Lipid Structure and Not Membrane Structure Is the Major Determinant in the Regulation of Protein Kinase C by Phosphatidylserine[†]

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Received May 13, 1998; Revised Manuscript Received July 17, 1998

ABSTRACT: This study addresses the molecular basis for protein kinase C's specific activation by phosphatidylserine. Specifically, we ask whether protein kinase C's phospholipid specificity arises from specific protein/lipid interactions or whether it arises from unique membrane-structuring properties of phosphatidylserine. We measured the interaction of protein kinase C βII to membranes that differed only in being enantiomers to one another: physical properties such as acyl chain composition, membrane fluidity, surface curvature, microdomains, headgroup packing, and H-bonding with water were identical. Binding and activity measurements reveal that protein kinase C specifically recognizes 1,2-*sn*-phosphatidyl-L-serine, independently of membrane structure. High-affinity binding and activation are abolished in the presence of enantiomeric membranes containing 2,3-*sn*-phosphatidyl-L-serine, 2,3-*sn*-diacylglycerol, and 2,3-*sn*-phosphatidylcholine. Our data also show that the stereoselectivity for 1,2-*sn*-diacylglycerol is not absolute; 2,3-*sn*-diacylglycerol modestly increases the membrane affinity of protein kinase C provided that 1,2-*sn*-phosphatidyl-L-serine is present. We also find that the stereochemistry of the bulk phospholipid, in this case phosphatidylcholine, has no significant influence on protein kinase C's membrane interaction. These data reveal that specific molecular determinants on protein kinase C stereospecifically recognize structural determinants of phosphatidylserine.

Local alterations in membrane architecture afford a widely used mechanism to modulate the function of membrane-interacting proteins. Modulation of protein function can occur via specific protein/lipid interactions or as a result of changes in physical properties of the membrane. Some proteins, for example CTP/phosphocholine cytidylyltransferase, are sensitive to membrane physical properties such as net charge and the presence of packing defects, rather than having specific lipid structural requirements (*1*). Others, such as phosphatidylcholine butyrate dehydrogenase, are activated by interaction with a specific phospholipid (*2*).

Nowhere is modulation by membrane interactions more apparent than in signal transduction (*3*). Most noteworthy is the translocation of signaling proteins to the membrane as a result of changes in membrane composition, or, in some cases, protein structure (e.g., Ca^{2+}-induced conformational changes, acylation). The resulting protein/membrane interactions regulate protein function either conformationally or by localizing the signaling protein near its substrates or other regulators. Often, specific membrane-targeting modules on the protein respond to changes in lipid composition. For example, PH domain-containing proteins are acutely regulated by interaction with poly-phosphoinositides: the newly discovered protein kinase B kinase is activated by a stereospecific interaction with phosphatidylinositol-3,4,5-trisphosphate (*4*).

Protein kinase C has served as a paradigm for lipid-regulated proteins ever since the discovery two decades ago that it is activated by diacylglycerol and phosphatidylserine (*5*). This family of serine/threonine kinases transduces the myriad of signals that promote phospholipid hydrolysis (*6*). Generation of diacylglycerol, typically in the plasma membrane, recruits cytosolic protein kinase C to the membrane where an additional interaction with phosphatidylserine results in the removal of an autoinhibitory pseudosubstrate domain from the active site (*7*). This membrane interaction is mediated by two separate membrane-targeting modules, the C1 domain which binds diacylglycerol, and the C2 domain which binds anionic lipids and, for conventional protein kinase Cs, Ca^{2+} (*3*, *8*). Although either domain alone can recruit protein kinase C to membranes, both domains must be membrane-bound to supply the energy required to release the pseudosubstrate from the active site.[1]

A hallmark of protein kinase C's lipid regulation is the remarkable specificity for its lipid activators (*3*). Extensive biochemical studies have established that the enzyme is

[†] This work was supported by grants from the National Institutes of Health (GM 43154 (A.C.N.) and GM 47230 (D.L.D.)), the American Heart Association, Indiana Affiliate (D.L.D.), an Established Investigatorship of the American Heart Association (D.L.D.), and a National Science Foundation Young Investigator Award (A.C.N.). J.E.J. was supported in part by a Medical Research Council of Canada Postdoctoral Fellowship.

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[1] Protein kinase C's pseudosubstrate can also be released by a number of membrane-unrelated events, including interaction of the kinase with short-chained phospholipids, fatty acids, or protamine-sulfate (*7*).

S0006-2960(98)01107-6 CCC: $15.00
Published on Web 08/14/1998

maximally activated by 1,2-*sn*-diacylglycerol and 1,2-*sn*-phosphatidyl-L-serine both in detergent/lipid mixed micelles (*9*−*11*) and in model membranes (*12*). Most remarkably, the enzyme binds surfaces containing diacylglycerol and phosphatidyl-L-serine with over an order-of-magnitude higher affinity than surfaces containing diacylglycerol and other anionic lipids, including phosphatidyl-D-serine (*13*). In the absence of diacylglycerol, the enzyme binds anionic membranes with no significant headgroup selectivity beyond the requirement for negative charge. Thus, protein kinase C's specificity for phosphatidylserine requires diacylglycerol.

The molecular basis for protein kinase C's remarkable specificity for phosphatidylserine has been the subject of much debate. One possibility is that phosphatidylserine possesses unique properties allowing it to optimally present diacylglycerol to the C1 domain of protein kinase C. Thus, the observed specificity would arise because of unique membrane-structuring properties of the phospholipid which better present the activating ligand, diacylglycerol, to protein kinase C. A second possibility is that protein kinase C contains determinants that specifically recognize the structure of the L-serine headgroup. These determinants may only become exposed upon interaction of the C1 domain with membranes, perhaps comprising surfaces on both the C1 and C2 domains. The persistence of specificity in detergent/lipid micellar systems, where as few as half a dozen molecules of phosphatidylserine activate protein kinase C, reveals that a membrane bilayer structure is not required for protein kinase C's activation. Nonetheless, protein kinase C activity is modulated by bulk properties of the membrane, leading several groups to propose that the presence of phosphatidylserine and diacylglycerol together may optimize the structural organization of the membrane in such a way as to present an ideal binding surface for protein kinase C, in the absence of specific protein/phospholipid interactions (*14*, *15*).

This study addresses the molecular basis for protein kinase C's exquisite specificity for phosphatidylserine. We have synthesized enantiomeric lipids, allowing us to present protein kinase C with membranes that differ only in being mirror images of one another; importantly their physical properties are identical. We take advantage of these enantiomeric membranes to ask whether protein kinase C recognizes membrane structure or lipid structure. Binding and activity assays reveal that protein kinase C recognizes specific structural determinants unique to 1,2-*sn*-phosphatidyl-L-serine, independently of membrane structural organization. Thus, the highly specific regulation of protein kinase C by phosphatidylserine arises from stereospecific recognition of this lipid by determinants on protein kinase C, and does not reflect unique membrane-structuring properties of this lipid.

MATERIALS AND METHODS

Materials. D-Serine, phospholipase A_2 (*Naja naja naja*), phospholipase C (*Clostridium welchii*), phospholipase D (*S. species*), oleic anhydride, ATP, and protamine sulfate were purchased from Sigma Chemical Co. (St. Louis, MO). 1-Palmitoyl-*rac*-glycero-3-phosphocholine was purchased from Alexis, Corp. (San Diego, CA). 1-Palmitoyl-2-oleoyl-*sn*-phosphatidyl-L-serine, 1-palmitoyl-2-oleoyl-*sn*-phosphatidylcholine, and 1-palmitoyl-2-oleoyl-*sn*-glycerol were purchased from Avanti Polar Lipids. Silica gel HL thin-layer chromatography (TLC) plates were purchased from Analtech Inc. (Newark, DE). The protein kinase C-selective peptide substrate [Ac-FKKSFKL-NH_2] was kindly synthesized by Dr. E. Komives, UCSD. [γ-^{32}P]ATP (3000 Ci mmol^{-1}) and [^{3}H]dipalmitoylphosphatidylcholine (DPPC)[2] (30 Ci mmol^{-1}) were from NEN Life Sciences. All other chemicals were reagent grade. Protein kinase C βII was purified to homogeneity from the baculovirus expression system and was stored at −20 °C in 10 mM Tris (pH 7.5 at 4 °C), 0.5 mM EDTA, 0.5 mM EGTA, 150 mM KCl, 0.5 mM DTT, and 50% (v/v) glycerol, as described (*16*).

Synthesis of 2-Oleoyl-3-palmitoyl-sn-phosphatidylcholine (2,3-sn-POPC), 2-Oleoyl-3-palmitoyl-sn-phosphatidyl-D-serine (2,3-sn-POP-D-S), and 2-Oleoyl-3-palmitoyl-sn-glycerol (2,3-sn-DG). 1,2-*rac*-POPC was synthesized by acylation of 1-palmitoyl-*rac*-glycero-3-phosphocholine with oleic anhydride according to the method of Mangroo and Gerber (*17*) and purified by flash silica chromatography. 2,3-*sn*-POPC was isolated by stereoselective cleavage of the 1,2-*sn*-POPC component of the racemic mixture with phospholipase A_2 (*18*). 1-Lyso-palmitoylphosphatidylcholine was removed by sequential flash silica gel and CM-52 chromatography (*19*), and the structure of the product, 2,3-*sn*-POPC, was confirmed by FAB mass spectra (m/z [M-CH_2]$^-$ = 744; ref *20*), 300 MHz NMR, and optical rotary dispersion measurements ($[M]_{300} = -232$). 2,3-*sn*-POP-D-S was synthesized by transphosphatidylation of 2,3-*sn*-POPC with D-serine (*21*) and purified by CM-52 chromatography (*19*). FAB mass spectra (m/z [M−H]$^-$ = 760), 300 MHz NMR, and optical rotary dispersion measurements ($[M]_{250} = -441$) were consistent with the predicted structure. Phospholipids were analyzed on silica gel HL thin-layer plates by elution with chloroform/methanol/formic acid (98%) (65:25:5; v/v). TLC-separated phospholipids were quantified by inorganic phosphate analysis (*22*). 2,3-*sn*-DG was synthesized by phospholipase C cleavage of 2,3-*sn*-POPC, by a method previously reported (*23*). After separation from the phosphocholine by chloroform/methanol extraction, 2,3-*sn*-DG was further purified by thin-layer chromatography using chloroform/acetone (95:5; v/v). TLC-purified 2,3-*sn*-DG was quantified by densitometric analysis (Molecular Dynamics scanner, ImageQuant software) of an iodine-stained chromatograph containing 2,3-*sn*-DG and standard amounts of 1,2-*sn*-DG. All lipids were >98% pure.

Analytical Methods. Negative ion FAB mass spectrometry data were collected by Dr. Douglas Gage at NIH Mass Spectroscopy Laboratories at Michigan State University, East Lansing, MI. ORD of lipid stereoisomers was measured in chloroform solution at 20 °C at 250 nm with a Jasco J20A recording spectropolarimeter (*24*). NMR data were collected on a 300 MHz Varian superconducting NMR spectrometer.

Lipid Vesicles. Sucrose-loaded large unilamellar vesicles containing trace [^{3}H]DPPC were prepared by drying mixtures of lipids in chloroform under a stream of nitrogen, followed by evacuation under vacuum. Lipids were suspended in 20

[2] Abbreviations: DPPC, dipalmitoylphosphatidylcholine; DTT, dithiothreitol; 1,2-*sn*-DG, 1-palmitoyl-2-oleoyl-*sn*-glycerol; 2,3-*sn*-DG, 2-oleoyl-3-palmitoyl-*sn*-glycerol; 1,2-*sn*-POPC, 1-palmitoyl-2-oleoyl-*sn*-phosphatidylcholine; 2,3-*sn*-POPC, 2-oleoyl-3-palmitoyl-*sn*-phosphatidylcholine; 1,2-*sn*-POP-L-S, 1-palmitoyl-2-oleoyl-*sn*-phosphatidyl-L-serine; 2,3-*sn*-POP-D-S, 2-oleoyl-3-palmitoyl-*sn*-phosphatidyl-D-serine.

mM HEPES, pH 7.5, 170 mM sucrose and then subjected to 5 freeze−thaw cycles followed by extrusion using a Liposofast microextruder (Avestin, Inc.), as described (*25*).

Protein Kinase C Membrane-Binding Assay. The binding of protein kinase C to sucrose-loaded vesicles was measured as described previously (*25, 26*). Briefly, protein kinase C (0.8 nM protein kinase C) was incubated with sucrose-loaded vesicles (50 μM lipid) in the presence of 0.3 mM $CaCl_2$, 0.3 mg mL^{-1} BSA, 100 mM KCl, 1 mM DTT, 5 mM $MgCl_2$, 20 mM HEPES, pH 7.5. Membrane-bound protein kinase C was separated from free enzyme by centrifugation at 100000*g* for 30 min at 25 °C. The fraction of sedimented vesicles was determined from radioactivity; the fraction of protein kinase C that sedimented with the vesicles was determined by assaying both the activity of protein kinase C in the supernatant and that associated with the pellet under identical conditions using the cofactor-independent substrate, protamine sulfate (0.2 mg mL^{-1}). The apparent membrane affinity was calculated as the ratio of free/bound protein kinase C divided by the total lipid concentration, as described (*25*).

Protein Kinase C Activity Assay. Protein kinase C activity toward a synthetic peptide (Ac-FKK$\underline{S}$FKL-NH_2) was measured as described previously (*16*). The standard reaction contained sucrose-loaded vesicles (50 μM lipid) in the presence of 100 μM [γ-^{32}P]ATP (250 mCi $mmol^{-1}$), 100 μM peptide substrate, 0.3 mM $CaCl_2$, 0.3 mg mL^{-1} BSA, 100 mM KCl, 1 mM DTT, 5 mM $MgCl_2$, 20 mM HEPES, pH 7.5. One unit of activity is defined as one nanomole of phosphate transferred per minute at 30 °C under the standard assay conditions described above.

RESULTS

The mechanism of protein kinase C's specific requirement for phosphatidylserine was addressed by examining the contribution of membrane structure versus lipid structure in providing a surface for protein kinase C to bind and become activated. To this end, we synthesized the enantiomeric counterpart of the lipids present in normal "activating membranes": phosphatidylserine, diacylglycerol, and phosphatidylcholine. Figure 1 shows the structure of the two enantiomers of phosphatidylserine, the naturally occurring 1-palmitoyl-2-oleoyl-*sn*-phosphatidyl-L-serine (1,2-*sn*-POP-L-S) and its enantiomer 2-oleoyl-3-palmitoyl-*sn*-phosphatidyl-D-serine (2,3-*sn*-POP-D-S). Note that there are two chiral centers in this lipid, one in the central carbon of the glycerol backbone and the other in the serine headgroup; the two molecules in Figure 1 differ only in the chirality at each of these positions. We also synthesized the enantiomers of 1,2-*sn*-diacylglycerol (1,2-*sn*-DG) and 1-palmitoyl-2-oleoyl-*sn*-phosphatidylcholine (1,2-*sn*-POPC): 2,3-*sn*-diacylglycerol (2,3-*sn*-DG) and 2-oleoyl-3-palmitoyl-*sn*-phosphatidylcholine (2,3-*sn*-POPC). These lipids contain only one chiral center in the glycerol backbone.

The purity of the lipids used in this study was determined by thin-layer chromatography (Figure 2). As expected, the enantiomeric pairs of phospholipids migrate with the same mobility on thin-layer chromatograms (Figure 2A; compare lanes 1 and 2 for the two phosphatidylcholines and lanes 3 and 4 for the two phosphatidylserines). Similarly, the two diacylglycerol enantiomers migrate with the same mobility

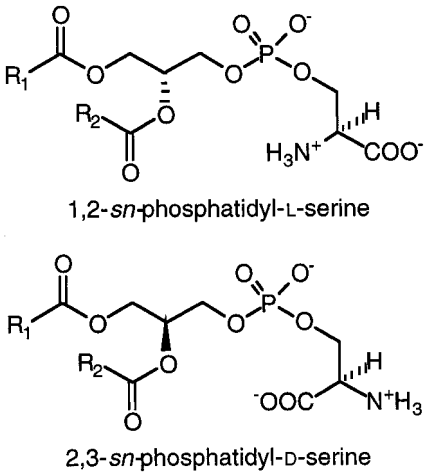

FIGURE 1: Structures of 1,2-*sn*-phosphatidyl-L-serine and its enantiomer 2,3-*sn*-phosphatidyl-D-serine. Note that these molecules differ only in the chirality at two positions, the *sn*-2 position of the glycerol backbone and in the serine headgroup. Acyl chain compositions of the two molecules are identical: R_1 = palmitoyl; R_2 = oleoyl.

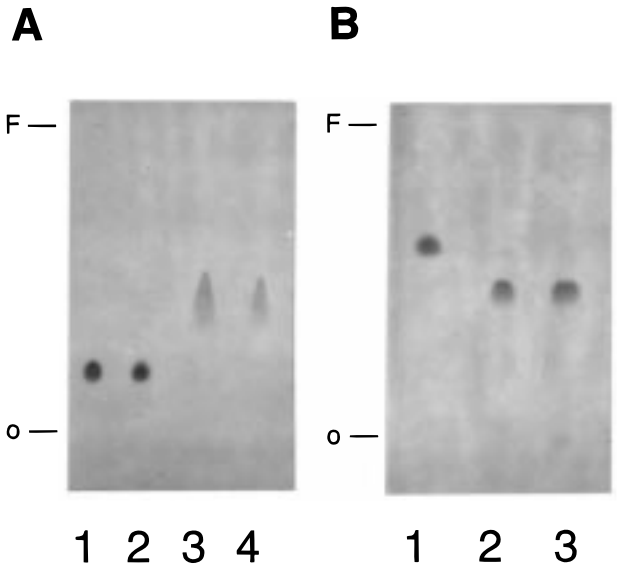

FIGURE 2: Thin-layer chromatographic analysis of lipids used in this study. (A) Phospholipids (50 nmol) were chromatographed in chloroform/methanol/formic acid (65:25:5, v/v): 1,2-*sn*-POPC (lane 1), 2,3-*sn*-POPC (lane 2), 1,2-*sn*-POP-L-S (lane 3), and 2,3-*sn*-POP-D-S (lane 4). (B) Diacylglycerols (50 nmol) were chromatographed in chloroform/acetone (95:5): 1,3-*sn*-DG (lane 1), 1,2-*sn*-DG (lane 2), and 2,3-*sn*-DG (lane 3). All lipids were visualized by iodine vapor. The origin (O) and solvent fronts (F) are labeled.

(Figure 2B; lanes 2 and 3), in contrast to the 1,3 stereoisomer which has different physical properties and migrates with a mobility different from that of the 1,2 or 2,3 enantiomers.

Large unilamellar vesicles composed of physiological lipids or their enantiomeric counterparts were made, and their ability to activate protein kinase C was measured (Figure 3A). Vesicles composed of the naturally occurring enantiomers 1,2-*sn*-POP-L-S, 1,2-*sn*-DG, and 1,2-*sn*-POPC (40:5:55 mol %) were potent activators of protein kinase C (column 1); the activity supported by these vesicles was 290 ± 30 units $nmol^{-1}$ and represented the maximal rate of catalysis of protein kinase C (on the order of 5 reactions/s (*27*)). In striking contrast, alteration in the stereochemistry of all three lipids (column 5) to form the mirror image membranes of

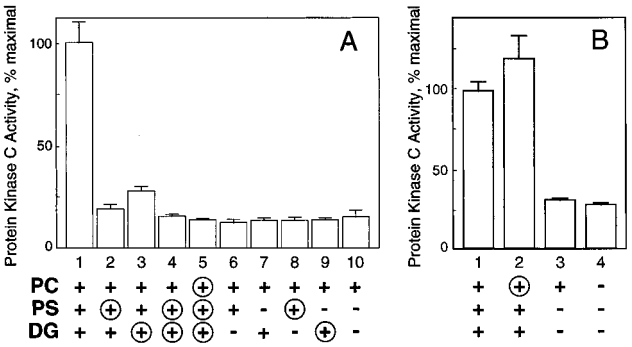

FIGURE 3: Stereospecific activation of protein kinase C βII by membranes containing both 1,2-*sn*-phosphatidyl-L-serine and 1,2-*sn*-diacylglycerol. The phosphorylation of a protein kinase C-selective peptide was measured in the presence of large unilamellar vesicles (50 μM total lipid) and 0.3 mM Ca^{2+} as described in Methods. (A) Vesicles were composed of (1) 1,2-*sn*-POPC, 1,2-*sn*-POP-L-S, and 1,2-*sn*-DG (55:40:5); (2) 1,2-*sn*-POPC, 2,3-*sn*-POP-D-S, and 1,2-*sn*-DG (55:40:5); (3) 1,2-*sn*-POPC, 1,2-*sn*-POP-L-S, and 2,3-*sn*-DG (55:40:5); (4) 1,2-*sn*-POPC, 2,3-*sn*-POP-D-S, and 2,3-*sn*-DG (55:40:5); (5) 2,3-*sn*-POPC, 2,3-*sn*-POP-D-S, and 2,3-*sn*-DG (55:40:5); (6) 1,2-*sn*-POPC and 1,2-*sn*-POP-L-S (60:40); (7) 1,2-*sn*-POPC and 1,2-*sn*-DG (95:5); (8) 1,2-*sn*-POPC and 2,3-*sn*-POP-D-S (60:40); (9) 1,2-*sn*-POPC and 2,3-*sn*-DG (95:5); and (10) 1,2-*sn*-POPC. (B) Vesicles were composed of (1) 1,2-*sn*-POPC, 1,2-*sn*-POP-L-S, and 1,2-*sn*-DG (55:40:5); (2) 2,3-*sn*-POPC, 1,2-*sn*-POP-L-S, and 1,2-*sn*-DG (55:40:5); (3) 1,2-*sn*-POPC (100%); or (4) in the absence of lipid. Data are normalized to the activity in column 1 and are expressed as the average ±SEM of 4−6 determinations, except in column 6 (A) and columns 1−4 (B), where the data are expressed as a mean ± range of duplicate measurements. Circled lipids indicate a 2,3-*sn*-backbone.

those in column 1 abolished cofactor-dependent protein kinase C activity (i.e., activity was reduced to that observed in the absence of any lipid (not shown) or in the presence of phosphatidylcholine alone (column 10)). Both 1,2-*sn*-POP-L-S and 1,2-*sn*-DG were required to activate protein kinase C. Alteration in the stereochemistry of either (columns 2 and 3, Figure 3A), or removal of either (columns 6 and 7, Figure 3A), resulted in membranes unable to significantly activate protein kinase C. In contrast, substitution of 1,2-*sn*-POPC (column 1, Figure 3B) with its enantiomer (column 2, Figure 3B) had no significant effect on protein kinase C activity. Thus, protein kinase C activity depended on the presence of both 1,2-*sn*-POP-L-S and 1,2-*sn*-DG, independently of bulk lipid composition. Interestingly, the requirement for the 1,2 isomer of diacylglycerol was not absolute: membranes containing 2,3-*sn*-DG supported slight activation of protein kinase C (approximately 27% of maximal cofactor-dependent activity (column 3, Figure 3A)). The requirement for the 1,2 isomer of phosphatidylserine was, however, absolute.

We next addressed whether the inability of enantiomeric membranes to activate protein kinase C resulted from the lack of recognition of these membranes by the kinase or from the inability of the membranes to promote activating conformational changes. Specifically, we asked how lipid structure affected the binding of protein kinase C to membranes. Figure 4 shows that protein kinase C bound to membranes containing 1,2-*sn*-POP-L-S and 1,2-*sn*-DG (40:5 mol %; remaining lipid 1,2-*sn*-POPC) with an apparent membrane affinity of 8.5×10^4 M^{-1} (column 1) similar to previously reported values (*15*, *28*, *29*). In marked contrast, no binding was detected to enantiomeric membranes (column

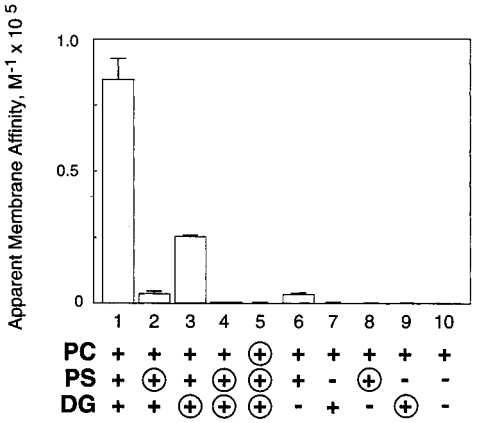

FIGURE 4: Stereospecific binding of protein kinase C βII to membranes containing both 1,2-*sn*-phosphatidyl-L-serine and 1,2-*sn*-diacylglycerol. The binding of protein kinase C to large unilamellar vesicles (50 μM total lipid) was measured in the presence of 0.3 mM Ca^{2+}. Vesicle compositions were as described in the legend to Figure 3A. The apparent membrane-binding affinity was calculated from the ratio of protein kinase C that was free in solution to the amount that was vesicle-associated, divided by the total lipid concentration, as described (*25*). Data are expressed as the average ±SEM of 4−6 determinations except column 6, which is expressed as a mean ± range. Circled lipids indicate a 2,3-*sn*-backbone.

5) under conditions where the only difference between assays was that the membranes in columns 1 and 5 were the mirror images of one another. Acyl chain composition, lipid composition, and binding conditions were identical.

Figure 4 also confirmed that the selective high-affinity interaction of protein kinase C for 1,2-*sn*-POP-D-S depended on diacylglycerol (*13*). The replacement of 1,2-*sn*-POP-L-S with its enantiomer, 2,3-*sn*-POP-D-S, in membranes containing 1,2-*sn*-DG resulted in a 20-fold drop in protein kinase C's membrane affinity, from 8.5×10^4 to 3.6×10^3 M^{-1} (Figure 4, columns 1 and 2). Binding to membranes containing 2,3-*sn*-POP-D-S either in the presence (column 2) or in the absence (column 8) of diacylglycerol was also weak. Note that the lipid concentrations used in Figure 4 (0.05 mM total lipid) were too low to accurately measure the low-affinity binding to anionic membranes. When similar experiments ($n = 2$) were conducted in the presence of 0.2 mM lipid, membrane binding was sufficiently high to allow calculation of the following apparent binding constants to membranes containing either phosphatidylserine enantiomer (40 mol %) and 1,2-*sn*-PC (60 mol %): $(4.24 \pm 0.06) \times 10^3$ M^{-1} to membranes containing 1,2-*sn*-POP-L-S and $(2.5 \pm 0.1) \times 10^3$ M^{-1} to membranes containing 2,3-*sn*-POP-D-S. Thus, protein kinase C bound with approximately equal affinity to the two phosphatidylserine enantiomers in the absence of diacylglycerol. In striking contrast, diacylglycerol caused over an order of magnitude increase in binding affinity that was specific for 1,2-*sn*-POP-L-S-containing membranes and not the enantiomer of this lipid.

Last, the data in Figure 4 show that the binding selectivity for 1,2-*sn*-DG is not absolute, in contrast to the absolute selectivity for 1,2-*sn*-phosphatidyl-D-serine. Although binding was selective for 1,2-*sn*-DG, replacement of this lipid in 1,2-*sn*-POPS membranes with 2,3-*sn*-DG resulted in only a modest 3.4-fold reduction in binding affinity from 8.5×10^4 (column 1) to 2.5×10^4 M^{-1} (column 3). Racemization

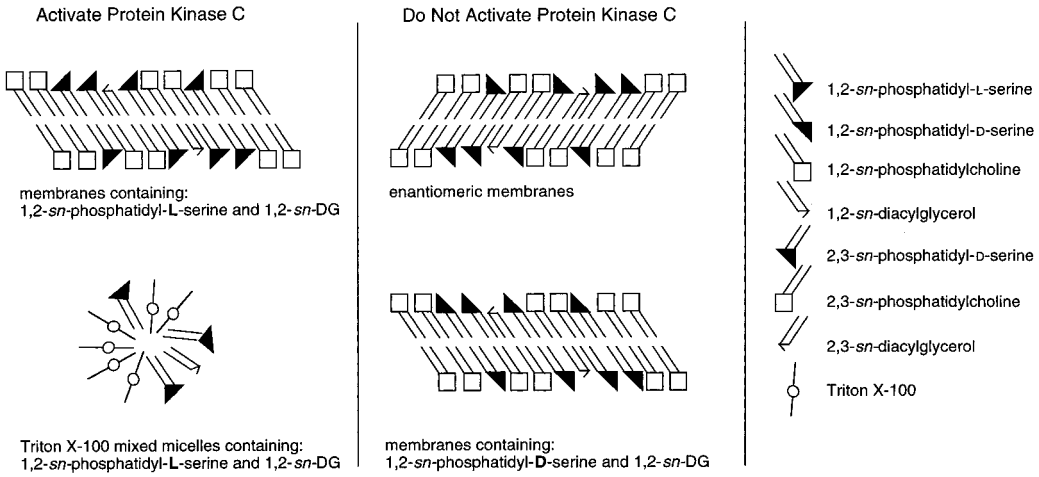

FIGURE 5: Lipid structure, rather than membrane structure, is the critical determinant in protein kinase C's phospholipid activation. Left panel: Maximal activation of protein kinase C requires both 1,2-*sn*-DG and 1,2-*sn*-phosphatidyl-L-serine independently of bilayer structure. Middle panel: Protein kinase C is not activated by, and does not bind with, high-affinity, enantiomeric membranes containing 2,3-*sn*-DG, 2,3-*sn*-phosphatidyl-D-serine, and 2,3-*sn*-PC, nor does it interact with 1,2-*sn*-phospholipid membranes in which phosphatidylserine contains the D-serine headgroup. Right panel: key describing lipid structures.

of the 2,3-*sn*-DG to 1,2-*sn*-DG is unlikely to account for the binding in column 3: first, no 1,3-*sn*-DG intermediate was detected by thin-layer chromatography (Figure 2B; ref *30*) and second, the 2,3-*sn*-DG was much less effective at promoting activation (Figure 3, column 3) compared with binding (Figure 4, column 3).

DISCUSSION

Molecular Basis for Protein Kinase C's Requirement for Phosphatidylserine. The requirement for phosphatidylserine and diacylglycerol in activating protein kinase C has been extensively documented (*10*−*13*, *31*, *32*). Nonetheless, the molecular basis for this selectivity has remained elusive. Structural studies have clearly established the presence of a specific binding site for diacylglycerol in the C1 domain, providing a molecular basis for the selective binding of both phorbol esters and diacylglycerol to the kinase (*33*). However, whether the specific interaction with phosphatidylserine arises from the presence of a specific binding site for this lipid has been the subject of much debate.

One hypothesis to explain the exquisite sensitivity of protein kinase C to phosphatidylserine is that this lipid has unique membrane-structuring properties that allow it to optimally present diacylglycerol to the C1 domain. Thus, it has been proposed that protein kinase C does not actually recognize the serine headgroup, but rather recognizes the membrane structure formed by this lipid. This model has been used to account for why modulators of membrane structure influence protein kinase C activity (*14*, *15*).

A second possibility is that protein kinase C possesses specific determinants on its surface that bind the L-serine headgroup. That is, a specific protein/phospholipid interaction drives the interaction of protein kinase C with membranes. This possibility is supported by the finding that lipid selectivity is independent of surface presentation of the lipid: the diacylglycerol-induced high-affinity binding to phosphatidylserine is observed both in membranes and in detergent/lipid mixed micelles (*13*) (see Figure 5, left panel).

To distinguish between these two possibilities, we measured the binding of protein kinase C to membranes that differed only by being enantiomers of one another (Figure 5). That is, all physical properties, including acyl chain composition, membrane fluidity, surface curvature, microdomains, headgroup packing, and H-bonding with water, were identical. Assay conditions were also identical: these included ionic strength, calcium and magnesium concentrations, substrate concentration, and vesicle size. Thus, events such as cation-induced aggregation or microdomain formation (*14*, *34*−*36*) would be expected to be the same in both assay conditions.

Our data reveal that lipid structure, rather than membrane structure, is the critical determinant in the regulation of protein kinase C by phosphatidylserine. Specifically, we show that the high-affinity binding to membranes containing 1,2-*sn*-phosphatidyl-L-serine, 1,2-*sn*-DG, and 1,2-*sn*-phosphatidylcholine is abolished upon substitution of these lipids with their enantiomeric counterparts. Furthermore, the stereochemistry of the bulk lipid does not influence the membrane binding or activation mediated by 1,2-*sn*-phosphatidyl-L-serine. Because enantiomeric membranes contain 2,3-*sn*-DG, it is possible that they do not activate protein kinase C because a stereospecific interaction between the C1 domain and diacylglycerol has been impaired. However, our study reveals that 2,3-*sn*-DG is almost as effective as 1,2-*sn*-DG in mediating the high-affinity membrane interaction of protein kinase C. Thus, protein kinase C would be expected to have an increased affinity for enantiomeric membranes if the role of phosphatidylserine is to better present the C1 domain ligand to the protein. The inability of enantiomeric membranes to activate protein kinase C indicates that specific lipid structural determinants, independently of membrane structural organization, provide the driving force in protein kinase C's interaction with phosphatidylserine.

An intriguing feature of protein kinase C's high-affinity binding to phosphatidylserine is that it requires diacylglycerol (*13*, *37*). This suggests that interaction of the C1 domain with membranes unmasks determinants on protein kinase C that bind phosphatidylserine. Such determinants are unlikely to reside exclusively on the C2 domain[3] because binding

driven by interactions with this domain alone (i.e., in the absence of C1 domain ligands) displays no headgroup selectivity beyond the requirement for negative charge (*13*). Consistent with this, mutagenesis of the C2 domain impairs Ca^{2+} and membrane binding without altering the diacylglycerol-induced selectivity for phosphatidylserine (*29*). The possibility that a binding site is formed by determinants contributed by both the C1 domain and the C2 domain is attractive given that phosphatidylserine selectivity appears to require membrane juxtaposition of both domains.

Membrane Properties Modulate Protein Kinase C But Are Not Responsible for Phosphatidylserine Specificity. Although the binding measurements described above establish that the selectivity of protein kinase C for phosphatidylserine results from its specific binding to this lipid, the activity of the protein is nonetheless sensitive to bulk membrane properties. Acyl chain composition (*15*, *38*, *39*), the presence of diacylglycerol-rich and diacylglycerol-poor microdomains (*14*, *35*), the presence of nonlamellar forming lipids such as phosphatidylethanolamine and unsaturated fatty acids (*40*−*42*), and the presence of membrane intercalators such as anesthetics (*43*) influence protein kinase C activity. Membrane changes promoting looser headgroup packing may be particularly important for regulating protein kinase C activity by facilitating intercalation of membrane-binding domains into the membrane core, an insertion proposed for the C1 domain (*33*, *44*). Optimal headgroup spacing could also facilitate protein/lipid interactions.

CONCLUSIONS

The above studies reveal that the key determinant in the phospholipid-dependent activation of protein kinase C is specific recognition of the 1,2-*sn*-phosphatidyl-L-serine molecule. This recognition is independent of membrane structural organization, but requires the binding of diacylglycerol to the C1 domain. Although membrane structure modulates the activity of protein kinase C, it is the specific binding of protein kinase C to its two lipid cofactors that provides the allosteric switch to activate protein kinase C.

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[3] Although the C2 domain was previously proposed to contain a phosphatidylserine-binding motif (*45*), mutagenesis of this sequence recently established that it is not involved in the lipid regulation of protein kinase C (*28*).

BI981107Q