry of the PKC–DG complex and saturation of the membrane-bound enzyme with DG at all studied mol % PS and DG.[4] The DAG-induced increase of the affinity of PKC to the membrane is hence likely caused by the direct binding of DAG to the enzyme rather than by an allosteric DAG-induced change of PKC affinity toward PS (Orr & Newton, 1992b).

[4] The mol % of DG employed here was at least 1 order of magnitude higher than the K_m values for DG for the three isoforms of the enzyme most abundant in a rat brain (Burns et al., 1990).

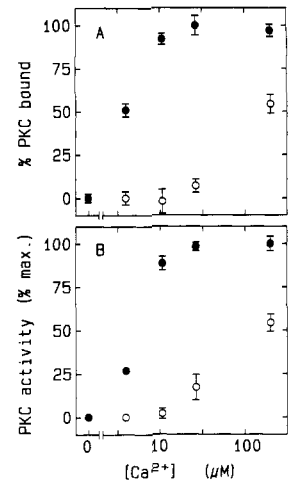

FIGURE 5: Effect of free calcium ion concentration on the PKC binding to the membrane of LUVs and its activity. (A) PKC binding to LUVs composed of 25 mol % POPS, 0 (open circles) or 1 mol % DG (filled circles). POPC constituted the remainder of 2.5 mM total phospholipid. Free calcium concentration was calculated from the known concentrations of reagents using the EGTA/Ca^{2+} system (Fabiato & Fabiato, 1981). The binding was determined in the SLV assay as described under Experimental Procedures. Points represent mean ± SD for two independent experiments, each in triplicate. (B) The relative activity of PKC toward 1 mg/mL histone with other conditions identical to those in the binding experiment (A). The activity was measured in the presence of 20 μM [γ-^{32}P]ATP as described under Experimental Procedures. A number of 52% was assigned to the PKC activity in the presence of LUVs with no DG at the highest calcium level (200 μM) since only such percentage of PKC was bound to the membrane at these conditions. Points represent mean ± SD for two independent experiments, each in triplicate.

DG increased the activity of the enzyme more significantly than it did the binding of the enzyme to the membrane (data not shown). This result is consistent with our suggested dual role for DAG in PKC activation. DAG increases the fraction of enzyme bound to the membrane (Figures 1, 2, and 5) but also increases the catalytic rate constant of PKC (Hannun & Bell, 1989).

Micromolar Concentration of Free Calcium Causes the Translocation of PKC to the Membrane Containing Physiological Amounts of POPS and Diolein. The calcium requirements for PKC binding to lipid vesicles reported by Bazzi & Nelsestuen (1987a, 1990) were above the physiologically relevant levels. We studied the effect of free calcium concentration on PKC activity and binding to 2.5 mM total lipid in the form of LUVs. This particular lipid concentration yields the surface/volume ratio encountered in a sphere of 10-μm diameter, somewhat larger than the diameter of an average cell. The vesicle membrane contained 25 mol % POPS, a percentage similar to that in the cytoplasmic leaflet of the erythrocyte membrane (Bishop & Bell, 1988), and 1 mol % of DG, also well within the physiological concentration range. Figure 5A shows that half of the enzyme binds to these LUVs at about 3 μM free Ca^{2+} if 1 mol % DG is included (filled symbols). Binding of a similar magnitude, in the absence of DG, required 2 orders higher concentration of free calcium (open symbols). The effect of calcium concentration on the PKC activity (Figure 5B) was very similar to the results obtained for binding.

This calcium-induced binding was also completely reversible. PKC was incubated for 15 min in the standard buffer containing 200 μM free Ca^{2+} in the presence of LUVs composed of 20, 79, and 1 mol % POPS, POPC, and DG, respectively (500 μM total lipid). A small volume (1% total) of concentrated EGTA solution was added to bring the chelator

Table I: Effect of Mg^{2+} on PKC Binding to the Membrane[a]

$[Mg^{2+}]$ (mM)	PKC bound/free	$[Ca^{2+}]_0$ (mM)	$\Psi(0)$ (mV)	$[PS]_{free}$ (%)
5.0	1.8 ± 0.2	1.5	−26	69
0.5	7.7 ± 0.4	7.0	−46	73

[a] The binding was measured by the SLV method as described in Experimental Procedures. LUVs were composed of 2:3 POPS/POPC mixture (0.5 mM total lipid). The concentration of Ca^{2+} in the bulk solution was 200 μM. Abbreviations and symbols: $[Mg^{2+}]$, concentration of Mg^{2+} in bulk solution; $[Ca^{2+}]_0$, concentration of Ca^{2+} at the membrane surface; $\Psi(0)$, electrostatic surface potential; $[PS]_{free}$, relative concentration of free PS in the membrane.

concentration to 0.5 mM in one aliquot of the sample. The other one served as a control. After 15 min, both samples were centrifuged and assayed in our standard SLV binding assay. No bound PKC was detected in the sample containing EGTA. In the control sample, as expected, about 90% of the enzyme was bound to the vesicles.

A 5-fold decrease in the total lipid concentration decreased the PKC bound/free ratio by the same factor of 5 at all calcium ion concentrations (data not shown). As reported earlier by Bazzi and Nelsesteuen (1990), an increase of POPS from the 25 mol % Figure 5 to 50 mol %, significantly increased the percentage of the enzyme bound to the membrane at the same total lipid and calcium ion concentrations (data not shown).

Mg^{2+} Decreases the Binding of PKC to the Membrane. Since the activity assay required the presence of 5 mM Mg^{2+}, it was also included in all binding experiments to allow a direct comparison between the PKC binding to the membrane and its activity. Earlier observations of the Mg^{2+} effect on the PKC binding to cellular membranes were contradictory (Wolf et al., 1985a,b; Sactor & Schwartz, 1990). A 10-fold decrease in the concentration of Mg^{2+} increased the PKC bound/free ratio 4-fold (Table I) in the presence of 500 μM total lipid, 3:2 POPC/POPS (LUVs). Mg^{2+} binds weakly to both PS and PC, with association constants of 10 and 2 M^{-1}, respectively (McLaughlin et al., 1981). The Gouy–Chapman–Stern theory of electrostatic potential of the membrane combined with the Boltzmann relation (McLaughlin, 1989) allows the calculation of the fraction of free anionic lipid, the surface potential of the membrane, and the concentration of cations at the interface. These theoretically calculated data are included in Table I. The binding of divalent cations to PS decreases the free fraction of this lipid available for PKC only slightly.[5] However, the magnitude of the surface potential is reduced and the change of the bound/free ratio of PKC correlates well with the calculated concentration of Ca^{2+} at the interface (Table I). Since the extent of PKC binding to the membrane depends on the free calcium concentration (Figure 5A), the surface potential-dependent increase in the local calcium concentration is likely to account for the stronger binding to PKC to the membrane at the lower concentration of magnesium ion.[6]

Dansylphosphatidylethanolamine Supports Both the Binding and Activity of PKC. PKC was reported to bind equally well to membranes composed of monovalent anionic lipids like PS, PA, PG, PI, and CL (Bazzi & Nelsestuen, 1987a; Rodriguez-Paris et al., 1989). A certain degree of lipid specificity was observed in PKC binding to mixed Triton X-100 micelles (Lee & Bell, 1989). Dansyl-PE is an anionic lipid used in the fluorescence energy transfer technique to study PKC binding to the lipid bilayer (Bazzi & Nelsestuen, 1987a, 1990; Orr & Newton, 1992a,b). In agreement with an earlier report (Bazzi & Nelsestuen, 1987a), at the total lipid concentration used in this kind of experiment (about 10 μM), PKC did not bind to membranes containing 10 mol % dansyl-PE and PC as the remainder. However, PKC also does not bind to membranes containing 10 mol % PS at the total lipid concentration similar to, or even higher than, that used in the energy transfer assay (Figure 1B). Figure 6 demonstrates that the membranes containing 1 mol % DG and the same mol % PS (curves) or dansyl-PE (filled symbols) bind PKC equally well at the same total lipid concentration. In the absence of DG, PKC binding to dansyl-PE (Figure 6, open symbols) or to PS containing LUVs (Figure 1A,B, open symbols) decreased to a negligible level at identical total lipid concentrations. Also, equimolar mixtures of dansyl-PE and PS exert the same effect on PKC binding as the equivalent mol % PS (data not shown).

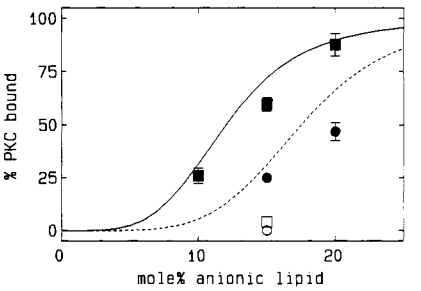

FIGURE 6: PKC binding to the membranes containing dansyl-PE. The SLV were composed of the indicated mol % dansyl-PE (open symbols) or dansyl-PE with 1 mol % DG (filled symbols), with POPC as the remainder. Total lipid concentration was 500 (squares) or 50 μM (circles). The details of the binding assay are given in Experimental Procedures. Points represent the mean ± SD for two independent experiments, each in triplicate. Dipalmitoyl-dansyl-PE from Molecular Probes (Eugene, OR) and dansyl-PE made by transphosphatidylation of egg PC (Avanti, Pelham, AL) yielded identical results in the limits of experimental error. The data presented are the average for both types of dansyl-PE. The curves, which represent the binding of PKC to LUVs containing POPS at 500 (solid) or 50 μM (dashed), instead of dansyl-PE at otherwise identical conditions, were taken from parts A and B of Figure 1, respectively.

Dansyl-PE also supported the activity of PKC to a level close to that obtained with PS and higher than that observed with two other anionic lipids, PG and PA (Figure 7A). The micelle assay displays much higher lipid specificity for PKC activation (Lee & Bell, 1989; Burns et al., 1990). In agreement with these reports, the activity of PKC supported by PS was 5–10 times higher than that in the presence of other anionic lipids when the mixed micelle assay was employed (Figure 7B). Dansyl-PE, however, still supported the activity to a greater extent than PA or PG.

DISCUSSION

Effect of Diolein on PKC Binding to Membranes. Our results show that the binding of PKC to DG in the membrane contributes significantly to the total affinity of the enzyme toward membranes. The contribution of the DG–PKC interaction to the overall binding energy is sufficient to cause translocation of the enzyme to the membrane at micromolar calcium concentrations and physiologically relevant mol %

[5] Although the fraction of free PS in the membrane decreased by no more than a couple of percent, the presence of multiple binding sites on PKC for PS (Hannun et al., 1985; Hannun & Bell, 1986; Newton & Koshland, 1989) may amplify the effect of this small change on the enzyme binding to the membrane (Mosior & McLaughlin, 1992a).

[6] A similar effect of Mg^{2+} was found for Ca^{2+}-dependent exocytosis. McLaughlin and Whitaker (1988) showed that the level of Ca^{2+}-dependent exocytosis in the sea urchin egg membrane was parallel to the magnitude of the surface potential of the membrane and was regulated by the concentration of magnesium or other polyvalent cations.

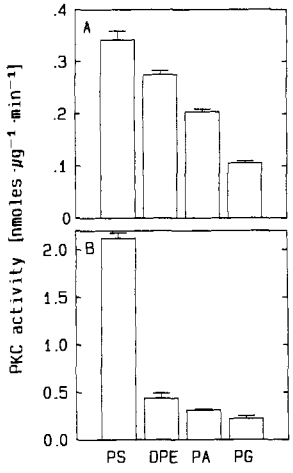

FIGURE 7: Anionic lipid specificity for the activation of PKC. Both vesicles (A) and Triton X-100 mixed micelles (B) contained 20 mol % of indicated anionic lipid and 1 mol % DG. The total lipid concentration was 500 μM (A). The concentration of Triton X-100 was 0.3% (B). KCl was excluded from the activity assay in the presence of mixed micelles. The histone III-S concentrations were 0.2 (A) and 1 mg/mL (B). Other assay conditions were identical to those described in Experimental Procedures. Bars represent mean ± SD for two independent experiments, each in triplicate.

POPS and DG as well as total lipid concentration. Three experiments, the dependencies of PKC binding to the membrane on the presence of DG (Figure 1) and free calcium ion concentration (Figure 5A) as well as the direct comparison of the total lipid concentrations required to bind the same fraction of the enzyme in the absence or presence of DG, are consistent with at least 2 orders of magnitude increase in PKC affinity toward the membrane caused by 1 mol % DG. This increase was proportional to the mol % DG in the membrane.

In the presence of Ca^{2+}, either DAG or phorbol ester induces the translocation of PKC to the cell membrane (Kraft & Anderson, 1983; Sactor & Schwartz, 1990; Crabos et al., 1991; Diaz-Laviada et al., 1990; Leach et al., 1991). Wolf et al. (1985a,b) showed an increase in the amount of PKC bound to inside-out erythrocyte membrane vesicles induced by phorbol ester. Bazzi and Nelsestuen (1987a) pointed out, however, that those data were consistent with the existence of two populations of PKC, one of which was most likely irreversibly bound to the membrane in a phorbol ester-dependent process (Bazzi & Nelsestuen, 1988). A detailed study on PKC binding to SUVs showed that DAG had no effect on this process (Bazzi & Nelsestuen, 1987a). This result is contradictory to our findings, and we cannot offer a satisfactory explanation for that difference. We observed the effect of DG on PKC binding to the membrane of either LUVs or SUVs, at very low (Figure 1) and high PKC/phospholipid ratios (Figure 2). Our results are, however, consistent with a recent finding by Orr and Newton (1992a) that DAG increases the binding of PKC to PS-containing mixed micelles. The observation that the binding of PKC to the mixed micelles required DAG was also published earlier (Lee & Bell, 1989).

Affinity of PKC toward PS. The dependence of PKC binding to lipid bilayers or to mixed micelles on the mol % PS and DAG, as well as on the Ca^{2+}, does not allow the determination of a unique dissociation constant for the formation of a PKC–PS complex. PKC binding to the membrane or its activation also depend on the ionic strength of the experimental buffer, as well as on cation binding to anionic lipids or nonspecific partitioning into the lipid bilayer (Mori et al., 1980; Bazzi & Nelsestuen, 1987a,b,c; Hannun & Bell, 1989; Trudell et al., 1989), all of which affect the electrostatic potential of the membrane (McLaughlin, 1989).

Bazzi and Nelsesteuen (1987a) estimated that the dissociation constant of PKC toward membranes is lower than 5 nM, since even at 5 mol % PS in the membrane and micromolar concentrations of lipid the enzyme appeared to bind either with the complete saturation of acidic lipid or with steric saturation of the membrane. In our experiments, little bound enzyme was observed at this mol % PS (Figure 1), despite a huge excess of lipid over the protein (molar ratio of 10^5 or equivalently 1 enzyme per 2 vesicles). The contradiction is, however, only apparent. As we have shown, dansyl-PE present in the membrane when the binding is measured by the fluorescence energy transfer (Bazzi & Nelsestuen, 1987a) binds PKC equally well as does PS (Figure 6). Furthermore, a 10-fold higher concentration of DG than that used in our experiments, as well as the absence of Mg^{2+} in the fluorescence energy transfer binding assay (the experimental conditions of Bazzi & Nelsestuen (1987a), increases the apparent binding constant of PKC toward the membrane by 2 orders of magnitude. Indeed, the SLV binding assay showed that over 90% PKC was bound to the membrane composed of 5 mol % POPS, 10 mol % dansyl-PE, 10 mol % DG, and 75 mol % POPC at the total lipid concentration as low as 5 μM with 200 μM Ca^{2+}.

Coincidence of PKC Binding to the Lipid Bilayer and Its Activity. At almost all experimental conditions used in this study, the membrane-bound form of the PKC was able to phosphorylate its substrates. In the absence of DG, PKC activity was strictly parallel to the fraction of the enzyme bound to the membrane (Figure 2). Also, the calcium dependencies of both the binding to the membrane and activity were very similar (Figure 5). In the presence of DG, the activity and binding to the membrane were not as well correlated (compare filled squares in Figure 1A with either filled diamonds or squares in Figure 4); specifically, little activity was observed when the mol % PS was below 15, whereas a considerable fraction of the enzyme was bound to vesicles at the same condition. On the other hand, at 30 mol % PS and higher, the enzyme was bound completely but a significant increase of PKC activity occurred at higher PS levels. The simplest explanation is that the substrate binding to the membrane depends on the mol % anionic lipid. Binding of a substrate to the membrane is a prerequisite for its efficient phosphorylation by PKC (Bazzi & Nelsestuen, 1987b,c). Basic peptides, including peptides that mimic the PKC pseudosubstrate region, bind to membranes with a highly sigmoidal dependence on the mol % anionic lipid (Mosior & McLaughlin, 1991, 1992b). Hence, if the affinity of the substrate for anionic lipids is weaker than that of PKC, the dependence of activity on the mol % PS could reflect the binding of the substrate to the membrane rather than that of the enzyme. We were not able to confirm this phenomenon in a direct experiment. Under the conditions used in the activity assay, histone induced vesicle aggregation whereas the synthetic peptide bound to the membrane too weakly. Our findings are consistent with those by Orr and Newton (1992a), who observed that the activation of PKC and its binding to Triton X-100 micelles were parallel.

We found that very similar concentrations of calcium ion were required for both the enzyme activation and its binding to the membrane (Figure 5). Wolf et al. (1985a,b) found that PKC bound to the membrane of inside-out erythrocyte membrane vesicles at a concentration at least 1 order of magnitude lower than that required for the phosphorylation of its substrates on the membrane. However, different substrates of PKC have different requirements for calcium as

an activator (Bazzi & Nelsestuen, 1987b).

Cooperativity. Binding of PKC to the membrane of LUV exhibits a highly sigmoidal dependence on the mol % anionic lipid (Figures 1 and 6). Orr and Newton (1992a,b) recently reported an identical finding for the PKC binding to Triton X-100 micelles containing either PS or PA. The Hill coefficients found in both systems are similar and are in the range of 4–7 (Orr & Newton, 1992a,b). This sigmoidal dependence of binding and a similar sigmoidal dependence of PKC activation on the mol % PS (Hannun et al., 1985; Newton & Koshland, 1989, 1990; Burns et al., 1990) were interpreted as a consequence of the cooperative sequestering of PS molecules by the enzyme (Newton & Koshland, 1989; Orr & Newton, 1992a,b). Evidence for the existence of this phenomenon was recently provided by Orr and Newton (1992a). However, McLaughlin and co-workers demonstrated, in a series of recent papers, that basic peptides of very simple structure also bind to the membrane with a highly sigmoidal dependence on the mol % anionic lipid (Kim et al., 1991; Mosior & McLaughlin, 1991, 1992b). They interpreted this phenomenon as a consequence of the nonspecific accumulation of charged molecules at the interface by the surface potential and the change of dimensionality (from 3D solution to 2D membrane surface). One of the predictions of the model employed in the description of these phenomena was the dependence of the apparent cooperativity, as measured by the Hill coefficient, on the total lipid concentration (Mosior & McLaughlin, 1992a). A decrease in the total lipid concentration should increase the Hill coefficient, calculated for the dependence of either the fractional saturation or the binding on the mol % anionic lipid. The decrease of total lipid concentration from 500 to 50 μM increased the Hill coefficient from 4.2 to 6.0 for the dependence of % of PKC bound to the membrane on the mol % PS. This increase suggests that the phenomena responsible for the appearance of the apparent cooperativity in binding of simple peptides to anionic lipids (Kim et al., 1992; Mosior & McLaughlin, 1991, 1992a,b) also play some role in the sigmoidal dependence of PKC binding to the membrane and the dependence of its activity on the mol % anionic lipid.

There is no direct evidence that the positively charged regions of the enzyme interact with the electrostatic potential of the membrane produced by anionic lipid as was proposed (Mosior & McLaughlin, 1991). However, the concentration of calcium in the layer immediately adjacent to the membrane depends dramatically on the surface charge of the membrane due to nonspecific accumulation of cations by the surface potential (McLaughlin, 1989). Dissociation of PKC from the membrane induced by Mg^{2+} (Sactor & Schwartz, 1990; Table I) and the dependence of PKC binding on the Ca^{2+} concentration suggest that the electrostatic potential of the membrane is likely to contribute to the sigmoidal dependence of binding on the mol % anionic lipid through the greater nonspecific accumulation of Ca^{2+} at the interface at the higher mole fraction of PS.

Model of PKC Activation by PS, DAG, and Ca^{2+}. Most of the recently proposed models of PKC activation separate the binding of PKC to the membrane from its activation (Burns & Bell, 1992; Stabel & Parker, 1991; Zidovetzki & Lester, 1992; Orr & Newton, 1992a). The enzyme is assumed to be activated by the binding of DAG or phorbol ester to the membrane-bound PKC. However, PKC is active when bound to membranes containing PS and no DG (Figures 3 and 4). Our results suggest a dual role for DAG (and phorbol ester by implication). DAG increases the apparent binding constant of the enzyme to the membrane by 2–3 orders of magnitude (proportionally to its mol %) (Figures 1 and 5). In addition, as was shown before (Hannun & Bell, 1989), DAG also increases the catalytic rate constant of phosphorylation, which in our experiments resulted in a 2-fold increase of the maximal rate of phosphorylation in the presence of 1 mol % DG.

PS, DAG, and Ca^{2+} bind to separate sites on PKC (Bell & Burns, 1991; Burns & Bell, 1992). PS and DAG binding sites are located in close proximity on the C1 region of the regulatory domain of the enzyme (Burns & Bell, 1991). The Ca^{2+} requirements of structurally different isoforms of PKC (Nishizuka, 1988; Kikkawa et al., 1989), as well as that of different regions of the enzyme expressed in the baculovirus system (Burns & Bell, 1991), suggest that the binding site(s) for Ca^{2+} are located at the carboxyl end of the C2 domain. The synergistic effects of PS, DAG, and Ca^{2+} seen in both the activation (Nishizuka, 1988; Hannun & Bell, 1986; Huang, 1989) and the binding of the enzyme to a lipid membrane (or micelle) (Orr & Newton, 1992a,b) imply that despite separate binding sites, all of these activators and cofactors exert the same effect on the enzyme; i.e., they change its conformation from an inactive to an active state. This occurs simultaneously with the binding of PKC to the membrane. The transition to the active state presumably requires a removal of the pseudosubstrate domain from the substrate binding site (House & Kemp, 1987; Makowske & Rosen, 1989). The highly basic pseudosubstrate region may bind to acidic lipids, contributing to the overall enzyme–membrane binding energy and stabilizing the active form of the enzyme (Mosior & McLaughlin, 1991). Indeed, Orr et al. (1992) have recently shown that the binding of PKC to PS and DAG caused a conformational change in the enzyme exposing the pseudosubstrate region to proteolytic cleavage.

At physiological concentrations of the activators, cofactors, and substrate, a combined energy of their interactions is necessary to activate the enzyme. However, at a high mol % PS in the membrane either DAG (Bazzi & Nelsestuen, 1987a; Figure 3) or Ca^{2+} (Epand et al., 1992) are dispensable. In the presence of some substrates neither DAG nor Ca^{2+} is required for activation (Bazzi & Nelsestuen, 1987b). Interestingly, in such cases DAG still slightly increased the activity of PKC. Also, the irreversibly bound form of PKC is active in the absence of DAG (phorbol ester) and Ca^{2+} and this activity is augmented about 2-fold by phorbol ester (Bazzi & Nelsestuen, 1988). Both of these findings are in agreement with our observation that 1 mol % DG increases the maximal speed of phosphorylation by a factor of 2 when the enzyme is completely bound to the membrane (Figure 4), presumably due to the DAG-induced increase of the catalytic rate constant (Hannun & Bell, 1989). Although the mechanism of this effect is not known, it may be related to the fact that DAG is a hexagonal phase promoter (Epand, 1985) and that the other hexagonal phase promoters also increase the maximal speed of phosphorylation catalyzed by PKC whereas the membrane "stabilizers" exert the opposite effect (Epand, 1987; Epand & Lester, 1990).

Our study on PKC binding to a membrane was limited to the DAG- and Ca^{2+}-dependent case, reversed by chelation of Ca^{2+}. Since the levels of DAG and Ca^{2+} required for the PKC activation and binding to the membrane found in this study occur in a cell only transiently, the model of PKC activation proposed above may only account for the short-term response of PKC to an external stimulus. Such a mode of PKC regulation was indeed found, for example, in *Pheochromocytoma* cells (Heasley & Johnson, 1989).

CONCLUSIONS

Diacylglycerol plays two distinct roles in the activation of PKC. Physiologically relevant concentrations of diolein increase the affinity of PKC to the membrane by 2–3 orders of magnitude. This strong effect allows an association of the enzyme with the membrane containing low mol % PS at micromolar concentrations of Ca^{2+}. DG also increased the maximal rate of phosphorylation catalyzed by the membrane-bound PKC. The activity of the enzyme was closely associated with the extent of PKC binding to the membrane containing PS or PS and DG. However, the role of substrate in the regulation of PKC activity requires further study. The increase of the Hill coefficient for the PS dependence of PKC binding to the membrane found for the decreasing concentration of the total lipid is consistent with the apparent cooperativity in binding being due, at least in part, to a reduction of the dimensionality of the enzyme–lipid complex. The affinity of PKC toward the membrane depends on the Ca^{2+} concentration at the lipid/solution interface. The latter depends approximately exponentially on the mol % PS in the membrane due to a nonspecific accumulation of cations at the membrane surface by the electrostatic potential produced by PS.

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APPENDIX

Binding Assay. We studied binding of PKC to LUVs composed of POPS, POPC, and DG. Our principle binding assay was a modification of a procedure used by Rebecchi et al. (1993) employing SLV to study the interaction of the phospholipase $C\delta_1$ with LUVs. The duration and speed of centrifugation of vesicles in the presence of PKC was limited by the stability of the enzyme and by the rate of sedimentation of the enzyme alone. The loss of PKC activity toward histone due to sedimentation (30 min) and other operations carried out at ambient temperature (30 min) was less than 10%.

The efficiency of sedimentation of SLVs depended on their composition. For 30 mol % POPS (and higher), more than 98% of the vesicles were sedimented under the conditions used in the binding assay. However, the LUVs consisting of 100% POPC, only about 80% were spun down under the same conditions. Substitution of POPC by egg PC improved the sedimentation efficiency to 90%. Addition of 5 mol % DG to vesicles containing 25 mol % POPS (POPC constituted the remainder) decreased the sedimentation from 96% (no DG) to 88%. Resuspension of the SLV in the standard 100 mM KCl buffer used throughout this study was complete as judged from the recovery of ^3H-labeled phosphatidylcholine. The presence of 5 mM $MgCl_2$ in the buffer caused, however, aggregation of vesicles containing 70 mol % or more POPS, as was observed by others (Boni & Rando, 1985). As a consequence our binding study was limited to membranes containing 60 mol % POPS or less. Vesicle aggregation was monitored with right-angle light scattering at 600 nm and negative stain electron microscopy. The sucrose-loaded LUVs resuspended in the standard solution after centrifugation had a size distribution similar to that of those which had been freshly extruded, as determined using electron microscopy with negative staining. Addition of PKC did not cause aggregation of vesicles or change their size distribution.

If the activity of the enzyme toward histone in the presence of LUVs was significantly higher than the basal activity (no lipid present), the kinase activity of both fractions toward histone was also determined. The relative activities of the bottom and top fractions after centrifugation were identical for both histone and protamine sulfate, providing that the concentrations of lipids in the enzyme assay were the same.

All binding experiments were conducted at equilibrium. Incubation of a suspension of PKC and SLVs for 30 min prior to separation by centrifugation did not change the extent of the enzyme binding to the vesicles. In the control experiments using ultrafiltration, the separation of the free and membrane-bound enzyme took only 5 min, yet the results were identical to that obtained after the 30-min–1-h-long separation by the centrifugation. Also, in the fluorescence energy transfer experiments the increase of the fluorescence emission was stable with time.

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