High Cooperativity, Specificity, and Multiplicity in the Protein Kinase C-Lipid Interaction*

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The number of phosphatidylserine molecules involved in activating protein kinase C was determined in a mixed micelle system where one monomer of protein kinase C binds to one detergent:lipid micelle of fixed composition. Unusually high cooperativity, specificity, and multiplicity in the protein kinase C-phospholipid interaction are demonstrated by examining the lipid dependence of enzymatic activity. The rates of autophosphorylation and substrate (histone) phosphorylation are specifically regulated by the phosphatidylserine content of the micelles. Hill coefficients of 8–11 were calculated for phosphatidylserine-dependent stimulation of enzyme activity, with a maximum occurring in micelles containing ≥12 phosphatidylserine molecules. The high specificity that exists is illustrated by the fact that phosphatidylethanolamine and phosphatidylglycerol, but not phosphatidylcholine or phosphatidic acid, can replace only some of the phosphatidylserine molecules. We propose that Ca"+ and acidic phospholipids cause the protein to undergo a conformation change revealing multiple phosphatidylserine binding sites and resulting in the highly cooperative and specific interaction of protein kinase C with phosphatidylserine. Consistent with this, the proteolytic sensitivity of protein kinase C increases approximately 10-fold in the presence of phosphatidylserine and Ca"+ compared to Ca"+ alone. The high degree of cooperativity and specificity may provide a sensitive method for the physiological regulation of protein kinase C by phospholipid.

The enzymes, receptors, and transport proteins of biological membranes are surrounded by a lipid environment which provides structural integrity. The specificity of the lipid-protein interaction is not clear because many lipids can usually support the function of a particular membrane protein. Several membrane proteins, such as the aspartate receptor of bacterial chemotaxis, regain maximum function when reconstituted with heterogeneous mixtures of lipid (1). Specificity is important in some instances (2, 3). Activity of reconstituted human erythrocyte glucose transporter is sensitive to both head group and acyl chain length of phospholipids (4). Similarly, the Na"+K"-ATPase requires anionic phospholipids (5), cytochrome c oxidase requires cardiolipin (6), and β-hydroxybutyrate dehydrogenase requires phosphatidylcholine for activity (7). Nonetheless, specificity in the lipid-protein interaction has been demonstrated for a surprisingly small number of membrane enzymes.

The Ca"+-activated, phosphatidylserine-dependent protein kinase C is an unusual membrane protein. It is present in the cytosol of most cells under resting conditions, but it becomes tightly associated with the plasma membrane in response to receptor-mediated hydrolysis of phosphatidylinositol bisphosphate (for review, see Ref. 8). Protein kinase C binds to acidic membranes in the presence of Ca"+ but is not activated unless diacylglycerol is present in the bilayer. Although enzyme activity is dependent on phosphatidylserine (PS), other phospholipids modulate the PS-stimulated activity. Phosphatidylethanolamine (PE) further activates the enzyme, whereas choline lipids such as sphingomyelin and phosphatidylcholine (PC) inhibit the PS-stimulated activity (9).

Bell and co-workers (10) have developed a mixed micellar assay which allows systematic variation of the number and species of lipids interacting with one monomer of protein kinase C. Studies using mixed micelles containing Triton X-100 established that one molecule of diacylglycerol/protein kinase C monomer is sufficient to activate the kinase, whereas several molecules of PS are required for enzymatic activity (10).

This contribution extends the work of Bell and co-workers in exploring the nature of the protein kinase C-phospholipid interaction using physically defined micelles. The autophosphorylation (11), which has been shown to occur by an intrapeptide reaction (12), is particularly informative as this activity depends only on the kinase-micelle interaction, rather than the three component substrate-kinase-micelle interaction. This system leads to the surprising finding of a high degree of cooperativity and a high degree of specificity in the protein-lipid interaction. Cooperative regulation of enzyme activity by multiple, specific phospholipids may be a feature which will be seen in a number of similar membrane proteins.

EXPERIMENTAL PROCEDURES

Materials

Bovine brain L-a-phosphatidylserine, egg L-a-phosphatidylcholine, egg L-a-phosphatidylethanolamine, 1-palmitoyl-2-oleoyl-phosphatic acid (PA), 1-palmitoyl-2-oleoyl-phosphatidylcholine (PG), and L-a-dioleoylglycerol (DG) were purchased from Avanti Polar Lipids, Inc. Triton X-100 (10% aqueous solution low in carbonyl and peroxide content) was from Pierce Chemical Co. Alkaline phosphatase-conjugated rabbit anti-mouse IgG, 5-bromo-4-chloroindolyl phosphate, histone H1, phenyl-Sepharose CL-4B, polylysine-agarose, trypsin (L-1-

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1 The abbreviations used are: PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PA, phosphatic acid; PG, phosphatidylcholine; DG, dioleoylglycerol; EGTA, [ethylendeoxy(oxythelylenenitrilo)tetraacetic acid; SDS, sodium dodecyl sulfate.
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Methods

Protein Kinase C—Protein Kinase C was purified from cytosolic extract of homogenized rat brain (Sprague-Dawley) by sequential DEAE, phenyl-Sepharose, gel filtration (AC54), and polylysine-agarose chromatography, as described (13). Separation of isoforms was achieved by chromatography of polylysine-agarose-purified protein kinase C on hydroxyapatite, as described by Huang and co-workers (14). For some experiments, membrane-bound protein kinase C was solubilized from rat brain particulate with 1% Triton X-100 and partially purified by DEAE chromatography (11). Protein kinase C (1-10 μg ml⁻¹) was stored at −20 °C in buffer containing 50% glycerol, 0.5 mM dithiothreitol, 0.5 mM EDTA, 0.5 mM EGTA, 10 mM Tris, pH 7.4. Hydroxylapatite-purified samples contained 0-100 mM KPO4.

Lipids—Mixed micelles containing 0-7.5 mol% DG, 0-15 mol% appropriate phospholipid (as noted), and 77.5-98.5 mol% Triton X-100 were prepared by drying lipids under N₂, hydrating in 3% (w/v) 44 mM Triton X-100, and incubating for 10 min at 30 °C with occasional vortexing (10). Micelles were diluted to 1% Triton X-100 with distilled water. Micelles were stored at 4 °C for up to 2 weeks with no detectable changes in ability to activate protein kinase C. Refrigerated micelles were warmed to 30 °C before use to allow micelles to regain initial size (15). The number of lipid molecules/micelle was calculated using an average of 140 molecules of Triton X-100/micelle and assuming that micelle size increases linearly with added lipid (15), as in Equation 1:

\[
\text{molecules of PS/micelle} = \frac{140(\text{PS})}{1 - (\text{PS} + \text{DG})}
\]

where \(x\) is the mole fraction of PS or DG. Sonicated dispersion of PS (1.4 mM) and DG (38 μM) in 20 mM Tris, pH 7.4, was prepared as described (12).

Protein Kinase C Assay—Substrate phosphorylation was assayed by measuring the initial rate of [γ³²P]ATP incorporation from [γ³²P]ATP into saturating amounts of histone (20 μM). Protein kinase C (50 μl of a 0.5-fold dilution stock enzyme into 2 mM dithiothreitol, 20 mM Tris, pH 7.4), micelles (8 μl of 1% Triton X-100 mixed micelles), and CaCl₂ (5 μl of 20 mM solution) were combined. The reaction was initiated by addition of 16 μl of solution containing histone H1 (100 μM), 0.14 Ci mmol⁻¹ ATP, 75 mM MgCl₂, 20 mM Tris, pH 7.4, and samples were incubated at 30 °C for 3 min. Thus, the incubation medium contained 0.1% Triton X-100 (0-0.28 mM phospholipid, 0-0.14 mM DG), 1.3 mM CaCl₂, 20 μM ATP, 15 mM MgCl₂, 6% glycerol, 0.07% EDTA, 0.07 mM EGTA, 15 mM Tris, pH 7.4. Mixed micelles were replaced with PSD stock solution for standard assay measurements. The reaction was quenched by addition of 25 μl of a solution containing 0.1 M ATP, 0.1 M EDTA, pH 7, and aliquots (75 μl) were spotted on Whatman No. 3 MM filter paper (3 cm²), washed in trichloroacetic acid, and prepared for liquid scintillation counting, as described (16). For some experiments, aliquots were spotted on Whatman P-81 ion exchange paper and washed four times in 0.4% (v/v) phosphoric acid with no detectable difference. Autophosphorylation was assayed as described above, except that histone was omitted from the reaction mixture. Reactions were terminated after incubation at 30 °C for 3 min by addition of 25 μl of SDS-polyacrylamide gel electrophoresis sample buffer (0.265 M Tris, 8.4% SDS, 42% glycerol, 0.008% bromphenol blue, 20% β-mercaptoethanol, pH 6.8). In one experiment, histone phosphorylation was quenched similarly. Samples were heated at 100 °C for 3 min and analyzed by SDS-polyacrylamide gel electrophoresis (10%, 17%) followed by autoradiography (Kodak X-Omat film).

Protein Kinase C bands were excised from dried gels, added to 10 μl of scintillation fluid (Scint A, Packard Instrument Co.), and [³²P]-detected by liquid scintillation counting. Determination of [³²P] incorporation into histone by this method or by the spotting/precipitation method yielded similar results.

Trypsin Digestion—Protein kinase C was equilibrated with (a) 1.2 mM CaCl₂, (b) 1.2 mM CaCl₂ and 100 μM PS, or (c) 1.2 mM CaCl₂, 100 μM PS, and 2.6 μM DG by incubating 200 μl of stock enzyme with 30 μl of appropriate CaCl₂ and lipid solution for 2 min at 30 °C. The incubation media contained 0.45 mM EDTA and 0.45 mM EGTA. Samples were treated with 100 mM trypsin (4 units ml⁻¹) at 30 °C for the indicated times. Proteolysis was quenched by addition of SDS-polyacrylamide gel electrophoresis sample buffer. Proteins were separated by SDS-polyacrylamide gel electrophoresis (10% polyacrylamide), electrophoretically transferred to nitrocellulose (18), and protein kinase C-labeled with two monoclonal antibodies specific for the regulatory and catalytic domains of protein kinase C isozyme 3. Blots were incubated with alkaline phosphatase-conjugated anti-mouse IgG, and primary antibody binding was detected by monitoring the insoluble product of 5-bromo-4-chloroindolyl phosphate hydrolysis (19). Trypsin hydrolysis of the fluorogenic substrate N-benzoyl-L-arginine ethyl ester was measured as described (20) and was found to be identical under the conditions described in (a), (b), and (c) above.

Data Analysis—The dependence of protein kinase C activity on the PS content of micelles was analyzed by a nonlinear least squares fit to a modified Hill equation:

\[
y = a + \frac{b x^n}{k^n + x^n}
\]

where \(y\) is the measured activity of protein kinase C, \(a\) is the activity in the absence of phospholipid (background), \(b\) is the phospholipid- 

stimulated activity, \(x\) is the concentration of PS, \(k\) is the concentration of PS resulting in half-maximal activity, and \(n\) is the Hill coefficient. Standard errors of \(n\) and \(k\) are reported.

RESULTS

Phosphatidylinerine Dependence of Autophosphorylation and Histone Phosphorylation—The dependence of protein kinase C autophosphorylation on the PS content of Triton X-100:DG mixed micelles is presented in Fig. 1A. The number of molecules of each component can be calculated (see "Methods") assuming that protein kinase C does not significantly alter the size of micelles, an assumption verified by Bell and co-workers (10). In the experiment described in Fig. 1A, the DG content of micelles was 3-4 molecules/micelle (open circles, 2.5 mol%), 7-9 molecules/micelle (filled circles, 5.0 mol%), and 11-14 molecules/micelle (open squares, 7.5 mol%). The PS content of the micelles ranged from 0 to 27 molecules/micelle (0-15 mol%). The rate of autophosphorylation was highly cooperative with respect to PS. A nonlinear least squares fit of the data to a modified Hill equation (see "Methods") revealed a Hill coefficient of 8 ± 2 for micelles containing 7.5 mol% PS and lower for slightly less DG. Hill coefficients of 7.8 ± 0.7 and 5.6 ± 0.4 were calculated for micelles containing 5.0 and 2.5 mol% DG, respectively. At the highest (saturating) DG concentration, half-maximal activity was supported by micelles containing 6.13 ± 0.08 mol% PS, and activity plateaued (98% maximal) at 9.9 mol% PS (17 molecules/micelle). At the lowest DG concentration, almost twice as much PS was required to maximally activate the kinase (17 mol%, corresponding to 30 molecules of PS/micelle).

The DG dependence of autophosphorylation at two PS concentrations is presented in Fig. 1B (10 mol% PS, open circles; 15 mol% PS, filled circles). Unlike the PS dependence, the DG requirement for autophosphorylation is not cooperative. Protein kinase C was fully active when micelles contained three or more DG molecules (2 mol%) if the PS concentration was 15 mol% but required more DG molecules for lower PS concentrations. As indicated in Fig. 1A, DG modulates the PS dependence of autophosphorylation.

The rate of substrate (histone) phosphorylation was also highly cooperative with respect to PS (Fig. 2A), agreeing with
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Bell and co-workers (10) who reported a Hill coefficient of 4.8. However, the degree of cooperativity was significantly greater in Fig. 2A, with a calculated Hill coefficient of 8.7 ± 0.9. Half-maximal activity \((k)\) was supported by micelles containing 6.13 ± 0.08 mol% PS, and activity saturated (98% maximum activity) at 9.6 mol% PS (15 molecules of PS/micelle). Increasing the DG content above 2.5 mol% did not alter significantly the PS dependence. As shown in Fig. 2B, one molecule of DG (0.7 mol%) in micelles containing either 10 or 15 mol% PS was sufficient for maximum protein kinase C activity. Thus, histone phosphorylation requires less DG than autophosphorylation.

The possibility that the different lipid dependences for autophosphorylation and substrate phosphorylation result from histone-induced alterations of micelle structure was investigated in the experiment described in Fig. 3. Histone is a basic protein and has been shown to associate with membranes containing acidic phospholipids (21). Thus, any substrate-induced aggregation or fusion of micelles might increase the number of lipid molecules/micelle and, consequently, the number of PS and DG molecules available to protein kinase C monomers. The autoradiogram presented in Fig. 3A shows the \([3^2P]phosphate incorporated into protein kinase C, in 3 min, in the absence \((a)\) or presence \((b)\) of histone. Although the rate of autophosphorylation in the presence of histone decreased approximately 2.5-fold relative to that in its absence as a result of substrate inhibition (16), autophosphorylation continued to increase in the presence of histone well after histone phosphorylation had plateaued. Quantitative analysis of the data (Fig. 3B, circles) revealed that the half-maximal rate of histone phosphorylation occurred at 4.8 ± 0.1 mol% PS, with activity saturating (98% maximum) at 8.1 mol% PS (13 molecules/micelle). The rate of autophosphorylation in the absence of histone (Fig. 3B, squares) was half-maximal at 9.1 ± 0.1 mol% PS and was at 98% maximum rate at 15.3 mol% PS (26 molecules/micelle). The same dependence was observed when autophosphorylation was measured in the presence of histone: activity continued to increase well after histone phosphorylation had plateaued, with half-maximal activity supported by micelles containing approximately 9 mol% PS. Thus, histone does not perturb the physical structure of the micelles in such a way as to reduce the PS requirement for protein kinase C activity. Rather, the different lipid requirements for autophosphorylation and substrate phosphorylation are intrinsic properties of the enzyme.

**Phosphatidyserine Dependence of Protein Kinase C Isozymes**—Fig. 4 shows the PS modulation of histone phosphorylation catalyzed by three different preparations of protein kinase C. Hill coefficients and values for \(k\) are summarized in Table I. Maximum activity required 12, 15, or 16 molecules of PS/micelle, respectively, for preparations enriched in Type I protein kinase C (but containing some Type II; nomenclature of Huang and co-workers (14)), Type III protein kinase C, and protein kinase C isolated from the membrane fraction of rat brain. Thus, protein kinase C iso-
enzymes have distinct PS dependences.

Effects of Other Phospholipids on Phosphatidylserine Requirement—Fig. 5 shows the PS dependence of protein kinase C-catalyzed histone phosphorylation supported by micelles containing PS as the only phospholipid (open circles) or equimolar PC and PS (filled circles). The neutral phospholipid had little effect on the concentration of PS required for histone phosphorylation: activity saturated at approximately 8 mol% PS (12 molecules of PS/micelle) regardless of whether PC was present in the micelles. However, the Hill coefficient for PC-containing micelles was 6.3 ± 0.5, which is lower than that for micelles containing PS as the sole phospholipid (9.2 ± 1.6), suggesting a decrease in the strength of the PS-PS interactions. These data indicate that the number of phospholipids required to support protein kinase C activity does not reflect a minimum membrane surface area necessary for protein kinase C binding, but rather reflects a specific head group requirement.

The effect of amino or acidic phospholipids on the PS requirement for histone phosphorylation is shown in Fig. 6.

Fig. 4. Protein kinase C isozymes have different PS dependences for activity. Rate of histone phosphorylation catalyzed by protein kinase C isozymes: Types I and II (±1 mix; ●) and Type 3 III (○) isolated from the cytosol of rat brain homogenate, and protein kinase C isolated from Triton X-100 solubilized rat brain membrane (□). Mixed micelles contained Triton X-100 (0.1%), DG (2.5 mol%), and 0–10 mol% PS. 100% activity was obtained as in Fig. 3B.

Table I

| Protein kinase C | Histone phosphorylation |
|------------------|-------------------------|
| Type III         | 8.2 ± 0.1               |
| Membrane         | 8.6 ± 0.9               |
| Types I + II     | 10.5 ± 1.2              |
| n, Hill coefficient | 4.75 ± 0.01       |
| k, concentration of PS resulting in half-maximal activation | 6.1 ± 0.1 |
|                  | 7.05 ± 0.07             |

a, n, Hill coefficient. k, concentration of PS resulting in half-maximal activation.
Equimolar PE (open squares) or PG (filled circles) in PS-containing micelles caused k for PS to decrease from 5.2 ± 0.1 to 3.9 ± 0.1 and 3.62 ± 0.03 mol%, respectively. Similarly, small decreases in the Hill coefficient from 9.6 ± 1.6 for pure PS to 8.3 ± 0.6 (PE) and 7.7 ± 0.5 (PG) were calculated. Higher concentrations of PE (7.5 mol%) inhibited protein kinase C activity. In contrast to PG, the acidic phospholipid PA did not significantly affect the concentration of micellar PS required for half-maximal stimulation (5.0 ± 0.2 mol%) but did cause the Hill coefficient to decrease to 4.8 ± 0.7. This suggests that PA cannot substitute for PS in activating protein kinase C and inhibits the cooperative interaction between PS and protein kinase C.

Conformational Changes Resulting from Phosphatidylserine-Protein Kinase C Interaction—Conformational changes attendant to the interaction of protein kinase C with PS were examined by investigating the proteolytic sensitivity of soluble and PS-bound enzyme. Protein kinase C is cleaved by trypsin to yield two major fragments: the catalytic domain (48 kDa) and the membrane-binding, regulatory domain (35 kDa) (13, 16, 22). Fig. 7 shows the generation of these fragments as a function of incubation time of protein kinase C with trypsin in the presence of Ca²⁺ (lanes 1-5), Ca²⁺ and PS (lanes 6-10), and Ca²⁺, PS, and DG (lanes 11-15). Intact protein kinase C and proteolytic fragments were probed using monoclonal antibodies to Type III protein kinase C. Membrane-bound protein kinase C was approximately 10-fold more sensitive to trypsin than soluble kinase (compare lanes 5 and 8; the relative quantities of proteolytic domains to native kinase generated after 10 min of trypsin treatment of soluble protein kinase C (lane 5) were similar to those generated after 1 min of trypsin treatment of PS-bound protein kinase C (lane 8)). The presence of DG in the bilayer had no detectable effect on the trypsin sensitivity of the PS-bound protein kinase C (compare lanes 8 and 13). Control experiments established that trypsin hydrolysis of the soluble, fluoroogenic substrate N⁵-benzoyl-L-arginine ethyl ester was not affected by the addition of lipid. Thus, the increase in proteolytic sensitivity that occurs when protein kinase C interacts with PS is not a result of lipid affecting trypsin activity nor does the increased rate of proteolysis arise from membrane binding altering the effective concentration of protein kinase C: enhanced susceptibility to trypsin was observed for both bilayer-bound (Fig. 7) and monomeric micelle-bound (not shown) protein kinase C. Thus, the increased rate of proteolysis of protein kinase C observed in the presence of PS reflects increased susceptibility of membrane-bound kinase to trypsin, suggesting a significant conformational change upon membrane binding.

The data in Fig. 7 do not suggest any further increase in trypsin sensitivity of Type III protein kinase C when DG is present in the bilayer. Nishizuka and co-workers (23) have reported that protein kinase C is more sensitive to the proteolytic enzyme calpain in the presence of PS and that this sensitivity is further increased in the presence of DG. It is possible that the calpain cleavage site is more sensitive to a DG-induced conformational change than the trypsin site or that the effect is isoform-dependent. Additionally, the Ca²⁺ concentration in the present work is an order of magnitude higher than that used in the calpain experiments, perhaps resulting in a tighter binding of the kinase to the lipid matrix. It is perhaps also worth noting that calpain-catalyzed cleavage was detected by monitoring the appearance of Ca²⁺-lipid-independent activity, an activity that may have been affected by the presence of DG.

**DISCUSSION**

Cooperativity of Phosphatidylserine-Protein Kinase C Interaction—Phosphatidylserine activates protein kinase C in a
highly cooperative and specific manner. At saturating DG concentrations, $\geq 12$ molecules of PS/protein kinase C monomer are required to elicit maximum enzyme activity. Hill coefficients of 8-11 best fit the data for the PS-dependent activation of protein kinase C. A Hill coefficient sensitivity serves as an indication of both the strength of subunit interactions and the number of interacting sites. Hemoglobin, with four subunits and a Hill coefficient of 2.8 for oxygen binding, is a well-characterized example of a cooperative interaction (24). Hill coefficients of 5 are among the highest reported for protein subunits (25). Thus, a Hill coefficient of 8 for a system with a maximum of 12 interacting (lipid) subunits suggests remarkable cooperativity in the interaction of PS with protein kinase C.

Bell and co-workers (10) reported a Hill coefficient of 4.8 for the activation of protein kinase C by PS. This much lower number may have been obtained because a mixture of isoforms, each with distinct PS requirements, was employed in the studies. The concentration of PS resulting in half-maximal activation ($K$) varies from 4.8 to 7.1 mol% for the different isoforms (Table I); therefore, mixtures will result in a much broader apparent PS dependence.

Functional differences between the various protein kinase C isoforms are poorly characterized. Subtle differences in the degree of stimulation by Ca$^{2+}$, PS, and DG have been reported. Of the three hydroxylapatite-separated isoforms, Huang and co-workers (14) have shown that Type III displays the greatest PS-DG-Ca$^{2+}$ stimulation in bilayer assays. This isoform requires the least PS for maximal activity in the mixed micelle assay. Different cellular distributions for these isoforms have been reported in a number of systems (26, 27). It is interesting to note that Type I and I$\!^I$ require the highest concentration of PS for activity and that it is these isoforms that are enriched in the brain, where the PS content is highest. A reasonable hypothesis is that the PS dependence of each isoform is optimized with respect to its membrane environment.

The cooperative PS dependences of autophosphorylation and substrate phosphorylation are similar at saturating DG concentrations. However, the saturating DG concentration for autophosphorylation is approximately five times higher than for histone phosphorylation. The different lipid dependences for the two modes of activity do not result from substrate restructuring the micelles. Although histone may aggregate mixed micelles containing acidic phospholipids (21), any such restructuring does not alter the PS dependence of autophosphorylation. Subsaturating DG concentrations modulate the PS dependence of autophosphorylation, a phenomenon observed also for histone phosphorylation (10).

Molecules containing low concentrations of PS ($< 3$ mol%) do not activate protein kinase C despite a ratio of micelles to kinase of 10$^3$. A cluster of PS molecules (for instance $>4$) may be necessary to provide sufficient surface charge density to attract protein kinase C to the micelle surface, with the remaining PS molecules being sequestered in a cooperative manner. Alternatively, the cooperativity in activity may not reflect cooperativity in binding (28, 29). This could occur if the increase in enzymatic activity resulting from PS binding was not linearly proportional to the number of ligands bound, so that binding of one more lipid to the kinase would cause a disproportionate increase in activity.

**Specificity of Phospholipid-Protein Kinase C Interaction** —

The interaction of protein kinase C with phospholipid is head group-specific. For example, neither PE nor PC in DG/Triton X-100 mixed micelles activates protein kinase C (30). The effects of neutral, amino, and acidic phospholipids on the PS requirement for activity are consistent with strict specificity in this lipid requirement. Of the lipids tested, only PE and PG caused a slight reduction in the number of molecules of PS required for full enzymatic activity. The amino group of PE may not be recognized by a limited number of PS-interacting sites. Curiously, PA did not reduce the PS requirement for activity, although alone this lipid causes some activation of protein kinase C (30). PA may bind to protein kinase C and weaken the PS-PS and PS-protein kinase C interactions without contributing significantly to the PS-stimulated activity. This is particularly interesting because DG is rapidly phosphorylated in vivo to yield PA (31).

**Model** — One possible model for the interaction of protein kinase C with the membrane, suggested by this work, is illustrated in Fig. 8. In the cytosol, protein kinase C may adopt a conformation that masks the PS-binding sites and hydrophobic domain of the enzyme. The presence of Ca$^{2+}$ and acidic membranes may induce a gross conformational change that exposes the PS-interacting sites and hydrophobic domain of the protein. This conformational change results in a 10-fold increase in the trypsin sensitivity of the protein. At the membrane, the enzyme may sequester PS molecules around the perimeter of the membrane-interacting domain. The sequestration could result in a higher activity of the protein, but it is clearly only one possible model to explain the cooperativity that is observed. In addition to direct protein-lipid interactions, ionic interactions of the amino and carboxyl (or phosphate) moieties of neighboring PS head groups may account for formation of a PS-enriched domain around the kinase. Alternatively, Ca$^{2+}$ binding to the acidic lipids may be responsible for the PS-PS interaction. Indeed, Ca$^{2+}$ is known to induce phase separation of PS in PS-containing membranes (32). Hydrophobic interactions between acyl chains may strengthen both lipid-lipid and lipid-protein interactions. Membrane association can be reversed by removal of Ca$^{2+}$ (33), consistent with Ca$^{2+}$ playing an important role in the PS-PS and PS-protein kinase C interactions. Addition of DG to the membrane activates the enzyme, an event which may not involve a gross conformational change (the trypsin sensitivity of protein kinase C is not affected by DG in the bilayer). Rather, DG may increase the hydrophobic interaction between the lipid and kinase. Consistent with this are reports indicating that protein kinase C is not dissociated from membranes containing phospholipids in the presence of chelators (34, 35).

Activity measurements indicate that $\geq 12$ molecules of PS interact with the various protein kinase C isoforms. For a head group diameter of 0.9 nm (36), this number of lipids would encircle a cylinder of diameter equal to 3.4 nm. Thus,

![Fig. 8. Tentative model for interaction of protein kinase C with PS-containing membranes. Proposed hydrophobic domain (shaded area) and PS-binding sites (++) are unmasked in the presence of PS and Ca$^{2+}$ resulting in the sequestration of PS (solid head groups) around the perimeter of the membrane-interacting domain of the kinase. DG increases the hydrophobic interaction of the kinase with the membrane.](image-url)
the number of PS-interacting sites obtained from activation studies is consistent with a layer of PS encircling a membrane-inserted domain. However, this number of phospholipids is also reasonable for an interaction in the absence of membrane insertion because 12 molecules of PS would cover a surface area of approximately 10 nm². Although the nature and extent of the penetration of protein kinase C into the bilayer is unknown, interaction of the kinase with the hydrophobic core of the membrane is suggested from labeling studies with a lipid-soluble probe (37). Further support for membrane interaction of protein kinase C is provided by a recent report of Bazzi and Nelsestuen (38) showing increases in the surface pressure of phospholipid monolayers upon protein kinase C binding.

Lipid Dependence—Protein kinase C may represent a new group of lipid-dependent enzymes. Three groups of lipid-dependent enzymes have been proposed by Sandermann (29): those with a few high affinity sites and low specificity, those having multiple binding sites with low specificity (for example, the Na⁺/K⁺-ATPase), and those with multiple low affinity sites plus a few specific binding sites (β-hydroxybutyrate dehydrogenase specifically binds at least three molecules of PC (Hill coefficient of 2.4) and nonspecifically binds approximately 80 phospholipids (7)). In contrast to these enzymes, protein kinase C has multiple, highly specific lipid-interacting sites.

A number of proteins bind to acidic membranes in a Ca²⁺-dependent manner (39). One of these, lipocortin, has been suggested to inhibit phospholipase A₂ by sequestering the phospholipid substrate (40). Whether these proteins interact with lipid in the cooperative and specific manner described for protein kinase C remains to be determined. It is worth noting that protein kinase C does not share significant sequence homology with the Ca²⁺-phospholipid binding domain of those proteins (39, 41, 42).

Regulation—High cooperativity in activation by lipid provides great sensitivity in enzyme regulation, allowing a membrane protein to sense and respond to very small changes in the local intrabilayer concentration of a particular lipid. For most cells, PS accounts for approximately 15 mol% of the total lipid in the inner leaflet of the plasma membrane (43, 44). However, preferential binding of PS to membrane proteins (45, 46) and cytoskeletal components (47), as well as sequestering of PS by Ca²⁺-phospholipid binding proteins (39), may result in a much lower concentration of available PS. Because the affinity of protein kinase C for PS changes over a narrow range of PS, the kinase can respond to subtle changes in the local concentration of this phospholipid. Thus, the cooperative activation of protein kinase C by PS may provide a physiologically relevant mechanism for sensitive enzyme regulation.

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Addendum—Since this paper was submitted, two reports by Huang et al. (48, 49) have confirmed the distinct PS dependences of protein kinase C isozymes (48) and that the proteolytic sensitivity of Type III protein kinase C increase upon membrane binding (49).