The Chirality of Phosphatidylserine and the Activation of Protein Kinase C†

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ABSTRACT: The properties of phosphatidyl-L-serine (L-PS) and phosphatidyl-D-serine (D-PS) were compared. The two forms of PS have similar but nonidentical L to L phase transition temperatures. Mixtures of phosphatidylserine with phosphatidylethanolamine and cholesterol (molar ratio 1:1:2) show polymorphic behavior at higher temperatures and in the presence of Ca^{2+}. Mixtures with L-PS undergo conversion to nonlamellar phases at lower temperatures than do similar mixtures with D-PS. The aggregation of vesicles upon addition of histones is greater for L-PS than for D-PS. With fluorescence digital imaging microscopy we could show differences in the extent of formation of histone-induced domains enriched in PS or in diacylglycerol. The most enriched domains were induced with histone in membranes containing L-PS. The MARCKS peptide showed no differences in domain formation between L-PS and D-PS. The maximal activity of protein kinase C was greater in the presence of L-PS when histone, which could form more enriched domains, was the substrate. However, with a MARCKS peptide substrate, which formed domains of equal enrichment with L-PS and D-PS, the maximal activity of protein kinase C was the same with D-PS and with L-PS. These observations demonstrate that L-PS and D-PS have different physical properties. These differences likely contribute to the greater ability of L-PS to activate protein kinase C.

A number of enzymes function only when bound to a membrane. The role of membrane lipids in supporting the activity of such enzymes has been the subject of investigation for many years. There are at least two aspects by which lipids can affect enzyme catalysis. One is as a consequence of the structure of the lipid which can bind to specific sites on the enzyme. The other factor is the manner in which the membrane lipid modulates certain physical properties of the membrane which affect enzyme activity. This latter effect would not require a specific lipid structure nor would it require a high-affinity binding site on the enzyme.

Protein kinase C (PKC)[1] is an enzyme which has an important role in signal transduction (*1*−*5*). The phosphorylation of most substrates by this enzyme requires phosphatidylserine (L-PS) as a cofactor. There are conflicting reports in the literature about the specificity of the requirement for L-PS to support the activity of PKC. In particular, assays of the enzymatic activity of PKC using Triton X-100 micelles show a strong specificity for L-PS over other anionic lipids (*6*−*9*). However, it has also been reported that other anionic phospholipids can replace PS with varying degrees of effectiveness (*10*, *11*). In particular, it has been found that a structurally unrelated anionic lipid, *N*-dansylphosphatidylethanolamine, is equally effective to L-PS in promoting the membrane binding of PKC as well as the activity of this enzyme (*12*). This result was in contrast to the observation that PKC had different degrees of activation using the two structurally related anionic lipids, i.e., phosphatidyl-L-serine (L-PS) and phosphatidyl-D-serine (D-PS) (*13*). Although the different degrees of activation by the two stereoisomers of PS suggested a highly specific binding site for the L form of this lipid on PKC, there could also be differences in the physical properties of the L and D forms of this lipid. This is because there is another chiral center, besides the α-carbon atom of the serine, in PS. Thus L-PS and D-PS are not enantiomorphs (mirror images) but rather diastereoisomers. Since diastereoisomers are not mirror images of each other, they do not necessarily possess identical physical properties. We have thus undertaken a study to compare the physical properties of these two isomeric forms of PS. The comparisons were made by choosing systems which were relatively simple and had the potential for exhibiting differences between the two isomers of PS.

Two membrane physical properties that have been suggested to be associated with increased activity of PKC are the tendency of the membrane to form inverted phases (*14*) and the ability of the membrane to form laterally segregated large domains (*15*, *16*). It is possible that the two properties

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[1] Abbreviations: dansyl-DAG, 1-acyl-2-[11-(5-(dimethylamino)-naphthalene-1-sulfonylamino)undecanoyl]glycerol; DOPC, *sn*-1,2-dioleoylphosphatidyl-L-choline; DOG, *sn*-1,2-dioleoylglycerol; L-DOPS, *sn*-1,2-dioleoylphosphatidyl-L-serine; D-POPS, *sn*-1-palmitoyl-2-oleoylphosphatidyl-D-serine; L-POPS, *sn*-1-palmitoyl-2-oleoylphosphatidyl-L-serine; MARCKS, myristoylated alanine-rich C-kinase substrate peptide; NBD-PS, 1-acyl-2-[6-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-amino)caproyl]phosphatidyl-L-serine; PKC, protein kinase C; L-PS, D-PS, and DG, generic abbreviations for phosphatidyl-L-serine, phosphatidyl-D-serine, and diacylglycerol irrespective of the nature of the acyl chains; LUV, large unilamellar vesicle.

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are interrelated, since it is found that a strong promoter of hexagonal phase formation, diacylglycerol (DG), also partitions into L-PS-rich domains (*16*). We have compared membranes containing L-PS with those containing D-PS and demonstrate that they have different physical properties which are likely to contribute to the observed differences in PKC activity in the presence of these two diastereoisomers.

EXPERIMENTAL PROCEDURES

Materials. Phospholipids, DOG, and NBD-PS were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). The lipids gave one spot on TLC using 100 μg loading. Cholesterol was a >99% pure grade, purchased from Sigma Chemical Co. (St. Louis, MO). Dansyl-DAG was prepared from C11-dansyl-PC by action of phospholipase C according to Yang and Glaser (*16*). The 25 amino acid MARCKS peptide was synthesized by the Biotechnology Center Genetic Engineering facility at the University of Illinois. Histone, extracted from calf thymus, type IIA, was purchased from Sigma (St. Louis, MO). All other chemicals were of analytical grade.

Lipid Vesicles. Lipids dissolved in chloroform were dried under a stream of nitrogen and subsequently evacuated under high vacuum for at least 1 h. The lipid films were then suspended, by vigorous vortexing, in 20 mM PIPES and 150 mM NaCl, pH 7.4, containing 20 mg/L NaN_3 (PIPES buffer) with 1 mM EDTA or with the addition of $CaCl_2$ and/or $MgCl_2$, as indicated. For the preparation of large unilamellar vesicles (LUV), the lipid suspension was subjected to five freeze−thaw cycles, followed by 21−51 rounds of extrusion through two stacked 0.1 μm polycarbonate filters in a Lipofast microextruder (Avestin, Inc., Ottawa, Ontario, Canada). Large unilamellar vesicles for microscopy were prepared in a buffer containing 20 mM PIPES and 100 mM NaCl, pH 7.4, according to the procedure of Harvestick and Glaser (*17*). Only vesicles with a radius larger than 10 μm were used for microscopy.

Differential Scanning Calorimetry (DSC). The lipid suspensions, generally containing 3 mg of lipid in PIPES buffer, were loaded into a 1.2 mL fixed volume cell. In addition, a range of lipid concentrations was used in either the liquid cell or the solid sample cell of an MC-2 high-sensitivity scanning calorimeter (Microcal Co., Amherst, MA). For the solid sample cell, an equal amount of buffer was loaded into a matching reference cell (about 0.5 mL). A heating scan rate of approximately 30 K/h was generally employed. Sequential heating scans on the same sample gave similar results.

^{31}P NMR. The ^{31}P NMR spectra, from suspensions of about 50 mg of lipid in PIPES buffer, were obtained using a Bruker AM-500 spectrometer operating at 202.45 MHz in a 10 mm broad-band probe over a 30 kHz sweep width in 16 × 1024 data points. A 90° pulse width of 16.6 μs was used. Composite pulse decoupling was used to remove any proton coupling. Generally, 800 free induction decays were processed using an exponential line broadening of 100 Hz prior to Fourier transformation. The probe temperature was maintained within ±0.1 °C by a Bruker B-VT 1000 variable temperature unit. Temperatures were monitored with a calibrated thermocouple probe placed in the cavity of the NMR magnet. The temperature was monitored both before and after a temperature or a kinetic run and found to be within ±0.2 °C of the original value.

Right Angle Light Scattering. There is a phenomenological correlation between the ability of micelles to be aggregated by histone and the activity of PKC in phosphorylating histone (*18*). We compared the aggregation of LUVs containing either L-PS or D-PS by histone. Vesicle aggregation was monitored by both right angle light scattering at 320 nm, using an Aminco-Bowman series II spectrofluorometer, and turbidity measurements, using a Perkin-Elmer Lambda 4B spectrophotometer set at 320 nm. The cuvette contained 600 μg of phospholipid in the form of LUVs suspended in 1.5 mL of PIPES buffer at a room temperature of 23 °C. The light scattering from this mixture was monitored, and then 0.6 mL of a 1.0 mg/mL solution of histone was added. Despite the resulting dilution there was an immediate increase in light scattering as a consequence of vesicle aggregation which did not subsequently change significantly with time.

Fluorescence Digital Imaging Microscopy. The vesicles were observed with the fluorescence digital imaging microscope according to Yang and Glaser (*15*). Due to the overlap between the excitation spectra of NBD and dansyl, a filter cutting above 390 nm was used on the excitation path for the acquisition of the dansyl images. After subtraction of the background fluorescence, each image was normalized to a mean radiance of 100 and displayed using a pseudocolor scheme where the lowest radiance value was dark blue and the highest radiance value was red (see Figure 4). We defined a domain as a portion of a vesicle whose radiance is over 1.5 times the average radiance value of the vesicle. The concentration of a component in a domain is expressed as the ratio of the average domain radiance versus the average radiance value of the vesicle (average enrichment, Figure 5A) and as the ratio of the maximum domain radiance pixel versus the average radiance of the vesicle (maximum enrichment, Figure 5B). For every condition, enrichments were determined with a population of at least 20 vesicles. The errors in determining the enrichment of the domains were always less than ±5%.

PKC Activity Assay. PKC activity assay toward the MARCKS peptide or histone was performed using purified rat brain PKC with minor modifications to the published procedures (*19, 20*). The 250 μL reaction mixture contained 100 mM KCl, 5 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.0, 200 μM $CaCl_2$, 0.3 mg/mL BSA, 1 μM MARCKS peptide or 0.2 mg/mL histone, 100 μM lipid in the form of LUVs consisting of 10−50% PS, 2−5% DAG, and the remainder POPC, 20 μM [γ-^{32}P]ATP (0.2 μCi/mL), and 200 ng/mL PKC. The reaction was initiated by the addition of ATP and stopped after 10 min at 25 °C with either 75 μL of ice-cold 5% (v/v) acetic acid for the reactions with the MARCKS peptide or 2 mL of ice-cold 25% (w/v) TCA for the reactions containing histone. The samples containing the MARCKS peptide were then spotted on Whatman ion-exchange P-81 paper and washed four times with 0.4% (v/v) phosphoric acid for 5 min each. The samples containing histone were filtered through Whatman GF/C filters and washed four times with 2 mL of 25% (w/v) TCA. The filters were then dried and counted using efficiency-corrected Cherenkov counting.

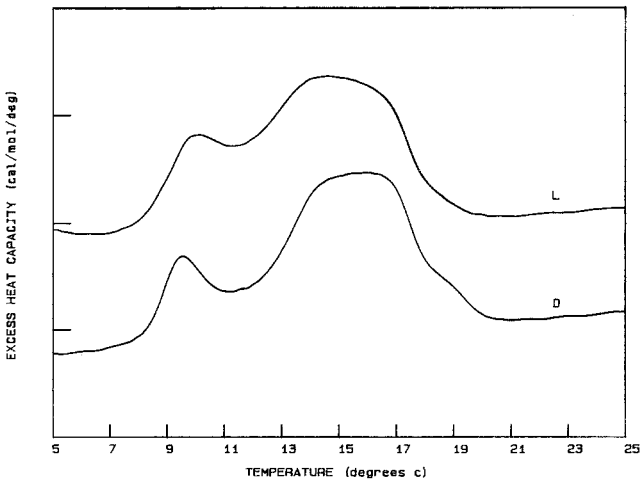

FIGURE 1: DSC curves of L-PS (L) and D-PS (D) at a concentration of 1.5 mg/mL in PIPES buffer without EDTA but containing 0.25 mM $CaCl_2$. Scan rate 24 K/h. The two scans are displaced along the vertical axis for clarity of presentation. Each tick mark along the vertical axis corresponds to 500 cal K^{-1} mol^{-1}.

RESULTS

DSC. DSC was used to compare the thermotropic transition temperatures of L-PS and D-PS either as a single lipid component or in lipid mixtures. The pure L-PS and D-PS have L_β to L_α transition temperatures at 9.8 and 9.4 °C, respectively, both with a transition enthalpy of 5 kcal/mol. The small difference in transition temperature between the two isomers is reproducible on repeated scans and with new samples. Calcium ion is known to markedly increase the phase transition temperature of phosphatidylserine and to cause the dehydration of this lipid (*21*, *22*). The small difference in transition temperatures between the two isomers of PS was not caused by a small contamination of Ca^{2+} in L-PS since DSC runs made in buffer in the absence or presence of EDTA gave identical results. The difference between the thermal transitions of the two isomers was also observed upon the addition of 0.25 mM Ca^{2+} (Figure 1). In the presence of calcium, there is a residual peak around 9−10 °C which probably reflects free PS. This peak retains the small difference in transition temperatures for the two isomers which were found in the absence of Ca^{2+}. However, the major component of the transition is shifted to higher temperatures. Interestingly, this component extends to higher temperatures for D-PS than for L-PS. This is opposite to the order found for the pure lipid. The results would be difficult to explain in terms of an impurity or positional isomerization of the acyl chains. These results provide further evidence for a difference in physical properties between L-PS and D-PS.

^{31}P NMR. Phosphatidylserine at neutral pH forms only lamellar phases, but mixtures of phosphatidylserine−phosphatidylethanolamine and cholesterol in the presence of Ca^{2+} are known to undergo transitions to an inverted hexagonal phase (H_{II}) (*23*). We used ^{31}P NMR to monitor the polymorphism of such lipid mixtures containing either L-PS or D-PS admixed with 1-palmitoyl-2-oleoylphosphatidylethanolamine (PE) and cholesterol in a 1:1:2 molar ratio in PIPES buffer with 2 mM Mg^{2+} and varying amounts of Ca^{2+}. At 30 °C in the absence of Ca^{2+} a bilayer powder pattern was observed with the major peak split, suggesting a small difference in the principal components of the chemical shift tensors for the two lipids. With increasing concentrations of Ca^{2+} there was an increase in the intensity of the upfield shoulder, probably reflecting the formation of some hexagonal phase. However, the major component of the powder pattern was similar to that of a bilayer phase up to the highest concentration of 10 mM Ca^{2+} used (data not shown). Samples with a lipid composition of 1:1:2 PE:PS:cholesterol with 10 mM Ca^{2+} were heated, and the ^{31}P NMR spectrum was measured as a function of temperature. Samples initially at room temperature were gradually heated and spectra obtained at a number of temperatures. Samples were heated to 60 °C and the ^{31}P NMR spectra measured at that temperature for the two cases; i.e., the mixtures with L-PS vs D-PS, are markedly different. The L-PS-containing sample is mostly H_{II} phase with some isotropic, while the D-PS-containing sample is still mostly in the bilayer phase (Figure 2). At 75 °C both samples are largely in the H_{II} phase, with some isotropic signal also present.

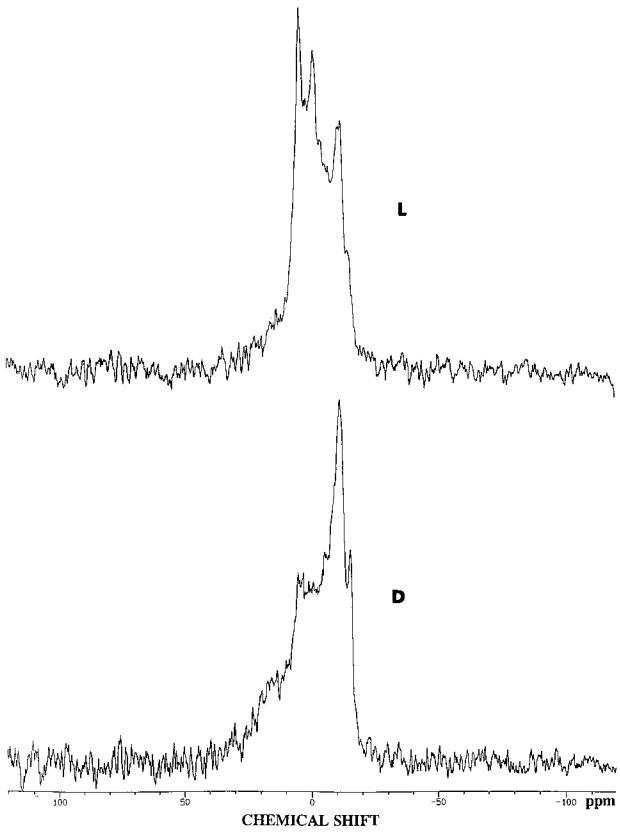

FIGURE 2: ^{31}P NMR spectra of a mixture of PE/PS/cholesterol in a 1:1:2 molar ratio heated to 60 °C and measured at that temperature in PIPES buffer, pH 7.4, with 2 mM Mg^{2+} and 10 mM Ca^{2+}. Upper spectrum (L), sample with L-PS; lower spectrum (D), sample with D-PS.

Vesicle Aggregation. Addition of 600 μg of histone to 600 g of a suspension of L-PS or D-PS in the form of LUVs, in PIPES buffer at 23 °C, at a final volume of 2.1 mL, resulted in an increase in either right angle light scattering or turbidity. Prior to the addition of histone the two types of LUVs had about equal light scattering. Upon addition of histone, within the time required for mixing, the samples with L-PS showed a 38 ± 5% greater increase in light scattering intensity than the samples with D-PS (Figure 3). Similar results were observed with turbidity measurements.

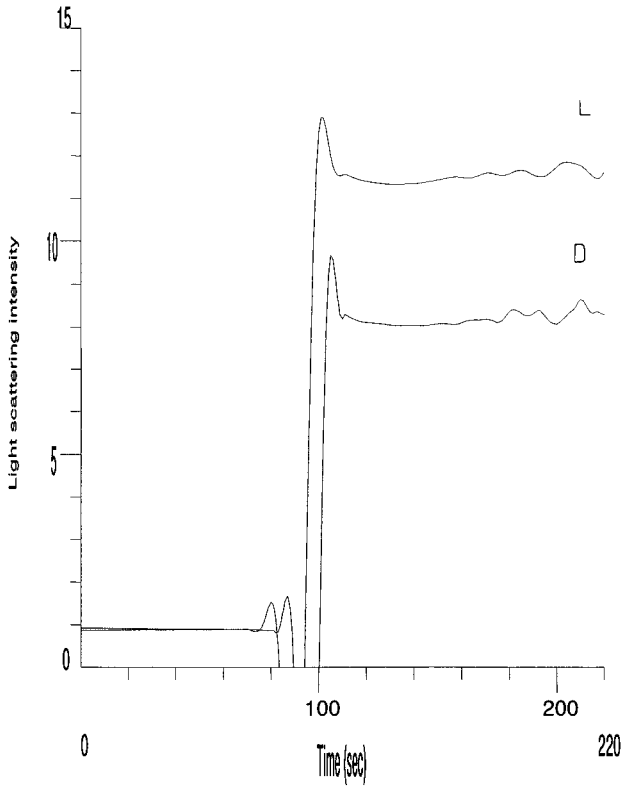

FIGURE 3: Right angle light scattering from a suspension of 600 μg of LUVs of 0.1 μm diameter of L-PS (L) or D-PS (D) in a final volume of 150 mM NaCl, 20 mM PIPES, 1 mM EDTA, and 20 mg/L NaN_3, pH 7.4. Histone (600 μg) was added at approximately 80 s. Right angle light scattering was recorded at 320 nm.

Lipid Domain Formation. We investigated here the influence of the chirality of the serine group of phosphatidylserine (L-DOPS versus D-DOPS) in the formation of large domains in lipid bilayers. Vesicles composed of 85% POPC and 10% of either L-DOPS or D-DOPS and 5% DOG and labeled with both dansyl-DAG (1%) and NBD-PS (0.3%) were formed in 100 mM NaCl and 20 mM PIPES, pH 7.4, and examined under the microscope in the presence of either 500 μM $CaCl_2$, 1 μM MARCKS peptide, or 300 μg/mL calf thymus histone IIA. The distribution of NBD-PS was uniform in the vesicles before addition of substrate or Ca^{2+} (Figure 4A).

Ca^{2+} at a concentration of 500 μM induced the formation of domains enriched in NBD-PS (Figure 4B). The average and maximum enrichments of the domains are plotted in panels A and B of Figure 5, respectively. For every condition, enrichments were determined with a population of at least 20 vesicles. The errors in determining the enrichment of the domains for the population of vesicles were always less than $\pm$5%. No significant difference appeared in either the average or the maximum enrichment, when the L-DOPS was substituted by the diastereoisomer D-DOPS (Figure 5). Moreover, no significant (that is, greater than 1.5-fold) enrichment in dansyl-DAG was observed with either phosphatidylserine isomers (not shown).

When introduced at a concentration of 1 μM, the MARCKS peptide induced the formation of domains enriched in NBD-PS (Figure 4C). The enrichment was equivalent to the effect of 500 μM $CaCl_2$ (Figure 5), and again no significant differences were observed between L-DOPS and D-DOPS (Figure 4C and Figure 5). Like Ca^{2+} under these conditions, the MARCKS peptide did not appear to trigger a significant enrichment of dansyl-DAG in domains (not shown). The results with calcium are different from previous data where calcium was able to segregate dansyl-DAG in the same domains with NBD-PS (*16*). The different ionic strengths of the buffers (100 mM NaCl and 20 mM PIPES, pH 7.4, in this study versus 10 mM Tris, pH 7, in ref *16*) and the resulting modifications of the electrostatic interactions of calcium with acidic lipids may account for this discrepancy.

When present at a concentration of 300 μg/mL, histones display very distinct features compared with calcium and the MARCKS peptides on PS-containing vesicles: (1) In vesicles containing L-DOPS, the enrichment of NBD-PS in domains induced by histones was consistently higher than in domains induced by calcium or MARCKS (Figure 5). (2) Dansyl-DAG was significantly enriched in the same domains with NBD-PS (Figure 4D and Figure 5). (3) A distinctive feature of the histones is the difference in enrichment of both NBD-PS and dansyl-DAG in domains when L-DOPS is substituted by D-DOPS (Figure 5). The enrichment of both fluorophores in domains was significantly higher in vesicles containing L-DOPS than D-DOPS, and they both exhibit the same relative variation of enrichment from L-DOPS to D-DOPS (Figure 5).

PKC Activity. We measured the activity of PKC in LUV as a function of the fraction of L- or D-DOPS in the membrane using histone as a substrate as described in Experimental Procedures. Our results were in general agreement with that previously published (*13*). We also performed the assay using the MARCKS peptide as substrate. We used two different concentrations of DG, viz. 2% and 5%. The former is more similar to recent work we have done, and the 5% value reproduces that used for the fluorescence microscopy. In both mixtures we find a significant increase in the rate of PKC-catalyzed phosphorylation of histone with L-PS compared with D-PS. This was not found when the MARCKS peptide was used as the substrate (Figure 6).

DISCUSSION

The two forms of PS have rather similar structures, with the same chemical groups and charge. If they were enantiomers, D-PS and L-PS would have to have identical phase transition properties as pure components. This would still not necessarily mean that they interact in an identical fashion, even without specific binding, with another chiral molecule such as PKC. In another case, it has recently been shown that gramicidin A channel function (a peptide having both L and D amino acids) is independent of phospholipid chirality (*24*). However, as pointed out in the introduction, D-PS and L-PS are diastereoisomers and not enantiomers. Therefore, it is possible that the small difference observed in the L_β to L_α transition temperature is accurate, indicating that L-PS and D-PS have different physical properties, in addition to their stereochemical differences. The ^{31}P NMR results with the lipid mixtures (Figure 2) show that when L-PS is present, there is a greater tendency to form inverted phases than with D-PS. The activity of PKC is promoted by lipids with an increased tendency to form H_{II} phases (*14*). For example, lipids with all of the acyl chains as oleoyl exhibit a greater activation of PKC than do 1-palmitoyl-2-oleoyl lipids with the same headgroups (*12*). The relationship of the physical properties of the membrane to its ability to support the

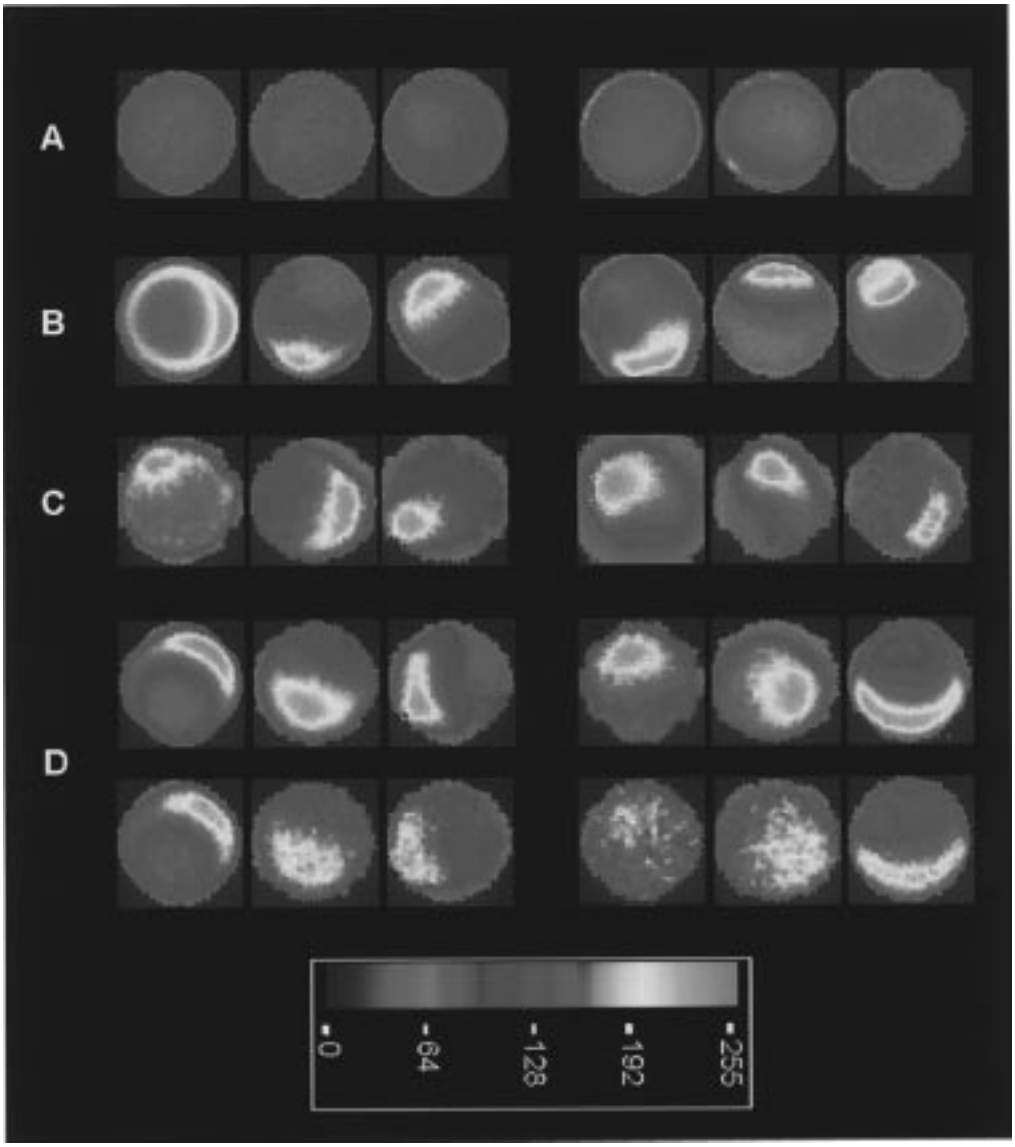

FIGURE 4: NBD-PS fluorescence of vesicles composed of DOPC (85%), DOG (5%), dansyl-DAG (1%), and NBD-PS (0.3%) with 10% of either L-DOPS (left three vesicles) or D-DOPS (right three vesicles) with no addition (control, A) or under the influence of 500 μM $CaCl_2$ (B), 1 μM MARCKS (C), or 300 μg/mL histone (D). The top row in (D) corresponds to NBD-PS fluorescence and the bottom row to dansyl-DAG fluorescence of the same vesicle. The pseudocolor scale indicates the radiance values.

activity of PKC is also suggested by the observation that L-PS vesicles are more extensively aggregated by histone than are vesicles composed of D-PS. The property of histone aggregation of micelles has been associated with their ability to support the activity of PKC (*18*).

It has been observed that molecular components that promote the activity of PKC are enriched in large membrane domains to which PKC preferentially binds (*15*, *16*). It is therefore likely that the formation of these domains results in an enhancement of the activity of this enzyme. The two forms of PS have rather similar domain-forming properties. Only in the presence of histone are marked differences found in the enrichment of both PS and DG in the domains. In this respect, histone behaves differently from the MARCKS peptide. We find that the enhanced activity of PKC with L-PS compared with D-PS is observed only with histone as a substrate and not with the MARCKS peptide. Thus, the formation of domains is well correlated with the observed enhanced activity.

There is, however, an additional factor to consider. It has been shown, using the sucrose-loaded vesicle assay (*25*), that PKC has a higher affinity for vesicles containing L-PS (with 1% DG, 25% POPS, and 74% POPC) than comparable vesicles with D-PS replacing L-PS (Mosior and Newton, personal communication). In addition, liposomes containing the enantiomeric forms of PS, PC, and DG do not bind PKC (Newton and Daleke, personal communication). A factor contributing to this is the high degree of stereospecificity known to be required for activation of PKC by DG. Nevertheless, these results demonstrate that lipid chirality also plays an important role in determining the relative binding affinity of PKC. These PKC binding assays are done in the absence of any substrate. Since PKC binding to the membrane is closely correlated with activation (*25*), it is likely that other factors in addition to histone-induced domain formation may contribute to the greater activation observed with L-PS.

In conclusion, it is clear that the physical properties of L-PS and D-PS are different. It is thus not justified to conclude that their different abilities to activate PKC demonstrate that there is a specific binding site for L-PS on the enzyme. The putative binding site for PS on PKC was

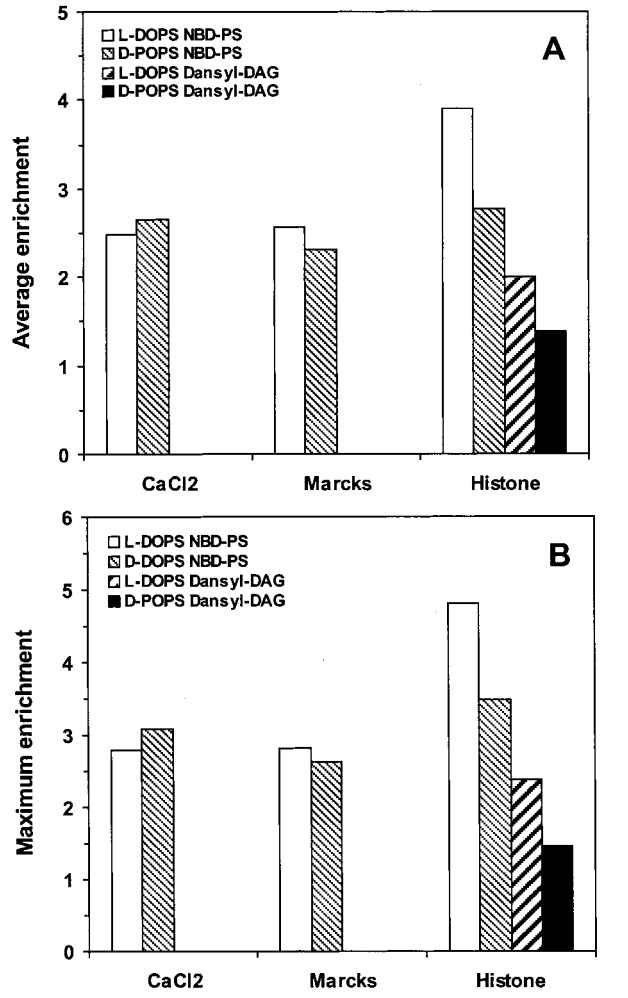

FIGURE 5: Average (A) and maximum (B) enrichment of NBD-PS and dansyl-DAG in domains under the influence of 500 μM $CaCl_2$, 1 μM MARCKS, or 300 μg/mL histone in vesicles composed of DOPC (85%), DOG (5%), dansyl-DAG (1%), and NBD-PS (0.3%) with 10% of either L-DOPS or D-DOPS. The error in determining the mean enrichment for the population of vesicles was less than ±5%.

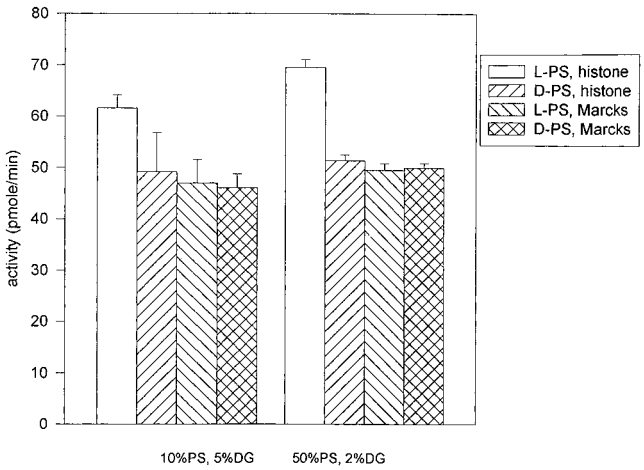

FIGURE 6: PKC phosphorylation of histone (□, ⧄) or the MARCKS peptide (⧅, ⊞) with either L-PS (□, ⊞) or D-PS (⧄, ⊞). The lipid composition of the LUVs was 10% PS and 5% DG (left) or 50% PS and 2% DG (right). Details of the assay conditions are given in Experimental Procedures.

shown not to be involved in the lipid regulation of the enzyme (*26*). Furthermore, the increased tendency of L-PS to form inverted phases as well as PS/DG-enriched domains would contribute to the greater activation of PKC with this stereoisomer of PS. The finding that L-PS and D-PS show similar activation of PKC when the MARCKS peptide is used as substrate, but not when histone is used as substrate, is good evidence for the importance of domain formation in the activation of PKC.

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