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Increased Activation of Protein Kinase C with Cubic Phase Lipid Compared with Liposomes[†]

Jennifer R. Giorgione, Zhi Huang, and Richard M. Epand*

Department of Biochemistry, McMaster University Health Sciences Centre, 1200 Main Street West, Hamilton, Ontario, L8N 3Z5 Canada

ABSTRACT: Protein kinase C (PKC) activation is measured using liposomes containing phosphatidylserine. Certain lipids display a wide range of polymorphism, depending on conditions. They can give rise to nonlamellar phases, such as hexagonal or cubic phases, as well as to lamellar phases. In this paper, we studied the activity and membrane binding of PKC in lipid bicontinuous cubic phases and hexagonal phases. The cubic phase lipid systems were (1) monoolein with 1-palmitoyl-2-oleoyl-3-phosphatidylserine (MO/PS) and (2) dielaidoylphosphatidylethanolamine/alamethicin (DEPE/alamethicin). Under fully hydrated conditions, both of the above lipid mixtures are bicontinuous cubic phases with a space group of *Pn*3*m* within certain concentration ratios and temperature ranges. Dioleoylphosphatidylethanolamine (DOPE) with up to 10 mol % PS exists in the hexagonal phase at room temperature. These cubic and hexagonal phases were able to support the PKC-catalyzed phosphorylation of histone. The amount of PKC bound to the MO/PS cubic phase showed little increase between 5 and 10 mol % PS. For both of the cubic phase systems studied, only a minor fraction of the PKC was bound to the membrane. This indicates that the specific activity of the enzyme bound to cubic phase membranes is much greater than that bound to phospholipid in the lamellar phase. Addition of up to 50 mol % MO to lipid in the lamellar phase had relatively small effects on the activity of PKC. The increase in PKC activity correlated well with an increase in PKC binding, resulting in little change in the specific activity of the membrane-bound form. These findings may be physiologically relevant due to the apparent presence of the cubic phase in certain biological structures. Also, these phases have little or no curvature strain, a property which has been shown to correlate with activation of PKC. Therefore, other factors, such as a curved morphology and/or interfacial polarity, must be responsible for the activation of PKC in these lipid systems.

Protein kinase C (PKC), which is widely distributed in tissues and organs, is a family of enzymes important in signal transduction (*1*). The mechanism of activation of PKC and its interaction with membranes have attracted much attention (*2−4*). PKC consists of several isoforms which are classified into subfamilies. The classical isoforms used in this study are regulated by Ca^{2+} and phospholipid, as well as by diacylglycerol. It is known that PKC activity is influenced by the presence of nonlamellar forming lipids (*5−8*). Furthermore, Stubbs and Slater (*9*) suggested that there is an optimal membrane curvature leading to maximally activated PKC.

Lipid systems used in PKC studies are in the form of vesicles, in which the lipids are in a lamellar phase. A great deal of experimental work has been done on the structure and phase behavior of phospholipid bilayers (*10*). The lamellar liquid-crystalline phase is an appropriate model for biological membranes. However, many studies have shown that the lamellar phase is only one of a large variety of phases presented by phospholipids. Many nonlamellar phases have also been found with purified phospholipids which are major components of biological membranes (*11*, *12*). These nonlamellar phases or, more likely, the propensity toward their formation can greatly affect the function and behavior of membranes (*13−16*).

Among nonlamellar phases, the cubic phase is an important family. As its name states, this phase has a three-dimensional periodic molecular arrangement with cubic symmetry. Recently, cubic phases formed by phospholipid systems have received a great deal of attention. Structurally, cubic phases have two distinct families: bicontinuous cubic phases, which are based on underlying periodic minimal surfaces; and discontinuous cubic phases, consisting of complex packing of discrete micellar or inverse micellar aggregates (*15*, *17−20*).

The bicontinuous cubic phase is of great interest and importance in biological systems, since it has been observed by microscopy in many biological specimens, including the infoldings of the plasma membrane, the smooth endoplasmic reticulum, and nuclear and mitochondrial membranes (*20*). The bicontinuous cubic phase can organize space within the cell, while at the same time allowing aqueous solutes to diffuse to any location within the aqueous compartments of the structure. In the present work, we studied the activation of PKC in two different lipid cubic phase systems. One

cubic phase used was monoolein which presents a cubic phase with a space group of *Ia3d* in low water content and another cubic phase of space group *Pn3m* in excess water (*21, 22*). Up to 10 mol % PS was added to MO while maintaining an isotropic phase as determined by ^{31}P NMR and a *Pn3m* space group by X-ray diffraction. Another cubic lipid system was a dielaidoylphosphatidylethanolamine (DEPE)/alamethicin mixture, which was reported to present a *Pn3m* bicontinuous cubic phase within a composition range of 1−10 wt % alamethicin over a large temperature range in excess water (*23*). Both these cubic phases have phospholipids with negative curvature lining aqueous channels, but less curvature strain than the corresponding lamellar phases of the same lipid (*24*). Bicontinuous cubic phases have zero mean curvature at the midplanes of bilayers, but locally have a continuous gradation of curvature; therefore, PKC can potentially select regions with optimal lipid packing for binding and activation. The mean curvature at the water/membrane interface is negative.

Hexagonal phases are not known to exist in biological membranes although they have been suggested to be an intermediate structure facilitating the movement of lipid to the air/water interface in the lung (*25*). The hexagonal phase system used in this work was dioleoylphosphatidylethanolamine/1-palmitoyl-2-oleoylphosphatidylserine (DOPE/PS). Bilayers containing PE with unsaturated acyl chains have negative curvature strain, but no curved morphology. In contrast, PE in the inverted hexagonal phase has almost no curvature strain, but does have a curved morphology. It was of interest to determine the relative importance of the curvature strain, or the curved morphology, in regulating PKC function.

MATERIALS AND METHODS

Materials. 1-Palmitoyl-2-oleoylphosphatidylserine (POPS), 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), 1,2-dielaidoylphosphatidylethanolamine (DEPE), 1,2-dioleoylphosphatidylethanolamine (DOPE), and 1,2-dioleoylglycerol (DAG) were purchased from Avanti Polar Lipids (Alabaster, AL). Monoolein (MO) was from Nu Chek. Alamethicin (a synthetic polypeptide), bovine serum albumin (BSA) fraction V, and protamine sulfate were obtained from Sigma (St. Louis, MO). Histone H1 was from GIBCO/BRL (Grand Island, NY). The MARCKS peptide (acetyl-FKKSFKL-amide) was purchased from the Institute for Biological Science, National Research Council (Ottawa, Ontario). [γ-^{32}P]ATP was from ICN, and [9,10-^{3}H]dipalmitoylphosphatidylcholine was from NEN. Other chemicals were from either Fisher or Sigma.

PKC Purification. Rat brain PKC was purified by a modified procedure of Huang et al. (*26*) as described previously (*27*). The purified protein displayed a single band on a silver-stained electrophoresis gel.

Lipid. Lipid films were made by dissolving phospholipids in 2:1 (v/v) chloroform/methanol and drying under a stream of nitrogen followed by desiccation under vacuum for 2 h. Films were suspended in standard buffer (100 mM KCl/5 mM MgCl$_2$/20 mM Tris-HCl, at pH 7.0), and subjected to 5 freeze−thaw cycles. Lamellar phase lipids were extruded through two 0.1 μm pore polycarbonate filters in a microextruder to form LUVs. Cubic and hexagonal phase lipids were not extruded.

PKC Binding Assays. Binding assays for the vesicular system were a modification of the sucrose-loaded vesicle assay of Rebecci et al. (*28*) as described elsewhere (*27*). For the cubic phase, lipids were suspended in standard buffer rather than sucrose buffer, and separated from the bulk solvent by centrifugation at 100000*g* for 45 min at 25 °C in 1.03 M sucrose. The lipid floated to the sides of the centrifuge tubes, and could be removed from the sucrose buffer. Lipid was resuspended in sucrose buffer, and centrifuged again under the same conditions, with PKC, Ca^{2+}, and BSA, to separate membrane-bound enzyme. The sedimented lipid/PKC and supernatant were assayed under identical conditions for activity toward protamine sulfate as previously described (*27*). For the hexagonal phase, it was sufficient to sediment the lipid in a benchtop Eppendorf centrifuge (12000*g*) for 10 min. No sucrose was necessary.

PKC Activity Assays. The activity of PKC toward histone was determined as previously described (*27*). Histone was added to a final concentration of 0.2 mg/mL. Lipid concentration was either 125 μM or 1.25 mM as indicated in the figure legends. [PKC] was 575 ng/mL. [γ-^{32}P]ATP (0.2 mCi/mL) concentration was 200 μM, and [Ca^{2+}] was 200 μM. The activity was assayed at 25 °C for MO/PS, 40 °C for DEPE/alamethicin, and 25 °C for DOPE/PS. For activity assays using the MARCKS peptide, the reaction was stopped using 75 μL of 5% acetic acid; 100 μL was spotted on Whatman P-81 filter paper (3 cm^2) and washed for 20 min with 0.04% phosphoric acid.

Western Blotting. The binding assay was performed with 10% PS/90% MO, as described above for the cubic phase. However, the activity of the top and bottom fractions was not determined. The total volume sedimented was 375 μL. This was removed after centrifugation, and the lipid was resuspended in 200 μL of sucrose buffer. These were loaded on a 7.5% polyacrylamide gel which was run overnight at 50 V. Protein was transferred to nitrocellulose (Hybond ECL nitrocellulose, Amersham). The gel and membrane were placed between 2 pieces of 3MM Whatman paper, and placed in an electroblotting cassette (Bio-Rad TransBlot) in 15.6 mM Tris, 120 mM glycine, 20% methanol. Transfer was for 3 h at 70 V. The nitrocellulose was incubated in blocking buffer which contained 3% BSA in 10 mM Tris, 150 mM NaCl (TBS) for 2 h, washed with TBS 3 times, and then incubated overnight with primary monoclonal antibodies to either PKCα, PKCβ, or PKCγ (Bio/Can Scientific, Ontario). The nitrocellulose was washed 3 times with TBS, and was then incubated with a polyclonal secondary antibody (peroxidase-linked goat anti-mouse IgG from Bio/Can Scientific, Ontario) for 3 h. The nitrocellulose was washed for 45 min in TBS with 3 changes. The ECL-chemiluminescent detection system (Amersham) was used to visualize the bands. These were exposed to Reflection NEF-496 Autoradiography Film (Mandel Scientific) for 1−10 s.

Microscopy. As an initial indication of the presence of the cubic phase, polarized microscopy was used. As an isotropic phase, a cubic phase would be dark when observed with a microscope under crossed polarizers whereas for the lamellar and hexagonal phases "birefringent" patterns would be observed. A polarizing microscope with two crossed polarizers equipped with a heat stage was used.

^{31}P NMR. ^{31}P NMR was used to determine the presence of lamellar, hexagonal, and isotropic phases. Lipid films

5%PS/95%MO
31P NMR in H2O   24.5 C

12%PS/88%MO
31P NMR in H2O   24.5 C
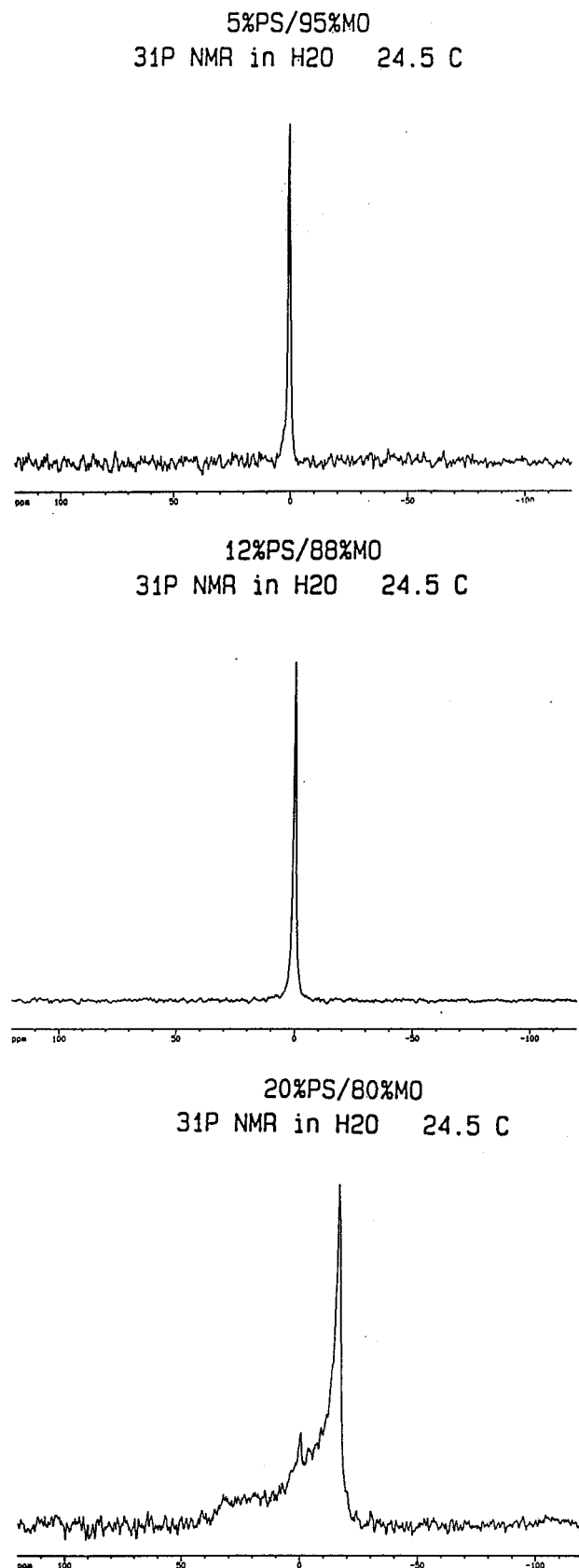
20%PS/80%MO
31P NMR in H2O   24.5 C

FIGURE 1: [32]P NMR spectra of MO/PS mixtures. Twenty-five milligrams of phosphatidylserine was used for each measurement, with an amount of MO to make up the desired composition. Scans were done at 25 °C.

were prepared as described above, and hydrated with standard buffer containing BSA and Ca^{2+} (and histone in one case as noted under Results). Twenty-five milligrams of phospho-

lipid was used in each case (total lipid varied accordingly, see figure legends), and was hydrated with 0.5 mL of buffer. The samples were freeze−thawed 5 times, and transferred to NMR tubes. A Bruker DRX 500 spectrometer operating at 202.45 MHz was used with a radio frequency pulse length of 9 μs with broad-band proton decoupling. The temperature was maintained within 0.1 °C with a Bruker B-VT 1000 variable temperature unit. Exponential line broadening of 100 Hz was applied prior to Fourier transformation.

X-ray Diffraction Experiments. Nickel-filtered CuKα (λ = 1.54 Å) X-rays were obtained from a Rigaku-Denki rotating anode. X-rays were focused using a Frank's type camera and recorded using a position-sensitive proportional counter (TEC Model 205) (*29*). Unoriented lipid dispersions were prepared by hydrating dried lipid films with a buffer similar to that used for PKC activity assays and containing 200 μM $CaCl_2$ and BSA but not histone or PKC. The lipid suspension was not extruded but was packed into 1.5 mm ϕ/glass capillaries using a metal wire. Some of the samples were also centrifuged in the capillary. Measurements were made at 25 ± 0.2 °C as used in the PKC assays. The X-ray exposure times were typically 10−15 min. Typical absolute lattice spacings, calibrated against freshly crystallized nona-decane (d = 26.2 Å), are accurate to ±0.5 Å for lattices up to ~80 Å and to ±1−2 Å for larger lattices.

RESULTS

The Pn3m Bicontinuous Cubic Phase. In excess water, MO was reported to present a *Pn3m* cubic phase with a lattice parameter of 105 Å at 22 °C (*21*). With the polarizing microscope, MO in excess Tris buffer was confirmed to exist in the cubic phase over a wide range of temperatures. To activate PKC, PS was incorporated into the MO cubic phase system. It is known that PS is a bilayer stabilizer with respect to inverted phases (*30*). It was important to determine the cubic to lamellar phase boundary of the MO/POPS system in the reaction buffer. Using a polarizing microscope, mixtures containing low amounts of PS at 25 °C revealed a dark view field, indicating the presence of the cubic phase. When the POPS content was increased to about 15 mol %, two coexisting phases were observed, and only a lamellar phase was seen with about 18 mol % or more POPS. Similar results were obtained using ^{31}P NMR. At 25 °C, mixtures containing either 5 or 12 mol % POPS were isotropic, while those containing 20 mol % had the characteristic bilayer shape powder patterns (Figure 1). We also studied this system using X-ray diffraction. Five reflections could be observed from samples of MO containing 0, 2, 5, or 10 mol % POPS. The relative spacings of the diffraction lines were $\sqrt{2}$, $\sqrt{3}$, $\sqrt{4}$, $\sqrt{6}$, and $\sqrt{8}$ for the five orders, corresponding to a *Pn3m* cubic phase. The first two orders were much stronger than the remaining three, and the third order was weakest, but observable. There was no evidence of additional peaks corresponding to coexisting ordered lamellar or hexagonal phases. The lattice spacings calculated from an average of the five reflections are given in Table 1. Very similar average values, but with higher precision, were calculated from the first two stronger orders (not shown).

This cubic phase was able to support the PKC-catalyzed phosphorylation of histone (Figure 2). The activity was determined as a function of PS concentration. The activity

Table 1: Lattice Spacings Observed by X-ray Diffraction

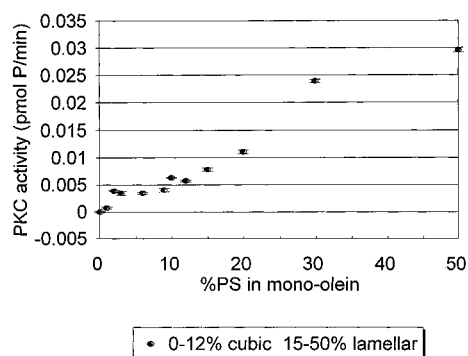|	*Pn3m* phase of MO		
% PS added	lattice spacing (Å)	calcd water tube diameter (Å)	H$_{II}$ phase of DOPE lattice spacing (Å)
0	103 ± 2	39	65.1 ± 0.2
2	109.5 ± 1	43	65.0 ± 0.1
5	121 ± 1	51.5	65.2 ± 0.2
10	136 ± 2	62	65.6 ± 0.2

FIGURE 2: PKC-catalyzed phosphorylation of histone increases as a function of PS concentration in a MO cubic phase. Lipid was composed of MO plus the indicated concentration of PS and used at a final concentration of 1.25 mM. Temperature, 25 °C. Rate of phosphorylation is expressed as picomoles of phosphate incorporated per minute. Data are expressed as the mean of triplicate determinations ± standard deviation.

was rather insensitive to PS mole fraction between 2 and 12%. Mixtures containing over 12 mol % PS were present in the bilayer phase in this assay, as extruded large unilamellar vesicles. The activity seen in the MO/PS cubic phase is higher at lower lipid concentrations (data not shown). When the lipid concentration is increased 5-fold from 125 to 625 μM, the level of activity drops about 2-fold. This is possibly a result of larger cubic aggregates forming, so that there is less surface area exposed (see Discussion) for PKC binding. Also, the enzyme may get trapped among the aggregates.

One mole percent diacylglycerol could be added to MO mixtures containing up to 10 mol % PS, and still remain as an isotropic phase, as shown by ^{31}P NMR (data not shown). DAG is known to increase the affinity of PKC for membranes, and cause maximal activation. In the bilayer phase, addition of 1 mol % DAG to 20 mol % PS containing membranes increased the activity by 1.9-fold (Figure 3). Addition of DAG to the cubic phase showed between 1.4- and 1.9-fold activation, with larger effects at greater PS concentrations.

Binding of PKC to cubic phase membranes was studied using a modified binding assay (see Materials and Methods). It was found that no more than 10% of the PKC was bound to MO/PS mixtures containing up to 10 mol % PS (Figure 4), despite the relatively high activity seen with these mixtures. The amount of enzyme bound to the lamellar phase (20−50 mol % PS + MO) increases greatly in comparison. For cubic phase lipid, both activity (Figure 2) and binding (Figure 4) are insensitive to the amount of PS present between about 2 and 10 mol %. This indicates that some PS is required for PKC activity and binding with cubic phases of MO, but that a maximal level of activity is reached at low mole fractions of PS. As a control, we performed
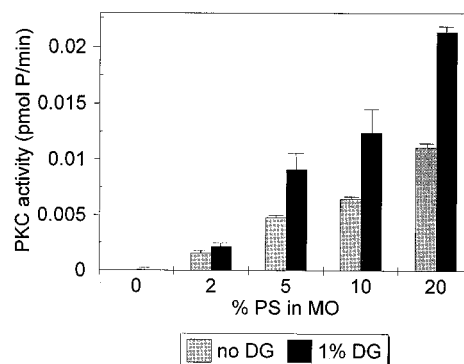

FIGURE 3: DAG increases the rate of PKC-catalyzed phosphorylation of histone when added to the PS/MO cubic phase. Lipid was composed of MO plus the indicated concentration of PS, with or without 1 mol % DAG, and used at a final concentration of 125 μM. Results from vesicles containing 20% PS in the lamellar phase, with and without DAG, are shown for comparison. Rate of phosphorylation is expressed as in Figure 2.
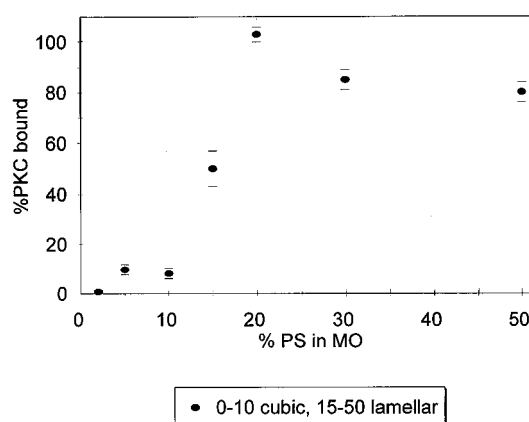

FIGURE 4: Binding of PKC to the PS/MO cubic phase. Lipid was composed of MO plus the indicated concentration of PS and used at a final concentration of 125 μM. Binding is expressed as a percentage of the total enzyme added.

Table 2: PKC Activity and Binding to Lamellar DOPC/POPS/MO Vesicles[a]

vesicle composition	PKC activity (pmol of P/min × 10^{-3})	% PKC bound to SLV
100% PC	1.98 ± 0.30	19 ± 2
90% PC/10% PS	2.01 ± 0.08	15 ± 1
80% PC/20% PS	5.66 ± 0.23	60 ± 5
80% PC/20% MO	1.36 ± 0.05	16 ± 1
70% PC/10% PS/20% MO	2.86 ± 0.14	27 ± 2
60% PC/20% PS/ 20% MO	6.37 ± 0.19	64 ± 5
50% PC/50% MO	0.92 ± 0.05	2 ± 0
40% PC/10% PS/50% MO	4.90 ± 0.05	47 ± 5
30% PC/20% PS/50% MO	7.90 ± 0.40	80 ± 8

[a] Selected points from curves of PKC vs % PS at 0, 10, and 20% PS which approximately correspond to PS-independent activity, midpoint of activity curve, and maximal activity at high PS. Conditions of assay are the same as described under Materials and Methods. The substrate is histone. % PKC bound is defined as the percentage of total enzyme added that is bound to the membrane fraction as measured by the SLV binding assay.

binding and activity assays using DOPC/POPS/MO mixtures, which exist in the lamellar phase (*31*). PKC activity and percent PKC bound to these vesicles at each composition are shown in Table 2. As the concentration of MO increases at a constant PS concentration, the level of PKC activity
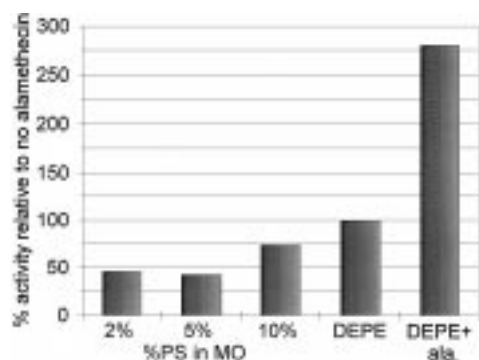
FIGURE 5: Increase in PKC activity in the DEPE/alamethicin cubic phase. For bars 4 and 5 (far right), lipid was composed of DEPE with or without 4 mol % alamethicin and used at a final concentration of 1.25 mM. Temperature, 40 °C. DEPE alone is lamellar, and DEPE with alamethicin is cubic. Standard deviation was less than 1%. For bars 1−3 (left), lipid was composed of MO/PS with 4 mol % alamethicin at 25 °C. All three were in the cubic phase. Standard deviation was 20, 13, and 5%, respectively. Activity is expressed as a percentage of that obtained (with MO and the indicated concentration of PS) in the absence of alamethicin for bars 1−3, and with DEPE alone for bars 4 and 5.

Table 3: Comparative Specific Activity of Membrane-Bound PKC[a]

	% PS	specific activity
set one		
cubic phase	2	39.1 ± 13.7
	5	3.77 ± 0.90
	10	8.19 ± 2.01
lamellar phase	15	1.60 ± 0.27
	20	1.08 ± 0.06
	30	2.83 ± 0.17
	50	3.72 ± 0.26
set two		
cubic phase	2	16.0 ± 6.3
	5	5.19 ± 1.09
	10	8.45 ± 0.23
set three		
lamellar phase	20% PS/30% PC/50% MO	1.00 ± 0.15
	20% PS/60% PC/20% MO	1.00 ± 0.11
	20% PS/80% PC	0.95 ± 0.12

[a] Specific activity is expressed as [(pmol of P/min)/(% PKC bound)] $\times 10^{-4}$. Set one used activity data from Figure 2 at 1.25 mM lipid and binding data from Figure 4 at 125 μM lipid; set two uses activity and binding data from Figures 3 (no DAG) and 4, respectively, at 125 μM lipid; set three uses activity and binding data from Table 2 at 125 μM lipid.

increases, as well as the percentage of PKC bound. However, the amount of PKC bound to this lamellar phase is much higher than the amount bound to MO cubic phases at the same PS level (compare Figure 4 and Table 2).

Addition of 0.5−10 mol % alamethicin to dielaidoylphosphatidylethanolamine (DEPE) has been found to promote formation of the *Pn3m* cubic phase in excess water, at temperatures from 38 to 75 °C (*23*). This cubic phase was also shown to exist when the phospholipid/peptide mixture was in excess buffer (data not shown). Without alamethicin, DEPE forms a lamellar phase up to 65 °C. Addition of 4 mol % alamethicin to DEPE at 40 °C caused a 2.8-fold activation of the measured PKC-catalyzed phosphorylation of histone, relative to DEPE alone (Figure 5). The binding of PKC to this cubic phase was less than 1%. [Binding was very weak, and values varied slightly. Values were always below 0.7% and went as low as 0 (not shown).] Since the measured activity with DEPE and alamethicin was 0.0024 pmol of P/min with less than 1% PKC bound, the specific activity is at least twice that observed with 10% PS/MO which supported activity levels of 0.0063 pmol of P/min with a minimum of 6% PKC bound. Alamethicin itself was not the cause for this activation, since 4 mol % alamethicin added to MO/PS mixtures in the cubic phase slightly decreased the PKC-catalyzed phosphorylation of histone, in comparison with that obtained with MO/PS itself. Addition of alamethicin to MO/PS mixtures did not change the phase, as determined by ^{31}P NMR (data not shown).

We compared the activity of PKC measured with MO/PS in the cubic phase using the small MARCKS peptide, instead of histone as the substrate for phosphorylation. The activity measured with MO containing 10% PS was similar for both the MARCKS peptide and histone (not shown).

We compare the specific activity of the membrane-bound form of PKC in cubic and lamellar phases (Table 3). Although in set one the amount of lipid used for activity and binding was different, the results are valid for comparative purposes. The specific activity in the cubic phase is manyfold higher than in the lamellar phase. Only at very high PS does the specific activity of the enzyme bound to

lamellar phase liposomes approach one of the points of the cubic phase system. The measured activity in the cubic phase is not related to the amount of lipid present. Thus, set two having the activity measured at lower lipid concentration gives a relative specific activity comparable to the cubic phase lipid used in set one. Furthermore, lamellar phase lipid with 20% PS (set three) has a comparable specific activity to the 20% PS mixture reported in set one and much lower than the specific activities of PKC in cubic phase lipids. These results confirm our conclusion that PKC bound to cubic phase lipid is more active than the enzyme bound to a liposome of comparable lipid composition.

Since only about 10% of the PKC binds to the cubic phase, we were interested in determining whether this was due to binding of one specific isoform. Western blotting with antibodies to each of the three PKC isoforms found in rat brain (α, β, γ), on top and bottom fractions after incubation of PKC with cubic phase lipid (10% PS/90% MO), showed that each isoform was bound in about equal amounts to the cubic phase (data not shown).

The Inverted Hexagonal Phase. Dioleoylphosphatidylethanolamine (DOPE) forms an inverted hexagonal phase at room temperature (*32*, *33*). Addition of up to 10 mol % PS to this phospholipid does not change the phase as determined by ^{31}P NMR (Figure 6), even in the presence of histone, BSA, Ca^{2+}, or low concentrations of ATP (data not shown). The presence of an H_{II} phase was confirmed by X-ray diffraction which showed three orders indexing in the ratio $1:\sqrt{3}:\sqrt{4}$. The H_{II} lattice spacing was rather insensitive to the presence of PS, increasing only slightly above the experimental error at 10% PS (Table 1). This hexagonal phase was able to support the PKC-catalyzed phosphorylation of histone (Figure 7). The activity was not due to the PE itself, since similar DEPE/PS mixtures which exist in the L_β gel phase at 25 °C did not show any increase in activity with increasing PS concentration (not shown). Binding of PKC to the hexagonal phase correlates with activity, with the percentage of enzyme bound increasing with the concentration of PS (Figure 7).
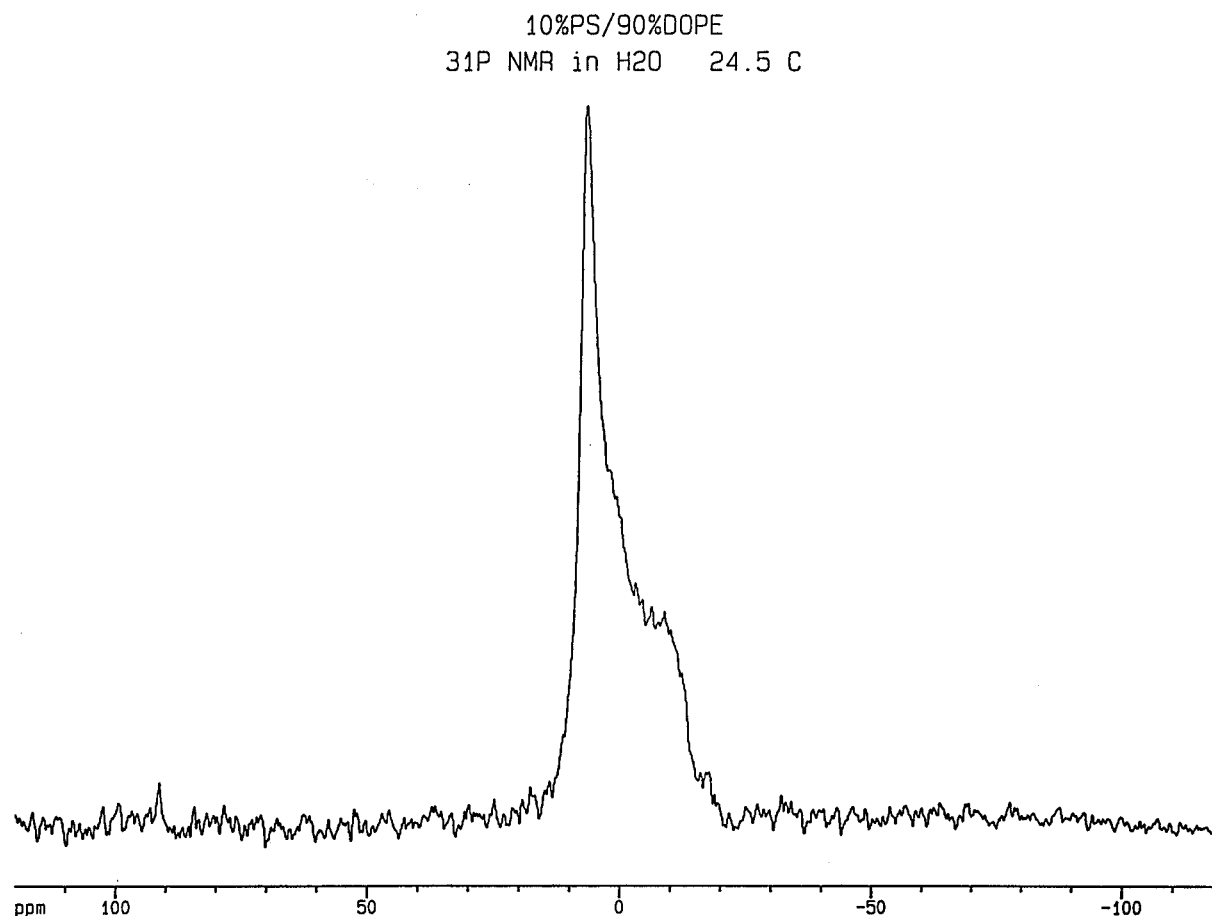
FIGURE 6: ^{31}P NMR spectra of DOPE/PS mixtures. Total phospholipid was 25 mg for each measurement. Temperature, 25 °C. Similar powder patterns were obtained at 15 °C.
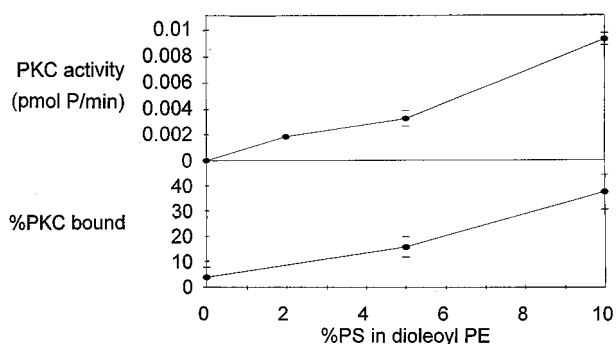

FIGURE 7: Hexagonal phases support PKC-catalyzed phosphorylation of histone and PKC binding. Phospholipid was composed of DOPE plus the indicated concentration of PS. Phospholipid was used at a final concentration of 125 μM. Temperature was 25 °C. Data are expressed as the mean of triplicate determinations ± standard deviation. Binding (bottom) is expressed as a percentage of the total enzyme added. Activity (top) is expressed as picomoles of P incorporated per minute.

DISCUSSION

It has long been known that membrane phospholipids or other additives which are hexagonal phase promoters activate PKC when added to bilayer phases (*34*). However, the physical property responsible for this correlation is not known. It has been suggested that the activity of membrane-bound enzymes may be modulated by curvature strain, i.e., an instability of the bilayer caused by the intrinsic tendency of the constituent monolayers to form curved structures (*35*). This formulation was modified in terms of the dependence

of lateral pressure on the position within the membrane (*36*). This mechanism of the lipid modulation of protein properties has been proposed for integral membrane proteins (e.g., see *37*). It may be less applicable for an amphitropic protein, such as PKC, which readily partitions between aqueous and membrane environments. Furthermore, PKC can be activated by Triton micelles containing PS (*38*). Such micelles would be devoid of negative curvature strain. It was therefore suggested that changes in the interfacial properties of amphiphile/water systems were the direct cause for the modulation of the activity of this protein kinase (*5*), although there certainly could be other differences in the nature of the interaction between PKC and a detergent micelle vs a bilayer.

One can test the relative importance of curvature strain/lateral pressure profiles of the phospholipid monolayers in modulating PKC activity with the use of nonlamellar phases. The curvature strain would be progressively reduced in going from the lamellar to the bicontinuous cubic and finally to the hexagonal phase (*24*). If this property were responsible for the modulation of PKC activity, the activity should progressively decrease in going through these three phases. The complication is that under a particular set of conditions the lipid will generally take up only one of the three phases. We therefore chose systems in which a minor change in the composition of the lipid resulted in a phase change, as well as performing appropriate controls.

We have studied two lipid systems that form bicontinuous cubic phases with a *Pn3m* space group. One of them is MO

(*21, 22*). This lipid alone does not support the activity of PKC (Figure 2). However, at % PS between 2 and 10, there is measurable activation of PKC. At these low mole fractions of PS, only a small fraction of the PKC is bound to these cubic phase lipids (Figure 4). Thus, the specific activity of the PKC bound to cubic phase membranes is much higher than that obtained with lamellar phase membranes of similar composition (Table 3). There is most likely optimal intrinsic monolayer curvature in the bilayer phase for PKC binding and activation (*9*). The phospholipid in the cubic phase likely contains this optimal curvature, since the aqueous channels are lined with headgroups arranged in such a way that they would have a continuous variation of curvature. In addition, at a composition of about 12% PS, there is a conversion from cubic to lamellar phase with increasing PS content. This results in an increased binding of PKC to the membrane, but there is no abrupt increase in PKC activity over the range of PS concentrations, i.e., 12−13%, at which the lipid undergoes a conversion of phase.

It is well-known that PS is required for the activity of PKC and its binding to membranes (*1, 27*). The amount of PS that can be mixed with MO, while maintaining the lipid in the cubic phase, is limited. However, we can assess the effect of MO addition to PS-containing vesicles in a bilayer phase (Table 2). MO increases the maximal activity observed at high concentrations of PS, and it shifts the dependence of the activity on PS to lower PS concentrations (full curves not shown). This is typical for the addition of a membrane component, like MO, which lowers the bilayer to hexagonal transition temperature (*39*). However, the activity of PKC, in all cases, correlates well with the fraction of PKC bound to a membrane. Thus, MO does not greatly reduce the requirement for PS, and it only slightly increases the activity of membrane-bound PKC.

The *Pn3m* cubic phase consists of a bicontinuous array of phospholipid, with the headgroups forming the lining of interconnected aqueous channels. These aqueous channels connect spherically shaped compartments of water which have a slightly larger diameter than the channels (connecting tubes). Despite this complication of nonuniform aqueous pore size, we can estimate the approximate average diameter of the aqueous channels of the cubic phase. The diameter of the water channel is (sin 45°)(lattice spacing) − (bilayer thickness). The thickness of an MO monolayer has been estimated at about 17 Å (*40*). From our measured unit cell dimensions for MO (Table 1), we estimate a water tube diameter of 39 Å. This is in good agreement with the estimate of Longley and McIntosh (*21*) of 40 Å. This is smaller than the diameter estimated for PKC as a globular protein of molecular mass 82 kDa and $\bar{v} \cong 0.72$, corresponding to a particle of diameter 57 Å. The cubic phase lattice parameter increases markedly with PS as does the diameter of the water cylinders (Table 1). At 2% PS, the calculated aqueous tube diameter, 43 Å, is still too small to accommodate PKC. However, at 5% PS, the tube diameter is 51.5 Å, which is not too much smaller than the 57 Å calculated for PKC as a globular structure. If PKC could become elongated or enter the polar region of MO or locally distort the lattice structure of MO, it could enter the cubic phase matrix. At 10% PS, the lattice spacing is large enough for PKC to enter. The fact that PKC binding does not increase between 5% and 10% PS (Figure 4) suggests that there is

no change in lipid accessibility between these two mole fractions of PS. However, between 2 and 5% PS, there is a large percent increase in PKC binding which likely reflects the greater accessibility of the lipid. However, the absolute amount of PKC bound to the cubic phase remains low because of the relatively low mole percent of PS required to maintain a cubic phase and the lack of affinity of PKC for MO. In contrast to binding, 2% PS does support some activity (Figure 2). This activity likely arises from PKC bound to the exterior of the cubic phase aggregates, and to the regions at the entrance to the water channels on the surface. Also, the observation that there was not a 2-fold increase in PKC activity when the amount of lipid was doubled can be explained by larger cubic aggregates forming, which do not expose twice as much outer surface area, even though there would be twice the headgroup area exposed inside the channels. This is true also for the 5 and the 10% PS mixtures, suggesting that surface-bound PKC contributes significantly to the activity observed, even in these cases in which the enzyme can enter the cubic phase matrix. Because of the low binding of PKC to MO with 2% PS, the specific activity of the bound enzyme must be very high. Thus, this membrane surface is very effective in activating PKC. This is also shown by the fact that in going from the cubic (10% PS) to the lamellar (15% PS) phase, the binding (Figure 4) increases much more than activity (Figure 2). Thus, the specific activity of the membrane-bound form of PKC is higher in the cubic phase. The binding to lamellar phase lipid is higher than to that of the cubic phase, despite the fact that half of the lipid in the lamellar phase is on the internal monolayer of the vesicle and not accessible to PKC. The greater binding to the lamellar phase lipid could have several causes. The dependence of binding on PS concentration is highly sigmoidal (*27*); hence, a small increase in PS can cause a large increase in binding. In addition, there may not be complete access to all the membrane/water interface in the cubic phase, and there may also be a difference in the binding affinity of PKC for cubic vs lamellar phase lipid.

An increase of PS from 2% to 5% or 10% increases the accessibility of the lipid surface to PKC, as a result of an increase in size of the aqueous tubes. Hence, there is an increase in binding, but the activity is not greatly changed. It would appear that increased PS above 2% allows more PKC to bind but that the specific activity of the bound enzyme is lower. The factors responsible for interfacial catalysis are complex and involve several binding and dissociation rates, in addition to the kinetics of the reaction itself. In particular, the rate of binding of histone (21 kDa) to the membrane-bound form of PKC and the diffusion of enzyme and substrates in and out of the cubic phase matrix may limit the rate of catalysis by PKC bound in the interior of the cubic phase matrix. Thus, even with 5 and 10% PS in MO, a large fraction of the activity of PKC may come from the enzyme bound to the surface of the cubic phase aggregate. This is not because there is a high concentration of the substrate, histone, at the surface of the cubic phase. Substitution of histone by a smaller substrate, the MARCKS peptide, had little effect on the observed rates. The MARCKS peptide has much lower binding affinity for anionic membranes than histone, but is an equally effective substrate. It is also not likely that substrate accessibility limits the rate of PKC phosphorylation since the MARCKS

peptide would have facile access to the aqueous pores of the cubic phase.

PKC activity in the cubic phase not only is a property of MO/PS systems but also was found with DEPE/alamethicin (Figure 5). Overall, a 2.8-fold increase in activity was found with the cubic phase compared to the lamellar phase, although the activity was not very high due to the lack of PS. Although PS is required to maximally activate PKC, it has been shown that PE can give rise to a lower level of activity (*7*, *41*). Formation of the cubic phase in DEPE is triggered by the addition of only a small mole fraction of alamethicin. The alamethicin itself shows some inhibitory activity against PKC with MO/PS cubic phases. Therefore, the enhanced activity of the alamethicin/DEPE cubic phase, compared with DEPE in the lamellar phase, shows that the cubic phase can support a higher level of activity than the lamellar phase. This is especially true when one considers the activity of PKC bound to the membrane. In the case of DEPE/alamethicin, very little PKC is bound to lipid, despite the increased measured activity of PKC. This again indicates that the specific activity of PKC bound to cubic phase membranes is very high. The lattice constant for this system was measured to be 160−180 Å (*23*), larger than the MO/ 10% PS mixture and sufficient to allow PKC to enter the structure.

An increase in monolayer curvature concomitant with a decrease in monolayer curvature *strain* occurs in the hexagonal phase. In the presence of small mole fractions of PS, this phase supports the activity of PKC (Figure 7). However, unlike the cubic phase, substantial amounts of PKC are bound to the hexagonal phase in the presence of PS, and this amount increases markedly with increased PS (Figure 7). Unlike the cubic phase systems, in which a relatively minor perturbation can convert the lamellar to the cubic phase, there is no other phase of comparable chemical composition to compare the hexagonal phase to. Nevertheless, it appears that the hexagonal phase can support some activation of PKC. From the hexagonal phase lattice spacings (Table 1), we calculate the diameter of an H_{II} cylinder to be 75.2 Å for DOPE. This is in reasonable agreement with previous measurements (*32*, *33*, *42*, *43*). The length of the hydrocarbon chain was determined to be 20 Å for this lipid (*43*), although it has also been suggested that the hydrocarbon chains are not completely segregated away from the polar headgroups (*44*). The internal core of an H_{II} cylinder has a diameter of 35.2 Å, much too small for PKC to enter. Curiously, the presence of PS did not make the cylinder diameters of the H_{II} phase substantially larger (Table 1). It is thus likely that, as in the case of the cubic phase, PKC binds to the entrances of the aqueous cores of the H_{II} phase. However, there is uncertainty about the nature of the ends of the cylinders. If they were blunt ends, they would expose the hydrophobic portions of the acyl chains, unless they were covered by a monolayer of micelle-type lipid or if they closed upon themselves to form a toroid, as has been shown in freeze−fractures of the disk membranes of frog retinal rod outer segments, observed by electron microscopy (*45*), as well as in bovine retinal rod outer segments (*46*, *47*). It is also not known how the hydrophobic exterior of an aggregate of hexagonal phase cylinders is protected from contact with water. It is possible that, as suggested with the ends of the cylinders, there is a covering of a lipid monolayer with positive curvature.

We can thus conclude that lipids in inverted phase structures can support the activity of PKC. This is particularly well demonstrated for cubic phase lipid in which we can show that lipid mixtures of essentially the same chemical composition in the cubic phase support considerably greater activity of membrane-bound PKC than in the lamellar phase. This may have relevance to biological systems since biological membranes have been shown to contain cubic phases (*20*).

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