Phosphatidyl-L-serine Is Necessary for Protein Kinase C's High-Affinity Interaction with Diacylglycerol-Containing Membranes†

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ABSTRACT: The contributions of phospholipid headgroup structure, diacylglycerol, and Ca^{2+} in regulating the interaction of protein kinase C βII with membranes or detergent/lipid mixed micelles were examined. Binding measurements revealed that, in the absence of diacylglycerol, protein kinase C displays no significant selectivity for headgroup structure other than charge: the enzyme binds with equal affinity to phosphatidyl-L-serine, phosphatidyl-D-serine, and other monoanionic lipids such as phosphatidylglycerol. In contrast, selectivity for headgroup occurs in the presence of diacylglycerol. This second messenger increases the affinity of protein kinase C for phosphatidyl-L-serine-containing membranes or micelles by 2 orders of magnitude, but has only moderate effects on the affinity of protein kinase C for surfaces containing other anionic lipids. Ca^{2+} does not affect the diacylglycerol-mediated increase in protein kinase C's affinity for phosphatidylserine, but does increase the enzyme's affinity for acidic phospholipids. Lastly, ionic strength studies reveal that electrostatic interactions are the primary driving force in the interaction of protein kinase C with membranes. In the absence of either diacylglycerol or phosphatidylserine, these interactions are sufficiently weak that little binding occurs at physiological ionic strength; thus, protein kinase C is unlikely to translocate to the plasma membranes in the absence of diacylglycerol, even if intracellular Ca^{2+} levels are high. Our data reveal that, although there is no specificity for binding acidic lipids in the absence of diacylglycerol, specific structural elements of the L-serine headgroup are required for the high-affinity binding of protein kinase C to diacylglycerol-containing membranes.

Protein kinase C isozymes transduce the plethora of signals that promote lipid hydrolysis (Nishizuka, 1986, 1992). These enzymes are recruited to the plasma membrane in response to diacylglycerol (DG)[1] and, for Ca^{2+}-regulated isozymes, increased Ca^{2+} levels. The "translocation" of protein kinase Cs from the cytosol to the plasma membrane is well documented for many stimuli and cell types (Kraft et al., 1982; Farrar et al., 1985). In addition, there are increasing reports on the differential translocation of protein kinase C isozymes (Baldassare et al., 1992; Ha & Exton, 1993; Szallasi et al., 1994). Mounting evidence indicates that the generation of DG from phosphatidylinositol bisphosphate hydrolysis (which is accompanied by increased intracellular Ca^{2+}) promotes the translocation of the conventional (Ca^{2+}-requiring) and novel (Ca^{2+}-independent) isozymes, whereas the slower generation of DG from hydrolysis of phosphatidylcholine (Exton, 1990) results in the translocation only of Ca^{2+}-independent isozymes (Ha & Exton, 1993).

Since the discovery by Nishizuka and colleagues that DG "synergizes" with a phospholipid cofactor, phosphatidylserine (PS), to activate protein kinase C (Kishimoto et al., 1980), extensive studies into the regulation of protein kinase C by lipid have resulted in the formulation of several similar models describing the molecular control of protein kinase C (Bell & Burns, 1991; Newton, 1993). On the basis of the strict structural requirements for the L-serine headgroup in the DG-dependent activation of the enzyme (Lee & Bell, 1989), Bell and co-workers proposed that protein kinase C has specific binding sites for several molecules of phosphatidylserine (PS). Occupancy of these would promote Ca^{2+}-binding, resulting in an inactive enzyme–$(PS)_n$–Ca^{2+} complex. Subsequent binding of 1,2-*sn*-DG was postulated to generate catalytic activity. Because activation by DG displays strict stereospecificity (Ganong et al., 1986; Rando & Kishi, 1992), it has been proposed to bind a specific structured site in the C1 domain conserved in all isozymes (Nishizuka, 1992).

Examination of the physical interaction of protein kinase C with lipid supports a two-step model for the activation of protein kinase C, but reveals that the initial binding to membranes does not require PS because the enzyme binds other acidic lipids in a Ca^{2+}-dependent manner (Bazzi & Nelsestuen, 1987a; Orr & Newton, 1992b). Furthermore, recent findings reveal that the role of DG is not merely catalytic; this second messenger increases the affinity of protein kinase C for PS-containing surfaces by over 2 orders of magnitude (Orr & Newton, 1992b; Mosior & Epand, 1993). The high-affinity binding to DG and PS produces an alteration in the structure of protein kinase C that exposes its autoinhibitory pseudosubstrate domain coincident with activation (Orr et al., 1992); this basic domain is retained in the substrate-binding cleft by a cluster of acidic residues in the catalytic core of the enzyme (Orr & Newton, 1994). The mechanism by which PS, DG, and Ca^{2+}, in concert, break the pseudosubstrate's interaction with the catalytic core remains to be elucidated. Furthermore, the relationship between these three cofactors has not been clearly established because the separate contributions of each are not fully understood.

This paper addresses the specific roles of DG, PS, and Ca^{2+} in regulating protein kinase C. We show that (1) protein

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[1] Abbreviations: AMP-PNP, tetralithium 5′-adenylyl imidodiphosphate; DG, diacylglycerol; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; PA, phosphatidic acid; PAGE, polyacrylamide gel electrophoresis; PC, phosphatidylcholine; PG, phosphatidylglycerol; PS, phosphatidylserine.

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kinase C binds anionic phospholipids, including PS, with equal affinity, (2) DG induces the specificity observed in activity assays by selectively increasing the affinity of protein kinase C for phosphatidyl-L-serine, and (3) Ca^{2+} does not affect the interaction with DG, but nonselectively increases the affinity for acidic lipids. Our data suggest a different model for protein kinase C in which phosphatidyl-L-serine, but not other acidic lipids, allows protein kinase C to bind DG-containing membranes with high affinity.

MATERIALS AND METHODS

Materials. Bovine brain L-α-phosphatidylserine (PS), *sn*-1-palmitoyl-2-oleoylphosphatidyl-L-serine (POP-L-serine), egg L-α-phosphatidylcholine (PC), and *sn*-1,2-dioleoylphosphatidylglycerol (PG) were purchased from Avanti Polar Lipids, Inc., and egg L-α-phosphatidic acid (PA) was from Matreya. *sn*-1-Palmitoyl-2-oleoylphosphatidyl-D-serine (POP-D-serine) was a generous gift from Michael Zimmerman and David Daleke. [^{14}C]-L-α-Dipalmitoylphosphatidylcholine (114 Ci mol^{-1}) and [γ-^{32}P]ATP (3000 Ci mmol^{-1}) were from Du Pont–New England Nuclear. *sn*-1,2-Dioleoylglycerol (DG), trypsin, tetralithium 5′-adenylyl imidodiphosphate (AMP-PNP), and ATP were from Sigma Chemical Co. A protein kinase C-selective peptide [FKKSFKL-NH_2 (Chakravarthy et al., 1991)] was synthesized by the Indiana University Biochemistry Biotechnology Facility. All other chemicals were reagent grade. Unless otherwise noted experiments were performed using 20 mM HEPES, pH 7.5 at 30 °C (HEPES buffer).

Protein Kinase C. Protein kinase C βII was purified to homogeneity from insect cells (Sf-21; Invitrogen) infected with recombinant baculovirus (generous gift from Daniel Koshland, Jr.) as described (Orr et al., 1992). The enzyme was stored at −20 °C in 10 mM Tris buffer, pH 7.5 at 4 °C, 150 mM KCl, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 0.5 mM dithiothreitol (DTT), and 50% glycerol.

Lipid. Phospholipid concentrations of chloroform stock solutions were determined by assay for phosphate concentration (Bartlett, 1959). The DG was analyzed by TLC (Kodali et al., 1990) and was >90% *sn*-1,2-dioleoylglycerol. Sonicated vesicles (5 mM lipid) in 20 mM Tris (pH 7.5 at 30 °C) were prepared as described by Newton and Koshland (1990). For some experiments, extruded sucrose-loaded vesicles of the compositions indicated in the figure legends were prepared as described by Mosior and Epand (1993). Trace [^{14}C]egg PC [0.025 μCi (μmol of lipid)$^{-1}$] was included with lipid; vesicles were suspended in 80 mM KCl in HEPES buffer and adjusted to a concentration of 4 mM on the basis of radioactivity. Triton X-100/lipid mixed micelles were prepared as described by Newton and Koshland (1989).

Protein Kinase C Activity Assay. Activity of protein kinase C was assayed by measuring the initial rate of [^{32}P]phosphate incorporation from [γ-^{32}P]ATP (50 μM; 3000 Ci mmol^{-1}) into saturating amounts (50 μg mL^{-1}) of a protein kinase C-selective peptide (see Materials), as described by Orr et al. (1992). Phosphorylation assays also contained 10 mM $MgCl_2$, 1 mM DTT, and the concentrations of $CaCl_2$ and lipid indicated in the figure legends.

Protein Kinase C Membrane-Binding Assay. Membrane-binding was determined using a trypsin-sensitivity assay (Orr & Newton, 1992a) or a centrifugation assay using sucrose-loaded vesicles (Rebecchi et al., 1992). The former takes advantage of the 100-fold increase in trypsin sensitivity of protein kinase C's hinge region that accompanies membrane-binding. For these protease-sensitivity experiments, protein kinase C (20–30 nM) was incubated with sonicated vesicles (500 μM lipid), 250–500 μM $CaCl_2$, 1 mM DTT, and 20 mM Tris, pH 7.5 (30 °C), for 3 min at 30 °C and then treated with trypsin (18 ng mL^{-1}; 12 units μg^{-1}) for 2.5 min at 30 °C. Reactions were quenched by the addition of 25 μL of sample buffer (8% SDS, 40% glycerol, 0.008% bromophenol blue, 20% β-mercaptoethanol, 0.25 M Tris, pH 6.8) and samples analyzed by SDS–PAGE and silver-staining (Poehling & Neuhoff, 1981). Gels were scanned with a Molecular Dynamics densitometer and relative amounts of protein kinase C quantified by integrating bands with the Molecular Dynamics ImageQuant software. Under these proteolysis conditions, soluble protein kinase C was resistant to proteolysis whereas 80% of membrane-bound protein kinase C was proteolyzed. Binding was related to the percent of protein kinase C that was in the proteolytically sensitive conformation, as described by Orr and Newton (1992a). For the centrifugation experiments, protein kinase C (approximately 50 nM) was incubated with sucrose-loaded vesicles (500 μM) in the presence of 50 μM AMP-PNP, 10 mM $MgCl_2$, 250 μM $CaCl_2$, 1 mM DTT, 50 μg mL^{-1} leupeptin, 20 mM HEPES, pH 7.5, and 12–312 mM KCl for 5 min at room temperature in a volume of 80 μL. The composition of the medium for the sucrose-loaded vesicle-binding assays was the same as for the phosphorylation assays except that peptide substrate was omitted and AMP-PNP replaced ATP. Vesicles were pelleted by centrifugation at 100000*g* for 30 min, and 70 μL of supernatants was quenched in 30 μL of sample buffer. Samples were analyzed by SDS–polyacrylamide gel electrophoresis (8% acrylamide), and protein kinase C was visualized by silver staining. Binding by the centrifugation or protease-sensitivity method yielded quantitatively and qualitatively similar data for comparable assay conditions.

Protein Kinase C Micelle-Binding Assay. Binding to Triton X-100 mixed micelles was measured with the trypsin-sensitivity assay described above. Proteolysis (catalyzed by 10 ng mL^{-1} trypsin; 30 °C) was allowed to proceed for 2 min for PS samples and for 6 min for PG samples because the rate of proteolysis was 3-fold slower for protein kinase C bound to PG-containing micelles.

Free Calcium Determinations. Concentrations of free Ca^{2+} were calculated using a computer program provided by Claude Klee (Jean & Klee, 1986) that takes into account pH, Ca^{2+}, Mg^{2+}, K^+, Na^+, EGTA, EDTA, and ATP concentrations.

Data Analysis. The dependence of protein kinase C binding or activity on the lipid composition of vesicles was analyzed by a nonlinear least-squares fit to a modified Hill equation described by Newton and Koshland (1989) using the program GraFit (Leatherbarrow, 1992). In some cases, data were fit to the Michaelis–Menten equation using the same program. The apparent K_a for binding of protein kinase C to vesicles was determined by the equation:

$$K_a = \frac{[\mathrm{PKC}]_{\mathrm{bound}}}{[\mathrm{PKC}]_{\mathrm{free}}[\mathrm{lipid}]} \quad (1)$$

where [lipid] represents the total lipid concentration (Mosior & Epand, 1993).

RESULTS

Figure 1 shows that DG caused a marked increase in the affinity of protein kinase C βII for PS in sonicated vesicles. DG (5 mol %; squares) decreased the concentration of PS required for half-maximal binding from 32 ± 1 to 9 ± 1 mol % in the presence of saturating Ca^{2+} (250 μM). For vesicles

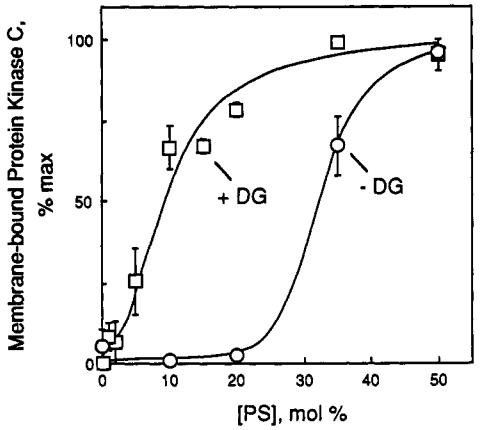

FIGURE 1: Diacylglycerol increases the affinity of protein kinase C for phosphatidylserine. Protein kinase C (20 nM) was incubated with 500 μM $CaCl_2$ and sonicated vesicles (500 μM lipid) containing 0–50 mol % PS, 45–50 mol % PC, and 0 (circles) or 5 mol % (squares) DG. Membrane-binding was assessed by monitoring the sensitivity of protein kinase C to proteolysis by trypsin, as described in Materials and Methods. Data for binding in the presence of DG represent the average and range of 2 separate experiments; data for binding in the absence of DG represent the mean ± SEM for 3 separate experiments. The curves shown are those predicted from a modified Hill equation (Newton & Koshland, 1989) using the program GraFit.

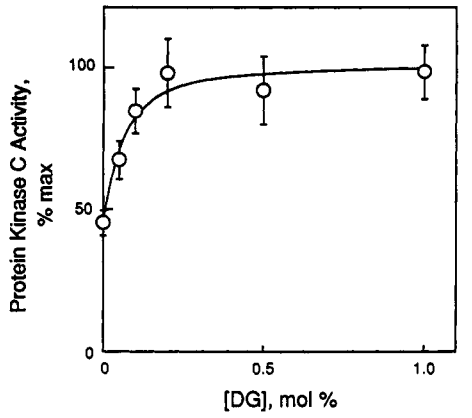

FIGURE 2: Diacylglycerol increases the catalytic efficiency of protein kinase C. The activity of protein kinase C toward phosphorylation of a synthetic peptide (see Materials and Methods) was measured in the presence of 500 μM $CaCl_2$ and sonicated vesicles (500 μM lipid) composed of 50 mol % PS, 49–50 mol % PC, and 0–1 mol % DG. Data represent the mean ± SEM of 3 separate experiments. The curve shown is that predicted from the Michaelis–Menten equation using the program GraFit.

containing 20 mol % PS, the inclusion of DG increased the apparent K_a approximately 200-fold, from 47 M^{-1} to 7.3 × 10^3 M^{-1}. The effect of DG on the affinity of the enzyme for PS-containing vesicles was on the same order (500-fold increase) as that reported by Mosior and Epand (1993) for sucrose-loaded vesicles. The sigmoidal character of the binding curves is consistent with the cooperativity in binding reported for PS in mixed micelles (Orr & Newton, 1992a) and sucrose-loaded vesicles (Mosior & Epand, 1993).

A second effect of DG was to increase significantly the catalytic activity of protein kinase C. Figure 2 presents the effect of DG on the activity of protein kinase C toward the phosphorylation of a synthetic peptide monitored in the presence of 50 mol % PS, a concentration resulting in maximal binding in the presence or absence of DG (see Figure 1). PS alone supported partial catalytic activity of protein kinase C (Figure 2); the degree of activity was proportional to the degree of binding so that concentrations below 20 mol % did not activate the enzyme (not shown). However the maximal rate

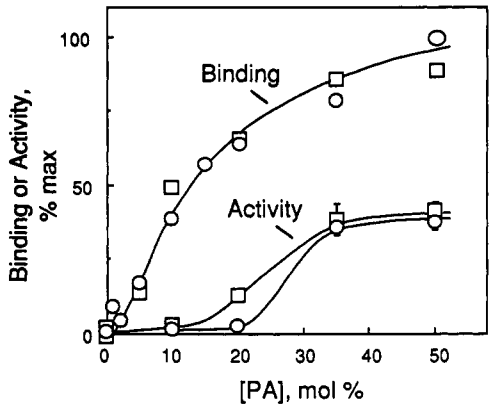

FIGURE 3: The interaction of protein kinase C with phosphatidic acid is relatively insensitive to diacylglycerol. Binding of protein kinase C to vesicles containing 0–50% PA with (squares) or without (circles) 5 mol % DG (remaining lipid PC) was measured in the presence of 500 μM $CaCl_2$ using the trypsin-sensitivity assay (see Materials and Methods). Activity was measured under similar conditions except that peptide substrate (50 μg mL^{-1}), ATP (50 μM), and $MgCl_2$ (10 mM) were present. Activity is expressed relative to that observed in the presence of saturating PS (30 mol %) and DG (1 mol %).

required DG. Under the assay conditions, the K_m for DG was 0.05 μM and the maximal increase in catalytic efficiency was 2.2-fold.

In marked contrast to its effects on the PS-dependent interaction of protein kinase C with membranes, DG had a much lower, if detectable, effect on the interaction of protein kinase C with another acidic lipid, PA. Figure 3 shows that the binding of protein kinase C to membranes containing egg PA was similar in the presence or absence of DG. Although PA was able to partially activate protein kinase C (41 ± 3% of maximal rate was observed in the presence of saturating PA), this activity was only slightly sensitive to DG. DG had a more pronounced effect on the affinity of protein kinase C for palmitoyloleoylphosphatidic acid, causing an approximately 10-fold increase in apparent binding constant (M. Mosior, unpublished data). Thus, the effect of DG on the affinity for PA, although varying with acyl chain composition, is at least an order of magnitude less than its effect on the apparent binding constant for PS.

To better address the role of headgroup structure in effecting the DG-dependent high-affinity protein–lipid interaction, we examined the binding of protein kinase C to lipids containing the L-serine headgroup or its stereoisomer, D-serine. Because the DG effect is independent of bilayer structure (see Discussion), these studies were conducted using the well-characterized mixed micellar assay developed by Bell and co-workers (Hannun et al., 1985). Figure 4A shows that POP-D-serine was considerably less effective at activating protein kinase C compared with POP-L-serine: 12 mol % POP-L-serine resulted in maximal activity of protein kinase C, whereas this concentration of POP-D-serine resulted in only 10% of the maximal rate. The relative effectiveness of these lipids in activating protein kinase C is consistent with that reported by Lee and Bell for dioleoyl-L-serine compared with dioleoyl-D-serine (Lee & Bell, 1989). No activity was detectable in the presence of either the D-serine- or L-serine-containing lipids in the absence of DG.

To determine whether the reduced activity reflected a reduced affinity for the D-serine headgroup or whether it reflected a reduced catalytic rate, we examined the binding of protein kinase C to both lipids. Figure 4B shows that protein kinase C bound with a significantly higher affinity to POP-L-serine compared with POP-D-serine in micelles containing

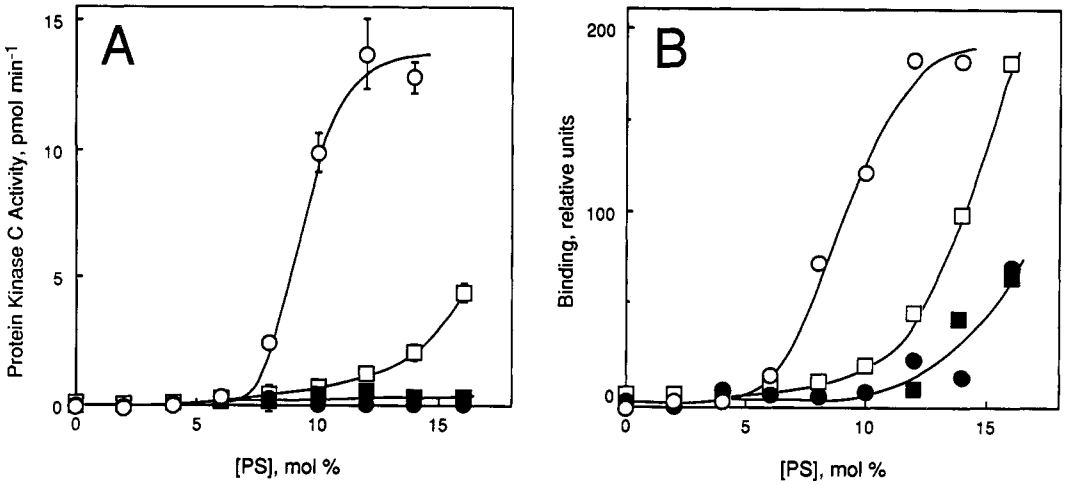

FIGURE 4: Diacylglycerol selectively increases the affinity of protein kinase C for phosphatidyl-L-serine compared with phosphatidyl-D-serine. (A) Activity of protein kinase C (3 nM) was measured in the presence of Triton X-100 (0.1%) mixed micelles containing the indicated amounts of POP-D-serine (squares) or POP-L-serine (circles) and 0 (filled symbols) or 5 mol % (open symbols) DG; 500 μM $CaCl_2$ was present in the assays. Symbols represent the mean ± SD of triplicate measurements. (B) Binding of protein kinase C (30 nM) to micelles described in part A was measured by the trypsin-sensitivity assay.

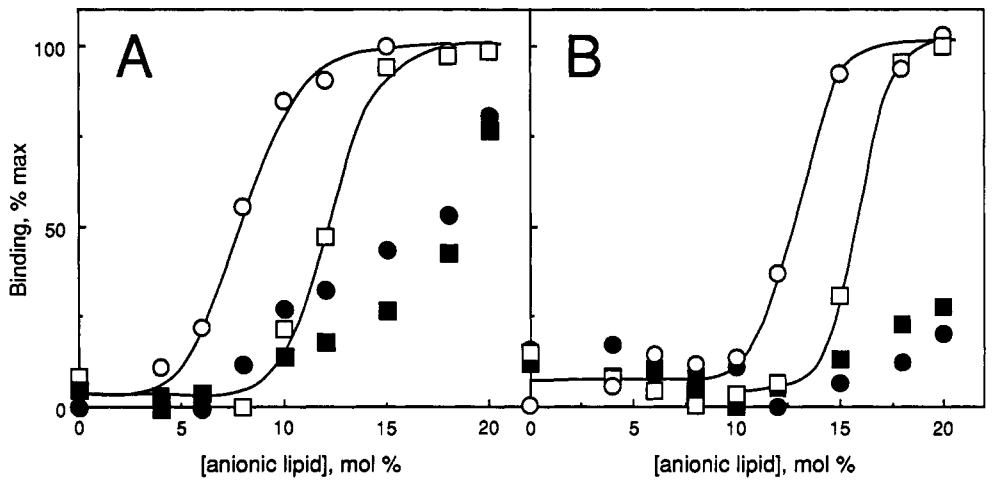

FIGURE 5: Diacylglycerol selectively increases the affinity of protein kinase C for phosphatidylserine in the presence of nonactivating or activating concentrations of Ca^{2+}. (A) Binding of protein kinase C (60 nM) to Triton X-100 (0.1%) mixed micelles containing the indicated amounts of PS (circles) or PG (squares) and 0 (filled symbols) or 5 mol % (open symbols) DG was measured in the presence of 250 μM $CaCl_2$ (activating concentration). (B) Binding to the micelles described in part A was measured in the presence of 100 nM $CaCl_2$ (nonactivating concentration). Binding was detected using the trypsin-sensitivity assay. Curves shown are predicted from a modified Hill equation (Newton & Koshland, 1989) using the program GraFit. The points in the absence of DG could not be fit to the equation; they represent the fraction of total protein kinase C that is membrane-bound, rather than the % max obtained from the fit (the two differ typically by <5%).

DG: relative binding constants were approximately 20-fold higher in the presence of 10 or 12 mol % POP-L-serine compared with those for equivalent concentrations of POP-D-serine. However, protein kinase C bound with the same affinity to both lipids in the absence of DG. Thus, protein kinase C does not discriminate between the stereochemistry of the headgroup in the absence of DG. In contrast, the presence of DG causes a selective increase in the affinity for micelles containing the L-serine headgroup.

Figure 5A shows that protein kinase C bound to micelles containing PS (filled circles) or PG (filled squares) with similar affinity in the absence of DG. In contrast, inclusion of DG caused protein kinase C to bind to micelles containing PS (open circles) with an approximately 10-fold higher affinity compared with PG (filled circles). This is similar to the selectivity observed for L-serine compared with D-serine (Figure 4B). Furthermore, the affinity for PG was similar to that for POP-D-serine (compare with Figure 4B), indicating that charge, rather than headgroup structure, determined binding in the absence of DG.

We next addressed the question: does the ability of DG to increase the affinity for PS require exposure of the pseudosubstrate and hence catalytic activity? In other words, can DG increase the affinity for PS under conditions where protein kinase C is in its inactive conformation? To test this, the effects of DG on the affinity for PS and PG were examined at a concentration of Ca^{2+} that does not activate protein kinase C βII (100 nM) (Newton et al., 1994). Figure 5B shows that when binding was measured in the presence of 100 nM Ca^{2+}, DG continued to differentially affect the affinity for PS versus PG. Once again, binding in the absence of DG was comparable for both lipids, whereas the affinity for micelles containing PS increased approximately 10-fold relative to micelles containing PG when DG was present. Thus, even though protein kinase C remains in an inactive conformation (pseudosubstrate bound to catalytic core), the combination of DG and the L-serine headgroup causes a high-affinity interaction. As reported previously, lowering the Ca^{2+} concentration decreased the affinity of protein kinase C for micelles (Orr & Newton, 1992b).

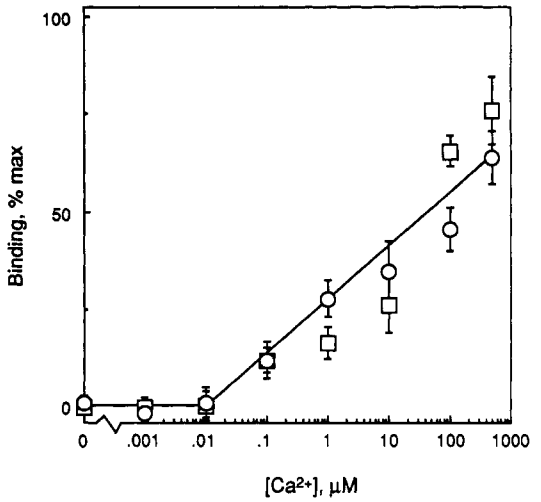

FIGURE 6: Ca^{2+} increases the affinity of protein kinase C for acidic lipids. The binding of protein kinase C (60 nM) to Triton X-100 (0.1%) mixed micelles containing 20 mol % PS (circles) or PG (squares) was measured in the presence of the indicated amounts of $CaCl_2$. Binding was assessed by the trypsin-sensitivity assay. Data represent the average and range of 2 separate experiments.

Analysis of the data in Figure 5 revealed that, for given concentrations of PS, DG caused the relative binding constant to increase by the same factor in the presence of 100 nM or 250 μM Ca^{2+}. The finding that a 3 orders of magnitude change in Ca^{2+} concentration had no effect on the DG-induced increase in micelle affinity indicates that Ca^{2+} does not affect the affinity of protein kinase C for DG. Thus, Ca^{2+}-binding and DG-binding sites on protein kinase C do not interact allosterically.

Figure 6 shows that Ca^{2+} caused a similar increase in binding of protein kinase C to micelles containing PS or PG. Thus, the Ca^{2+}-induced increase in affinity for PS reported previously (Bazzi & Nelsestuen, 1987a; Hannun et al., 1986) displays no significant selectivity for headgroup structure beyond the requirement for negative charge.

In order to address the contribution of electrostatic interactions in the DG-sensitive binding of protein kinase C to PS-containing membranes, the effect of ionic strength on binding was examined. Figure 7A shows that the binding of protein kinase C to saturating PS (40 mol %) was sensitive to increasing ionic strength, with 57% inhibition of binding at an ionic strength close to physiological (137 mM KCl, ionic strength of 188 mM). In contrast, the binding of protein kinase C to PS-containing membranes was significantly less sensitive to ionic strength (137 mM KCl reduced binding by only 5%) if 5 mol % DG was present. Qualitatively similar data were obtained for membranes containing 1 mol % DG. The ability of KCl to decrease membrane-binding, presumably by screening charge interactions, suggests that electrostatic forces provide a major driving force in the binding of protein kinase C to acidic lipids. The increased resistance to ionic strength observed with membranes containing PS and DG is consistent with protein kinase C binding to DG-containing vesicles with a higher affinity.

Figure 7B shows that the interaction of protein kinase C with non-PS acidic lipids displayed similar sensitivity to increased ionic strength whether or not DG was present. The binding of protein kinase C to vesicles containing 40 mol % PG, a monovalent acidic lipid, was inhibited approximately 60% by 262 mM KCl (ionic strength of 313 mM), regardless of whether or not DG was present in the vesicles. This degree of inhibition was similar to that observed for PS (no DG) at this KCl concentration. Note that DG may have some effect on the ionic strength sensitivity of the binding to PG, but that it may be masked by the error of the assay. However, relative to PS, PG-containing membranes were considerably more sensitive to ionic strength effects in the presence of DG.

DISCUSSION

Interaction with Phospholipid. Protein kinase C binds with equal affinity to membranes or micelles containing phosphatidyl-L-serine, its stereoisomer phosphatidyl-D-serine, or the structurally unrelated anionic lipid PG. This binding is

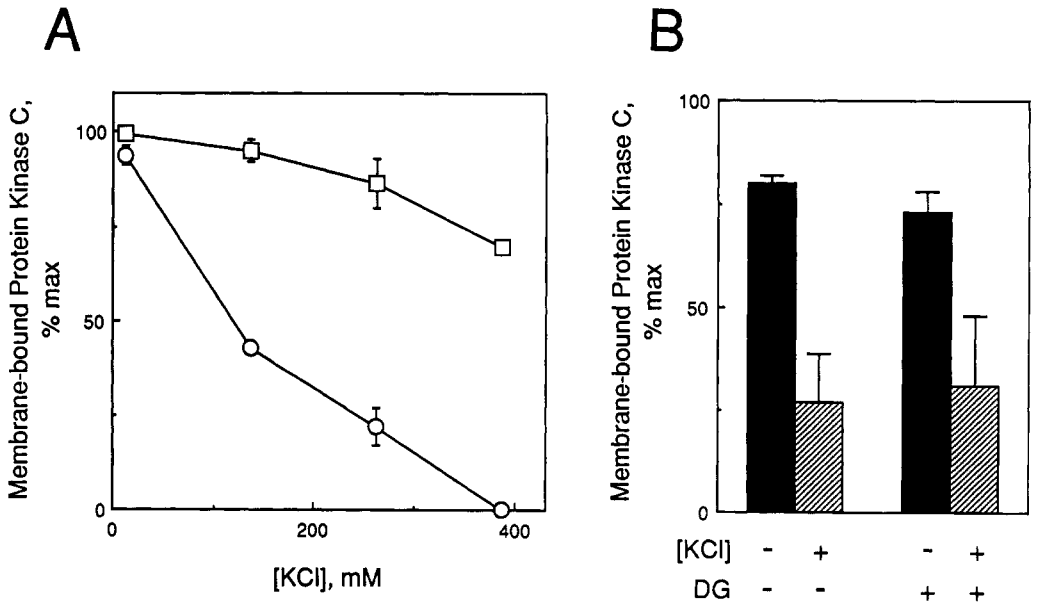

FIGURE 7: Diacylglycerol renders the interaction of protein kinase C with phosphatidylserine, but not other acidic lipids, relatively insensitive to increasing ionic strength. (A) The binding of protein kinase C (50 nM) to sucrose-loaded vesicles (500 μM lipid) composed of 40 mol % PS, 55–60 mol % PC, and 0 (circles) or 5 (squares) mol % DG was measured in the presence of 250 μM $CaCl_2$ and 12–387 mM KCl. Data show the average and range of a duplicate experiment. (B) The binding of protein kinase C to vesicles containing 40 mol % PG, 55–60 mol % PC, and 0 or 5 mol % DG was measured in the presence of 500 μM $CaCl_2$ and 12 mM (black) or 262 mM (cross-hatched) KCl. Data represent the weighted average ± SEM of 2 triplicate experiments.

of a relatively low affinity and is markedly reduced by even physiological ionic strength. The dependencies on membrane surface charge and sensitivity to ionic strength are consistent with electrostatic interactions providing the major driving force in the binding to anionic lipids. Ca^{2+} nonselectively increases the affinity of protein kinase C for these acidic lipids.

The finding that protein kinase C binds with a 2 orders of magnitude lower affinity to phosphatidyl-D-serine compared with phosphatidyl-L-serine in micelles containing DG accounts for why a previous study reported no detectable binding of protein kinase C to mixed micelles containing this lipid (and DG) using a gel filtration assay (Lee & Bell, 1989).

Effect of DG on Phospholipid Interaction. DG selectively increases the affinity of protein kinase C for membranes or micelles containing phosphatidyl-L-serine compared with phosphatidyl-D-serine or other acidic lipids. Specifically, DG causes a $\geq$100-fold increase in the apparent binding constant for membranes or micelles containing PS and a $\leq$10-fold increase in the affinity for surfaces containing non-L-serine acidic headgroups.

Two possible models could account for the headgroup selectivity in the presence of DG. First, interaction with phosphatidyl-L-serine could structure or expose the DG-binding site. The increased accessibility of the DG-binding site may not necessarily involve conformational changes (Mosior & Epand, 1993); PS may alter the disposition of protein kinase C to bring the DG site closer to the membrane. The increased accessibility, or altered conformation, caused by PS would thus increase the affinity of protein kinase C for DG. Alternatively, binding to DG could structure or expose specific binding sites for the L-serine headgroup. The present data do not distinguish between the two models. However, several lines of evidence suggest that phosphatidyl-L-serine may contain structural elements that allow it to better "present" DG to protein kinase C. Notably, activation by cofactors always requires DG, whereas DG-mediated activation can occur in the presence of non-PS activators. For example, the "anomalous" ability of dansyl-PE (Mosior & Epand, 1993), or short-chained phosphatidylcholines (Walker & Sando, 1989), to substitute for PS would suggest that specificity for headgroup reflects the ability to uniquely organize or expose DG to protein kinase C. Such a model would account for why lyso-PS, whose intermolecular organization (*e.g.*, packing, tilt of headgroup) would differ from that of PS, is unable to activate protein kinase C (Lee & Bell, 1989) and why activity is sensitive to membrane physical properties (Epand & Lester, 1990) such as acyl chain unsaturation (Bolen & Sando, 1992). Nonetheless, the ability of increasing ionic strength to selectively screen the interaction with non-PS acidic lipids but not with PS, when both lipids are in the same micelle, suggests some specificity in the direct binding of PS to protein kinase C (Orr & Newton, 1992b).

DG causes a similar increase in the affinity of protein kinase C for Triton X-100 mixed micelles (Orr & Newton, 1992b), sucrose-loaded vesicles (Mosior and Epand, 1993), and sonicated vesicles (see Figure 1) containing PS. The similar increase in affinity for PS in detergent/lipid mixed micelles or model membranes reveals that the effect of DG does not require a bilayer structure. Rather, protein kinase C is regulated by specific structural determinants in the lipid; these either bind to specific determinants on protein kinase C or indirectly regulate the enzyme because their intermolecular interactions provide a surface to better expose DG to protein kinase C (see above).

The headgroup-selective increase in membrane/micelle affinity induced by DG explains the apparent inconsistency of why phospholipid dependencies for [^{3}H]phorbol ester binding did not mirror those for activation in a study by Lee and Bell (1989). In particular, these researchers reported that the D-serine and L-serine headgroup supported comparable phorbol ester binding, in contrast to the different phospholipid dependencies for activity (Lee & Bell, 1989). These studies were performed in the absence of DG, and given the low concentrations of phorbol ester used (approximately 0.002 mol %), the lipid interaction examined would have been the low-affinity one. Our work shows that the phospholipid dependencies for binding do mirror those for activation, provided that the compositions of micelles used for both studies are identical.

Effect of DG on Catalytic Activity. Maximal catalytic activity of protein kinase C requires both the L-serine headgroup and DG. However, up to 50% of V_{max} can be achieved by high concentrations of PS alone or other acidic lipids [see also Bazzi and Nelsestuen (1987b), Lee and Bell (1992), and Mosior and Epand (1993)]. DG also increases the catalytic efficiency of protein kinase C bound to Triton X-100/PS mixed micelles (Hannun et al., 1985; Newton & Koshland, 1989), revealing that, as with its effects on the affinity for PS, a defined bilayer structure is not necessary.

We have recently shown that the combination of DG and PS results in the reversible exposure of the enzyme's pseudosubstrate (Orr et al., 1992), an autoinhibitory domain that has been proposed to occupy the substrate-binding cleft and prevent access of substrates to the active site (House & Kemp, 1987; Kemp & Pearson, 1991). Binding to conventional (*i.e.*, PS and DG) or nonconventional [*e.g.*, protamine sulfate (Takai et al., 1979), short-chained phosphatidylcholines (Walker & Sando, 1988)] activators results in removal of the pseudosubstrate domain from the active site of protein kinase C (Orr & Newton, 1994). The activity observed in the absence of DG reveals that the enzyme can be pushed into the active conformation by nonphysiologically high concentrations of PS.[2] However, because only partial catalytic activity is observed, the pseudosubstrate likely maintains a significant affinity for the active site. Maximal release of the pseudosubstrate mediated by PS would thus require binding of DG. Furthermore, the L-serine headgroup is essential for DG to mediate maximal release of the pseudosubstrate because even structurally related lipids such as phosphatidyl-D-serine are unable to maximally activate protein kinase C.

Ca^{2+} Effects. DG selectively induces a high-affinity interaction of protein kinase C with PS in the presence of nonactivating (100 nM) or activating (250 μM) concentrations of Ca^{2+}. This result indicates that induction of the strong interaction with PS occurs independently of the active conformation and does not require pseudosubstrate exposure.

The finding that DG causes the same relative increase in apparent binding constant over a 3 orders of magnitude range of Ca^{2+} concentrations reveals that Ca^{2+} does not induce sensitivity to DG. Thus, the Ca^{2+}-binding site(s) involved in membrane targeting and the DG-binding site do not interact allosterically. Rather, Ca^{2+} directly influences the affinity of protein kinase C for PS (and other acidic lipids). This would suggest that binding of Ca^{2+} to conventional protein kinase Cs exposes an "acidic lipid-binding domain"; this domain may

[2] The much greater dependence on DG for activity in Triton X-100 mixed micelles arises because concentrations of PS examined in micelles are relatively low—such low concentrations are unable to push protein kinase C into its active conformation.

already be exposed for the Ca^{2+}-independent protein kinase Cs. Consistent with a role for Ca^{2+} in converting conventional protein kinase Cs into a membrane-recognizing form, we have found that binding of a Ca^{2+}-independent isozyme (protein kinase C ϵ) to PS-containing vesicles or mixed micelles displays the same regulation by DG observed for βII, except that Ca^{2+} is not required (Newton et al., 1994).

Ionic Strength Effects. The binding of protein kinase C to acidic membranes is sensitive to ionic strength. However, the degree of sensitivity depends on the nature of the protein–lipid interaction. Specifically, the interaction with membranes containing PS is considerably more resistant to changes in ionic strength if DG is present. The relative resistance to ionic strength for membranes containing both PS and DG is consistent with a report from Bazzi and Nelsestuen showing that >300 mM NaCl is necessary to reduce the binding of protein kinase C to such membranes (Bazzi & Nelsestuen, 1987b). Two possible explanations could account for the differential sensitivity to ionic strength in the presence of both PS and DG compared with that for PS alone. First, binding could be driven primarily by electrostatic forces in the absence of DG, with DG promoting additional protein–lipid interactions involving van der Waals forces, hydrogen-bonding, and hydrophobic forces. However, the observation that protein kinase C quantitatively dissociates from membranes containing PS and DG at sufficiently high ionic strength is inconsistent with this possibility (Bazzi & Nelsestuen, 1987b). Alternatively, the binding of protein kinase C to PS could be driven by the same forces (primarily electrostatic) whether or not DG is present. The increased resistance to ionic strength caused by DG could reflect the 2 orders of magnitude increase in affinity for PS that is caused by binding DG. Indeed, if conditions are chosen such that protein kinase C is bound to PS-containing vesicles with the same affinity (achieved by adjusting the total lipid concentration), the ionic strength sensitivity is similar for binding in the presence or absence of DG (Mosior and Newton, unpublished data). Thus, the ionic strength sensitivity for binding is consistent with electrostatic interactions driving the protein kinase C–lipid interaction, and with DG increasing the strength of this interaction by 2 orders of magnitude.

An important observation from this work is that, at physiological ionic strength, significant binding of a Ca^{2+}-dependent protein kinase C to membranes occurs only if both DG and PS are present in membranes, even in the presence of high concentrations of Ca^{2+}. Thus, although some isozymes display an added requirement for Ca^{2+} for binding, DG is the necessary trigger for the specific, high-affinity interaction with PS that exposes the enzyme's pseudosubstrate. These results suggest that cellular protein kinase C will not be recruited to the plasma membrane in the absence of DG.

Conclusions. A model for the protein kinase C–lipid interaction based on the foregoing data is presented in Figure 8. Binding measurements are consistent with protein kinase C interacting with acidic membranes by an electrostatic mechanism that displays little selectivity for headgroup other than charge—binding to PG, phosphatidyl-D-serine, or phosphatidyl-L-serine occurs with similar affinity. In this mode, the enzyme is catalytically inactive because the pseudosubstrate blocks the active site (Orr et al., 1992; Orr & Newton, 1994), although nonphysiologically high concentrations of lipid are able to partially activate the enzyme, presumably by reducing the affinity of the pseudosubstrate for the catalytic core. In this regard, the ability of phosphoinositides to reduce the requirement for PS in activating protein kinase C may result from their high negative charge increasing membrane binding, particularly since inositide effects increase in proportion to the number of phosphates on the headgroup (Lee & Bell, 1991). In the absence of clusters of high negative charge, the low-affinity interaction is unlikely to be relevant in the cell because of its marked sensitivity to even physiological ionic strength.

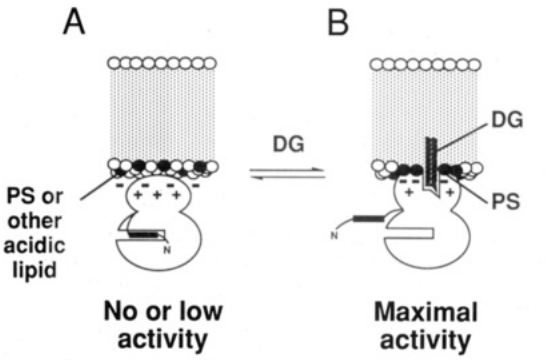

FIGURE 8: Schematic illustrating the low-affinity interaction with acidic lipids and the high-affinity interaction that requires phosphatidyl-L-serine and diacylglycerol. (A) In the absence of DG, protein kinase C binds to PS and other acidic lipids with comparable affinity. High concentrations of acidic lipids are able to partially activate protein kinase C, presumably by reducing the affinity of the pseudosubstrate (black rectangle) for the substrate-binding site (cleft). This interaction may not be physiologically relevant because it is inhibited significantly by physiological ionic strength. (B) A high-affinity interaction with membranes occurs in the presence of both phosphatidyl-L-serine and DG; if activating concentrations of Ca^{2+} are present, this interaction causes the pseudosubstrate to be removed from the substrate-binding site, allowing maximal catalytic activity. PS may structure, or allow access to, the DG-binding site, and hence increase the apparent affinity of protein kinase C for DG. Alternatively, DG binding may structure phospholipid-binding domains to selectively increase the affinity for the L-serine headgroup.

The presence of both PS and DG results in a remarkable increase in the affinity of protein kinase C for membranes (or micelles). This increase can occur in the absence of the active conformation of protein kinase C and in the absence of a defined bilayer. If Ca^{2+} levels are sufficiently high, PS and DG, in concert, activate protein kinase C by removing its autoinhibitory pseudosubstrate from the active site. Whether PS structures or exposes the DG-binding site, or whether DG structures or exposes the PS-binding sites, remains to be determined. What is clear, however, is that there is no selectivity for anionic phospholipid headgroups in the absence of DG.

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