Contributions to Maxima in Protein Kinase C Activation*

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In many lipid systems, the activity of protein kinase C (PKC) exhibits a peak followed by a decline as the mol % of one component is increased. In these systems, an increase in one lipid component is always at the expense of another or accompanied by a change in total lipid concentration. Here we report that in saturated phosphatidylserine (PS)/phosphatidylcholine (PC)/diacylglycerol (DAG) mixtures, increasing PS or DAG at the expense of PC revealed an optimal mol % PS, dependent on mol % DAG, with higher mol % PS diminishing activity. The decrease at high mol % PS is probably not attributable simply to more gel-phase lipid due to the higher melting temperature of saturated PS versus PC because a similar peak in activity occurred in unsaturated lipid systems. Increasing the total lipid concentration at suboptimal mol % PS provided the same activity as higher mol % PS at lower total lipid concentration. However, at optimal mol % PS, activity increased and then decreased as a function of total lipid concentration.

PKC autophosphorylation also exhibited an optimum as a function of mol % PS, and increasing the PKC concentration increased the mol % PS at which activity decreased, both for autophosphorylation and for heterologous phosphorylation. Formation of two-dimensional crystals of PKC on lipid monolayers also exhibited a peak as a function of mol % PS, and the unit cell size of the crystals formed shifts from 50 × 50 Å at low mol % PS to 75 × 75 Å at higher PS. Collectively, these data suggest the existence of optimal lipid compositions for PKC activation, with increased quantity of these domains serving to dilute out enzyme-substrate aggregates and/or enzyme-enzyme aggregates on the lipid surface.

Protein kinase C (PKC)1 (1), which constitutes a family of structurally related kinases, is defined by the phospholipid dependence of its activity, with all isoforms activated by acidic phospholipids, preferably phosphatidylserine (PS), and all but PKCα and PKCα/γ further stimulated by diacylglycerols (DAG) or phorbol esters. The originally identified α, β, and γ family members also require calcium binding to the C2 domain, which is distinct in these isoforms (for reviews, see Refs. 2–4). Although the role of some lipid components is becoming better defined, variable results in different systems leave many questions unanswered.

Structural analyses of DAG and phorbol ester analogues (5–7) and analysis of PKC deletions and mutations argue for specific phorbol ester and DAG binding to the Cys-rich, zinc-containing C1 domain (8–11). X-ray fluorescence (12), NMR (13), and x-ray crystallography (14) have revealed the structure of this domain. Binding of phorbol ester does not alter the conformation, but helps to present a hydrophobic surface on the domain that could facilitate insertion into the membrane to a depth of 7–8 Å (14). The two versions of the C1 domain, C1A and C1B, present in most PKC isoforms have structural differences (reviewed in Ref. 15) that may account for greatly differing affinities of fluorescent phorbol esters for the two sites (16).

Comparison of PS analogues supports a requirement for the 1,2-sn-1-PS head group for PKC activation (17), although other acidic lipids can substitute for part of the PS requirement (18–20). However, a specific PS-binding site on the enzyme has not yet been identified, and the requirement for acidic lipids can be bypassed with short chain phosphatidylcholine (PC) micelles (21) or PC/phosphatidylethanolamine systems at high calcium (22).

Activation of PKC by PC micelles, but not PC vesicles (21), and greatly enhanced activity with unsaturated saturated phospholipids (23) emphasized the importance of physical properties of the membrane as well as specific DAG and PS structures. Physical properties that have been considered include fluidity, head group spacing, surface curvature, and liquid crystalline versus gel versus hexagonal II (HexII) phase of the lipids (21, 23–26).

Some of the classical PKC activators as well as additional lipids and lipid-soluble molecules that can contribute to PKC activation increase activity up to a maximum and decrease activity with further increase of the additive. These include short chain PCs (21) and short chain PSs (27), lysophospholipids (28), steroids such as tamoxifen (29) and bile acids (30), and free fatty acids (31). In saturated phospholipid systems, a maximum is observed as a function of DAG (32, 33), suggesting several hypotheses having to do with the coexistence of compositionally distinct domains and/or interface regions in addition to alterations in mol % PS. Goldberg and Zidovetski (31) observed similar maxima as a function of DAG in unsaturated systems and noted a correlation between increasing activity and increasing frustration of the bilayer, but decreasing activity as bilayers were disrupted to form a HexII phase.

All of these lipid systems are complex, and the increase of one component is always at the expense of another or accompanied by a change in total lipid concentration. Here we show that activity also can decrease as mol % PS is increased beyond maximally effective concentrations, but that the mol % PS for maximal activity depends on the total lipid concentration and enzyme concentration. The unit cell size of PKC two-dimensional crystals changes in a corresponding manner as a function of mol % PS in lipid monolayers.
FIG. 1. Dependence of PKCα activation on mol % DMPS and DO in DMPC/DMPS/DO system. Phosphorylation of histone by PKCα was measured as described under "Experimental Procedures" with the indicated molar ratios of DMPC/DMPS/DO at a final concentration of 1 mM total lipid. The PKC concentration was 4 nM. Points represent means ± S.E. of duplicate determinations from a representative of six independent experiments.

EXPERIMENTAL PROCEDURES

Materials—All lipids (dimyristoylphosphatidylcholine (DMPC), dimyristoylphosphatidylerine (DMPS), dioleoylphosphatidylethanolamine (DOPE), dioleoylphosphatidylcholine (DOPC), and dioleoylphosphatidylserine (DOPE), and dioleoylphosphatidylcholine (DOPC), and dioleoylphosphatidylserine (DOPE)) were from Avanti Polar Lipids (Alabaster, AL). [γ-32P]ATP (7000 Ci/mM) was from ICN Pharmaceuticals (Costa Mesa, CA) and adjusted to a specific activity of 0.75–1.25 mCi/mmol for kinase assays. [3H]Phorbol 12,13-dibutyrate (19.6 Ci/mmol) was from NEN Life Science Products. Fetal calf serum and Grace’s insect medium were from Life Technologies, Inc., and yeastolate and lactalbumin were from Difco. Polyclonal antibodies to C-terminal epitopes in PKCα or PKCδ were from Santa Cruz Biotechnology (Santa Cruz, CA). Histone (type III-S), ATP, phorbol esters, and leupeptin were from Sigma. Organic solvents (chloroform, methanol, benzene, and hexane) were high performance liquid chromatography-grade and were obtained from Fisher. All other materials were reagent-grade and were obtained from Sigma, Fisher, or Calbiochem.

Peptide substrates for PKC corresponding to the pseudosubstrate sequences for PKCα or PKCγ, but with S replacing A, were synthesized in the Biomolecular Research Facility at the University of Virginia. The sequences were as follows: ε peptide, KRKQGSSVRVPH; and η peptide, RRKQHSRRVVH.

Lipid Preparations—Multilamellar vesicles of the various lipid mixtures were prepared from stock solutions in CHCl3, by evaporating the solvent under a stream of nitrogen gas, resuspending the mixture in hexane, and lyophilizing under vacuum overnight. Lipid purity was periodically confirmed by thin-layer chromatography, and concentrations of phospholipids were confirmed by phosphate assay (34).

PKC Purification and Assay—PKCs and PKCδ were expressed in SF9 cells from baculovirus constructs generously provided by Drs. Parker, Stabel, and Fabbrö. The enzymes were purified by fast performance liquid chromatography through Q-Sepharose and phenyl-Superose columns (Pharmacia Biotech, Piscataway, NJ) as described previously (28, 35). Purity was confirmed by polyacrylamide gel electrophoresis followed by silver staining and by Western blotting with isotype-specific antibodies. Enzyme concentration was determined by binding of [3H]phorbol 12,13-dibutyrate as described previously (36).

PKC activity was determined by its ability to transfer phosphate from [γ-32P]ATP either to lysine-rich histone or to substrate peptides corresponding to the pseudosubstrate sequences of PKCα or PKCγ with S-for-A replacements. Reactions were carried out in 75 μl containing substrate (0.2 mg/ml histone or 30 μM peptide), 5 mM MgCl2, 40 μM [γ-32P]ATP (10–20 mCi/mM), 20 mM MOPS (pH 7.4 at 30 °C), 0.8–4 nM PKC isozyme as indicated, and PC/PS/DO mixtures of varying compositions and final concentrations (0.10–10 μM) as indicated. Reactions were terminated after 3 or 5 min at 30 °C by spotting 50 or 60 μl onto P-81 ion exchange paper (Whatman). Papers were washed three times in 50 mM NaCl to remove unreacted ATP and dried. Bound 32P was determined by Cerenkov counting.

Autophosphorylation of PKC was carried out under similar conditions, except that the specific activity of the ATP was higher (100 mCi/mM), and reactions were stopped by addition of boiling SDS-containing sample buffer. 32P incorporation into PKC was determined by autoradiography after polyacrylamide gel electrophoresis using X-1 film. Several exposures were obtained to ensure linearity of detection, and autophosphorylation was quantitated by densitometry.

Growth and Analysis of Two-dimensional Crystals of PKC on Lipid Monolayers—Two-dimensional crystals of PKCδ were grown by depositing 25-μl aliquots containing 0.28 μM PKCδ in 20 mM MOPS (pH 7.4) into 30-μl Teflon wells and overlaying the solution with 1 μl of DOPC/DOPS/DO (95:5:5 molar ratio; 2 mM/g total lipid in CHCl3/hexane (1:1 by volume)) as described previously (37). After 1 h at 20 °C, monolayers were collected onto carbon-coated electron micrograph grids, stained with 1% uranyl acetate, and examined using a JEOL JEM 100× electron microscope (37). Electron micrographs were recorded on Kodak 4489 film. Grids were scored for total number of two-dimensional crystals formed and for the % of small (50 × 50-A unit cell size) and large (75 × 75-A unit cell size) crystals formed.

RESULTS

Recent experiments in a DMPC/DMPS/DAG system revealed a peak in PKC activity at DAG mol fractions that corresponded to a mixture of compositionally distinct lipid domains as determined by differential scanning calorimetry (32, 33). Potential explanations for the decrease in activity at high mol % DAG included (a) some requirement for interface regions between domains that would be maximal under maximal domain coexistence conditions; (b) a possible dilution of PKC multimers or PKC-substrate aggregates as an optimal domain is increased; and (c) the decrease in mol % PS that would occur as DAG was increased at the expense of PC and PS in those studies.

To examine the effect of varying the mol % PS, DMPS was varied at the expense of DMPC at several DO mol fractions. As shown in Fig. 1, PKCα activity was maximal at 60% DMPC with 15 mol % DO and then rapidly fell as PS was increased to 85%. When the mol % DO was higher, less DMPS was required to reach maximal activity (35 mol % DMPS with 25 mol % DO; ≤15 mol % DMPS with 35 mol % DO), but activity also decreased at lower mol % DMPS.

Since DMPS has a higher Tm than does DMPC (−39 °C (38) versus −24 °C (39)), increasing the mol % DMPS will increase the amount of lipid in gel versus liquid crystalline phase at the temperature of the PKC assays (30 °C). In an attempt to eliminate concerns about gel-phase domains, a similar experiment with varying mol % PS was conducted in the unsaturated system DOPC/DOPS/DO. As shown in Fig. 2, maximal histone phosphorylation by PKCα occurred at 40–60 mol % DOPS with 2 mol % DO and at 20% DOPS with 20% DO. A similar peak in activity occurred at 60 mol % DOPS in the absence of...
DO, but the maximal activity was only about half that with 2% DO. Some activity of PKC α in the absence of DAG has been observed previously in short chain PSs (27) and in unsaturated PC/PS vesicle systems with fatty acids (31). Very similar results were observed with PKC δ and a peptide substrate. As in the saturated system, maximal activity occurred at lower mol % PS (20%) with higher mol % DAG (20%). PKC δ activity, however, was completely DAG-dependent in this system. Since DOPS and DOPC both have T_m values well below 30 °C (40, 41), the decrease in activity at high mol % DOPS cannot be attributed to formation of gel-phase domains.

If suboptimal PKC activity at very low or very high mol % PS is due to insufficient amounts of an activating lipid domain(s) (including an interface as a potentially relevant domain), then increasing the total lipid concentration should increase the amount of the relevant domain(s) even at suboptimal compositions. As shown in Fig. 3, increasing the total lipid concentration at the lower mol fractions of PS did ultimately generate PKC δ activity similar to that achieved at lower total lipid concentrations with higher mol % PS. This occurred both in the unsaturated DOPC/DOPS system (Fig. 3A) and in the saturated DMPC/DMPS system (Fig. 3B). The lower maximal activity with higher mol % PS in the saturated system probably reflects the lesser amount of liquid crystalline phase with increasing mol % DMPS at the assay temperature (30 °C). Similar results were observed with PKC α using histone as substrate (data not shown). However, at the very high PS mol fractions, increasing the total lipid concentration resulted in decreasing PKC activity in both saturated and unsaturated systems. Thus, it seems that two phenomena are occurring. Increasing the total lipid concentrations at lower than optimal mol % PS does seem to increase the amount of relevant domain(s) to permit maximal PKC activation, but this does not explain the decrease in PKC activity at high lipid concentrations at higher mol fractions of PS.

If PKC activity requires some interaction between PKC molecules at a membrane surface, i.e. if PKC dimers or some other multimers have higher activity than do monomers, activity per unit enzyme would be expected to decrease as PKC concentration is diluted with respect to the activating surface. Thus, if enzyme concentration is increased, the decrease in activity per
unit enzyme should not occur until higher mol fractions of PS. As shown in Fig. 4 (A and B), this is indeed the case. With 20 mol % DO, increasing the PKC concentration 5-fold (from 0.8 to 4 nM) delayed the decrease in activity to higher mol fractions of PS at both 0.2 and 1.0 mM total lipid. At the lower concentrations of PKC, maximal activity was reached at lower mol fractions of PS as well, suggesting an optimal PKC/PS ratio. This trend was not so evident at low (2%) DO (Fig. 4 C), perhaps because 0.8 nM PKC is already excessive for this concentration of DO.

An alternative explanation for the decrease in activity at high mol % PS is that substrates that also bind to PS could be removed from PKC access with dilution on the PS-containing surface. To eliminate consideration of exogenous substrates, PKC autophosphorylation was examined as a function of mol % DOPS. As shown in Fig. 5, autophosphorylation also decreased at very high mol fractions of PS, and again, the PS requirement was diminished with increased DO. Increasing the PKC concentration from 0.8 to 4 nM was similarly associated with maintenance of maximal activity at higher mol fractions of PS at high mol % DO (Fig. 4B), but not at low mol % DO (data not shown). Although these results could argue for multimerization of the enzyme, they also are consistent with autophosphoryla-

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**Fig. 4.** Effect of PKCδ concentration on DOPS requirement for activity at two lipid concentrations and two DO concentrations. PKCδ activity was assayed as a function of mol % DOPS in DOPC/DOPS/DO (80–x:x:20) at 1.0 mM (A) or 0.2 mM (B) total lipid concentration and in DOPC/DOPS/DO (98–x:x:2) at 0.2 mM lipid (C). In all panels, the assay was conducted with 4.0 nM (●) or 0.8 nM (○) PKC. Error bars represent means ± S.E. from one of three independent experiments.

**Fig. 5.** Effect of varying mol % DOPS on PKCδ autophosphorylation at two DO concentrations and two PKC concentrations. A, autophosphorylation of PKCδ (4 nM) was carried out as described under “Experimental Procedures” as a function of mol % DOPS in DOPC/DOPS/DO (98–x:x:2 (○) or 80–x:x:20 (■)) at 1 mM total lipid. B, autophosphorylation of PKCδ was carried out in DOPC/DOPS/DO (80–x:x:20) with 4.0 nM (●) or 0.8 nM (○) PKC. PKC bands on autoradiographs were analyzed by densitometry, and results are expressed as % of maximal for each PKC or DO concentration. Points represent means ± S.E. of replicate gel lanes from a representative of three independent experiments.
Contributions to Maxima in PKC Activation

In the saturated DMPC/DMPS system, increasing the mol % PS will increase the amount of gel-phase domains since DMPS melts at a higher temperature than DMPC. Gel-phase lipids have been found to activate PKC much less efficiently than liquid crystalline-phase lipids (31–33); however, even in the unsaturated DOPC/DOPS/DO system, in which all of the lipid is above the phase transition at the temperature of the PKC assays, a peak in activity was still observed as a function of mol % PS (Fig. 2). Since all of the acyl chains in this system are the same unsaturated 18:1, some explanation besides gel-phase domains or % unsaturation in the whole system is required to account for the results.

The mol % PS required for maximal activity is dependent on the mol % DAG, with more PS requiring less DAG within certain limits. In the saturated DMPC/DMPS/DO system, DMPS/DO ratios varying from −1:2 to 4:1 provide near-maximal activity, and in the unsaturated DOPC/DOPS/DO system, ratios varying from 30:1 to 1:1 provide full activity. A simple fixed ratio of the two activators does not seem to explain the results. However, in the saturated system, activity correlates with 50–70 mol % DMPC, and in the unsaturated system, with 38–40 mol % DOPC. These results are suggestive of the involvement of PC in the activating domains, as had been suggested previously with the observation of a DMPC/DMPS/DO fixed ratio compound via Fourier transform infrared spectroscopy (32) and/or with a possible requirement for interface regions between compositionally distinct domains as proposed before (32, 33).

If PKC activation does correlate with the formation of lipid domains of defined composition (including interface as a potentially important domain), then increasing the lipid concentration at suboptimal compositions should increase PKC activity toward that observed with optimal compositions. In fact, this is what is observed, in both saturated and unsaturated lipid systems (Fig. 3). However, increasing the total lipid concentration at optimal compositions results in a decrease in PKC activity (Fig. 3). Bazzi and Neelbostuen (42) observed a similar decrease in PKC activity at high concentrations of PC/PS/DAG.

A potential explanation for the decrease in activity at high lipid concentrations is dilution of PKC-substrate or PKC-PKC complexes on the lipid surface. Increasing substrate concentrations should diminish dilution of PKC-substrate complexes; however, substrate/PKC and substrate/lipid interactions are complex. Several investigators have observed a peak in the dependence of PKC activity on substrate concentration with those basic-rich substrates like polylysine/serine, polylysine/arginine, protamine, and poly-l-arginine that can provide significant PKC activation in the absence of phospholipids (42, 43). It is possible that the decrease in activity at high concentrations of these activating substrates also is due to dilution of PKC multimers on the activating surface. A decrease in PKC activity at high histone concentrations has been observed, however, as well, both for intact PKC (44) and for its catalytic fragment, PKM (45). Furthermore, basic-rich substrates can bind PS and cluster PS in lipid vesicles, thus altering lipid structure (46), and acidic lipids can interact with and affect secondary structure of some PKC substrates, altering their ability to be phosphorylated (47).

To avoid the additional complexities of exogenous substrate as an added variable, we examined PKC autophosphorylation as a function of mol % DOPS. Autophosphorylation, too, exhibits a peak as a function of mol % DOPS, and the DOPS concentration required is dependent on the mol % DO just as it is with heterologous phosphorylation (Fig. 5). In support of the hypothesis that high lipid concentrations or high PS molar ratios dilute out PKC-PKC complexes, increasing PKC concentrations via an inter- versus intramolecular mechanism.

In other recent work, we used similar DOPC/DOPS/DO mixtures to generate two-dimensional crystals of PKC on a lipid monolayer (37). For both PKCa and PKCδ, three crystals of different unit cell sizes were observed. We thus examined two-dimensional crystal formation also as a function of mol % PS. As shown in Fig. 6A, the ability to detect crystals on the grids exhibited a similar peak at ~60 mol % PS with 5 mol % DO. However, the proportion of large unit cell (75 × 75 Å) versus small unit cell (50 × 50 Å) crystals also varied as a function of mol % PS (Fig. 6B). Few crystals form at 0% PS, and those that do are all of the small unit cell size. Formation of the larger unit cell crystals increased as a function of mol % PS up to ~40–50% PS.

### DISCUSSION

Numerous lipids and lipid-soluble molecules have been observed to affect PKC activity, and many, including short chain PCs, short chain PSs, lysophospholipids, tamoxifen, bile acids, free fatty acids, and DAGs, exhibit biphasic effects, with low concentrations activating PKC and higher concentrations decreasing activity (21, 27–33). Here we show that PKC activity also exhibits a peak as a function of mol % PS in PC/PS/DAG vesicles.

**FIG. 6.** Dependence of two-dimensional crystal formation on mol % DOPS. Two-dimensional crystals of PKCδ were formed on lipid monolayers consisting of DOPC/DOPS/DO (95–x:x:5) as described under “Experimental Procedures.” Two independently prepared grids were surveyed under each condition for total number of two-dimensional crystals (A) and for the % of large (75 × 75Å unit cell) or small (50 × 50Å unit cell) crystals (B). Points represent means ± S.E. of replicates.
delays the decrease in activity to higher molar ratios of PS, both for autophosphorylation and for histone phosphorylation (Figs. 4 and 5).

The PKC concentration dependence for the decrease in autophosphorylation at high concentrations of activating lipid is consistent with dilution of enzyme-substrate complexes where the phosphorylation is intermolecular. However, the possibility also exists that a PKC-PKC dimer or higher aggregate has greater activity and that such multimers are diluted out at higher concentrations of activating lipid. A similar model was proposed for the decrease in PKC autophosphorylation as a function of short chain PC concentration beyond optimal activating concentrations (48). Consistent with this possibility is the observation of different two-dimensional crystal species of PKCα as a function of mol % DOPS in lipid monolayers (Fig. 6). Very few crystals form on monolayers lacking PS, and those that do have a unit cell size of 50 × 50 Å. As discussed previously (37), this cell size is smaller than that expected to accommodate the diameter of a globular PKC molecule and suggests either crystal formation from small amounts of PKC fragments contaminating the enzyme preparations or formation of crystals with a more elongated PKC molecule attached to the lipid via its narrow end. As DOPS is increased to 40–50 mol %, more 75-Å crystals are seen per grid. This unit cell size can accommodate the diameter of a more globular PKC molecule, or it might be attributed to dimerization of PKC. Image resolution is not yet adequate to resolve this issue. However, the observation that the number of 75 × 75-Å crystals per monolayer and the PKC activity in multilamellar vesicles increase similarly as a function of increasing DOPS suggests that these crystals represent active PKC. The formation of more 75 × 75-Å crystals at the expense of the 50 × 50-Å crystals argues against the possibility that the smaller crystal form is a PKC fragment.

In summary, at least two explanations can be offered for the maxima observed in PKC activation in various lipid systems. First, an appropriate surface for enzyme activation must be provided. Although this surface may be provided by some negatively charged substrates or even glass, greater activation is achieved on lipid surfaces, where physical properties of the lipid in addition to negative charge and specific DAG and/or PS interactions contribute, possibly by facilitating an insertion event. Exposure of proteolytic cleavage sites in the V3 region with lipid versus non-lipid activating systems (49) is consistent with different conformations of active PKC in lipid versus non-lipid systems. Second, an optimal ratio of PKC to activating surface is suggested by the interdependence of these variables. The decrease in PKC activity at high concentrations of activating lipid, both for autophosphorylation and for heterologous phosphorylation, suggests the importance of PKC-PKC complexes.
