

Mechanism of the Apparent Cooperativity in the Interaction of Protein Kinase C with Phosphatidylserine[†]

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Received June 8, 1998; Revised Manuscript Received September 11, 1998

ABSTRACT: Protein kinase C displays high apparent cooperativity in its activation by phosphatidylserine. This contribution uses a novel approach to address the physical basis for this apparent cooperativity. We examine the binding of protein kinase C β II to large unilamellar vesicles as a function of increasing mole fraction phosphatidylserine and as a function of increasing total lipid concentrations. Binding data are subjected to an analysis, described in the Appendix, that allows calculation of the fractional saturation of phosphatidylserine binding sites with this ligand. This analysis reveals that (1) protein kinase C β II binds approximately eight phosphatidylserine molecules and (2) the binding of each lipid is not cooperative. Rather, the apparent cooperativity observed in protein kinase C's interaction with multiple phosphatidylserine molecules arises from effects specific to the interaction of a multivalent macromolecule with multiple membrane-associated ligands. Nor does diacylglycerol, which has been previously shown to dramatically increase protein kinase C's affinity for phosphatidylserine-containing membranes, induce cooperativity. Thus, protein kinase C binds multiple phosphatidylserine molecules in the absence of interaction between potential binding sites. The method presented for determining the stoichiometry and cooperativity in the interaction of protein kinase C with phosphatidylserine is applicable to any multivalent molecule binding to monovalent ligands incorporated into lipid membranes.

The protein kinase C family of enzymes epitomize the role of membrane lipids in regulating protein function. Shortly after its discovery 2 decades ago, protein kinase C was shown to depend on the lipid second messenger, diacylglycerol, and the anionic phospholipid, phosphatidylserine, for activation (1). Activation results from the binding of protein kinase C to membranes, an event that is mediated by two membrane-targeting modules: the C1 and C2 domains. Membrane interaction of both these domains induces a conformational change that removes an autoinhibitory sequence (the pseudosubstrate) from the active site (2).

The requirement for phosphatidylserine and diacylglycerol in the activation of protein kinase C has been well-documented (1, 3–7). Biochemical and structural studies have established that diacylglycerol binds a pocket in the C1 domain, an event which dramatically increases the surface hydrophobicity of this domain (8). The structural basis for the interaction with phosphatidylserine is still under investigation.

Both the enzymatic activity and membrane association of protein kinase C depend sigmoidally on the mole percent of phosphatidylserine in Triton X-100 mixed micelles (3, 6, 7, 9) or the membrane of large unilamellar vesicles (10–12). A fit of both binding and activation data to the Hill equation have yielded high Hill coefficients: up to 12 in the detergent micelle system and up to 6 in lipid bilayers. These high

numbers suggest that protein kinase C interacts with multiple phosphatidylserine molecules (3, 6). This cooperativity has been proposed to reflect an increase in the intrinsic association constants of protein kinase C for phosphatidylserine as binding sites are progressively occupied (3, 6). However, two other factors contribute to this observed cooperativity. Both reduction of dimensionality and the electrostatic surface potential contribute to the apparent cooperativity in the interaction of protein kinase C with anionic lipids (13, 14). Thus, although it is clear that protein kinase C interacts with multiple phosphatidylserine molecules, whether binding sites interact allosterically is not known.

The analysis of cooperativity in the interaction of protein kinase C with phosphatidylserine has relied on the application of the Hill equation to the dependence of either enzymatic activity or membrane association on the mole percent of this phospholipid in lipid membranes or lipid/detergent mixed micelles. However, the correct use of the Hill equation requires specific knowledge of the actual occupancy of binding sites by a ligand (i.e. fractional saturation of binding sites). Given the difficulty in measuring the fractional saturation of protein kinase C with phosphatidylserine, this task has not been accomplished for the association of phosphatidylserine with protein kinase C.

In this study we demonstrate how to determine the number of binding sites on a multivalent macromolecule that associates with a ligand restricted to a surface and how to assess the degree of cooperativity in this interaction. Using this approach, we show that protein kinase C β II interacts with eight phosphatidylserine molecules in the absence of interac-

[†] This work was supported by National Institutes of Health Grant GM 43154 and a National Science Foundation Young Investigator Award.

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tion between binding sites and that diacylglycerol does not induce cooperativity between these sites.

EXPERIMENTAL PROCEDURES

Materials. 1-Palmitoyl-2-oleoyl-phosphatidylserine (POPS¹), 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC), and 1,2-*sn*-dioleoylglycerol (DG) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). (All lipids were analyzed by TLC; only DG displayed a minor contaminant that was identified as 1,3-dioleoylglycerol; this contamination was small, and the lipid was used without further purification). Protamine sulfate, ATP sodium salt, EGTA, Tris (Trizma Base), 12-myristate 13-acetate phorbol (PMA), and 12,13-dibutyrate phorbol (PDBu) were supplied by Sigma. The peptide, Ac-FKKSFKL-amide (derived from the MARCKS protein (15)) was synthesized by the Indiana University Biochemistry Biotechnology Facility. γ [³²P]ATP (3000 Ci mmol⁻¹), [³H]-DPPC (42 Ci mmol⁻¹), and [³H]-PMA (20 Ci mmol⁻¹) were supplied by Du Pont-New England Nuclear (Boston, MA). Bovine serum albumin, fraction V, fatty acid-free, was obtained from Boehringer Mannheim (Indianapolis, IN). Protein kinase C β II, from the baculovirus expression system, was purified as described in ref 16 and stored at -20 °C in 10 mM Tris buffer, pH 7.5 (4 °C), 0.5 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM EGTA, 0.5 mM dithiothreitol, and 50% (v/v) glycerol. All salts were analytical grade and were supplied by J. T. Baker, Inc. All other chemicals were reagent grade.

Lipid Vesicles. Mixtures of lipids in chloroform were dried under a stream of nitrogen and subsequently evacuated under high vacuum. Lipids were then suspended in either 170 mM sucrose or 100 mM KCl, both buffered with 20 mM 1,4-piperazinebis(ethanesulfonic acid) (PIPES) to pH 7.0. Aliquots (0.5 mL) of 10–50 mM lipid were subjected to 5 freeze–thaw cycles, followed by 21 to 51 rounds of extrusion through two stacked 0.1- μ m polycarbonate filters in a microextruder Liposofast (Avestin, Inc., Ottawa, Ontario, Canada) to form large unilamellar vesicles (LUVs). All phospholipid concentrations in stock solutions were determined by phosphate analysis (17). At later stages of experiments, the lipid concentration was calculated from the amount of [³H]DPPC (DPPC = dipalmitoylphosphatidylcholine) that was routinely included in trace quantities in lipid mixtures.

Incorporation of Phorbol Esters in Vesicles. Appropriate aliquots of concentrated PMA in dimethyl sulfoxide were injected into vigorously vortexed suspensions of large unilamellar vesicles. Subsequently, vesicles were incubated for 15 min at 22 °C with occasional gentle vortexing. The partitioning of PMA into vesicles was complete (>98%) as assessed by centrifugation of sucrose-loaded vesicles and analysis of bound ³H-labeled PMA. Appropriate aliquots of suspensions of vesicles containing PMA were diluted severalfold in either activity or binding assay samples. The final content of dimethyl sulfoxide did not exceed 0.5% (v/

v) and did not change the association of the enzyme with vesicles to any appreciable degree.

Protein Kinase C Membrane Binding Assay. The sucrose loaded vesicle assay followed the procedure of Rebecchi et al. (18) adopted for protein kinase C, as described elsewhere (10). Membrane-bound enzyme was separated from unbound enzyme by centrifugation for 15 min at 25 °C. The maximal acceleration varied from 50000g for vesicles with >50 mol % POPS, to 200000g for POPC vesicles to ensure ~95% sedimentation of LUVs. Both the supernatant and the pellet were assayed under identical conditions for kinase activity toward protamine sulfate, as described previously (10, 19). For samples incubated at 22 °C for up to 3 h, the total activity was the same regardless of whether vesicles were added immediately prior to the assay or at various times before the assay. The vesicle-associated kinase activity, A_v , was calculated according to equation 1, where A_b and A_t are

$$A_v = \frac{\beta A_b + (\beta - 1)A_t}{\alpha + \beta - 1} \quad (1)$$

the measured activities of the bottom and top fractions, respectively. The fraction of sedimented vesicles, (α , was calculated from the distribution of ³H-labeled PC, which was included in trace amounts in all lipid mixtures. The fraction of kinase activity found in the supernatant in the absence of lipid, β , was equal, within the limits of experimental error, to the value expected for a nonsedimenting protein (i.e. 0.73 under the experimental conditions used). All experiments were performed in a standard solution composed of 100 mM KCl, 20 mM PIPES, pH 7.0, and 0.3 mg mL⁻¹ bovine serum albumin (BSA). Concentrations of additions that varied depending on experiment are given with appropriate results. The apparent membrane affinity of the enzyme with membranes, K_a , was defined as the ratio of membrane bound to free enzyme divided by the total lipid concentration.

Protein Kinase C Activity Assay. The activity of protein kinase C using protamine sulfate or synthetic peptides as substrates followed published procedures (7). The concentration of protamine was 0.2 mg mL⁻¹; the concentration of the peptides was 50 μ M. The phosphorylation reaction was initiated by the addition of 50 μ M [γ -³²P]ATP (150 Ci mol⁻¹), 10 mM MgCl₂, and the indicated concentrations of the substrates, to an 80 μ L reaction volume containing the concentrations of protein kinase C, lipid, phorbol ester, CaCl₂, and EGTA noted in the figure captions. The phosphorylation reaction was terminated after 8 min at 30 °C by the addition of 25 μ L of 5% (v/v) acetic acid. Samples (85 μ L) were spotted on P-81 ion exchange paper and subsequently washed four times in 0.5 l of 0.4% (v/v) phosphoric acid, followed by one wash in 95% ethanol. Radioactivity associated with papers was determined by liquid scintillation counting in Biosafe II.

Calculation of Free Ca²⁺ Concentration. The total concentration of Ca²⁺ in stock solutions of this cation, as well as stock solutions of other salts and BSA, was measured by plasma emission spectroscopy. The calculations of free Ca²⁺ concentration followed the established protocol (20) and took into account contamination of all solution components by calcium and the effect of ionic strength.

¹ Abbreviations: DG, diacylglycerol; DODG, 1, 2-*sn*-dioleoylglycerol; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; LUV, large unilamellar vesicles; MARCKS, myristolated alanine rich C kinase substrate; PMA, 12-myristate 13-acetate phorbol; POPC, 1-palmitoyl-2-oleoyl-phosphatidylcholine; POPS, 1-palmitoyl-2-oleoyl-phosphatidylserine.

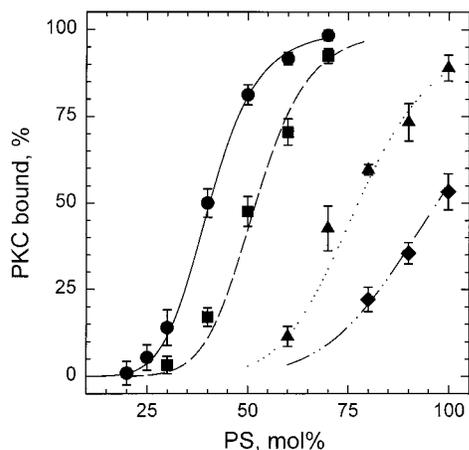


FIGURE 1: Dependence of protein kinase C binding on the mole percent of phosphatidylserine to membranes containing 0.2 mol % DG. The fraction of protein kinase C bound to sucrose-loaded vesicles was measured as described in Experimental Procedures. All vesicles contained 0.2 mol % DODG and the indicated mol % POPS; POPC constituted the remaining lipid. Total lipid concentrations were as follows: 10 μM (\blacklozenge), 50 μM (\blacktriangle), 1 mM (\blacksquare), or 5 mM (\bullet). Protein kinase C (1 nM) and lipid vesicles were suspended in a solution containing 0.3 mg mL⁻¹ BSA, 0.5 mM EGTA, 100 mM KCl, and 20 mM PIPES, pH 6.8. Data represent an average of two independent experiments; error bars indicate the range. Curves represent the best fit to the Hill equation, with Hill coefficients of 6.8 ± 0.4 (dotted-dashed line), 7.8 ± 1.0 (dotted line), 6.5 ± 0.6 (dashed line), and 6.4 ± 0.2 (solid line).

Ionic Strength Dependence. A suspension of vesicles and enzyme was incubated for 15 min in a solution containing 0.3 mg mL⁻¹ BSA, 0.5 mM EGTA, 20 mM PIPES, pH 7.0, and 100–400 mM KCl. Subsequently, membrane-bound enzyme was separated by the sucrose-loaded vesicle method.

Determination of the Fractional Saturation. The apparent membrane affinity, K_{app} , was calculated as a ratio of experimentally determined membrane-bound and free fractions of the enzyme divided by the total lipid concentration. The values of K_{app} obtained at various lipid concentrations were averaged. Subsequently, each point of the dependence of K_{app} on the mole percent of phosphatidylserine was fitted, together with two to four proximal points, with either a linear (F1), exponential (F2), or power (F3) function.

$$y = Ax + B \quad (\text{F1})$$

$$y = B \exp(Ax) \quad (\text{F2})$$

$$y = Bx^A \quad (\text{F3})$$

The appropriate function and the number of proximal points included in the fit were selected on the basis of the accuracy of the parameters obtained in the least-squares fitting procedure. This function and its parameters obtained in the fit were then used to compute the value of its derivative for a given mole percent of phosphatidylserine. The latter value was used to determine the fractional saturation of phosphatidylserine binding sites in protein kinase C β II according to eq A10 in the Appendix.

RESULTS

Figure 1 illustrates the well-characterized sigmoidal dependence of the association of protein kinase C with lipid vesicles on the mole percent of phosphatidylserine. A fit of the Hill equation to these data yielded Hill coefficients

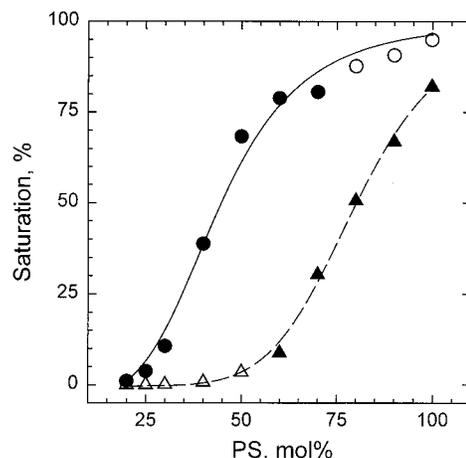


FIGURE 2: Saturation of phosphatidylserine-binding sites in protein kinase C by this lipid for vesicles containing 0.2 mol % DG. The membrane-association constant was calculated from the experimental data shown in Figure 1 and as described in Experimental Procedures; the percentage of saturated phosphatidylserine binding sites was then calculated from the dependence of the membrane-association constant of protein kinase C on the mole percent of phosphatidylserine as described in the Appendix. Symbols are the same as in Figure 1. Closed symbols indicate the points for which the fraction of membrane-bound kinase was measured at the same lipid concentration as used for calculating the fractional saturation. Since the calculations of K_a for <10% or >90% bound protein are not accurate, we used K_a values obtained at higher or lower total lipid concentrations, respectively, to calculate fractional saturation under those conditions (open symbols). Curves are a graphic representation of the least-squares fit of the data to the Hill equation. Hill coefficients obtained from this fit are 4.0 ± 0.4 (solid line) and 6.9 ± 0.3 (dashed line).

ranging from 6.4 to 8. The Hill coefficient did not depend significantly on the total lipid concentration, suggesting that almost complete saturation of binding sites for phosphatidylserine had been reached under all conditions. For the experiments in Figure 1, residual Ca²⁺ was chelated with 0.5 mM EGTA to yield a free concentration of approximately 1 nM: this concentration is well below the apparent dissociation constant of this cation from membrane-bound enzyme so that Ca²⁺ did not contribute to the membrane interaction (14). This negligible Ca²⁺ concentration prevented the appearance of the apparent cooperativity resulting from the effect of the electrostatic potential produced by anionic lipids on the interaction of Ca²⁺ with the enzyme (14). Thus, the sigmoidal dependence of the percent of membrane-bound enzyme on the mole percent of phosphatidylserine in Figure 1 resulted exclusively from the interaction of protein kinase C with phosphatidylserine.

The data in Figure 1 only indicate the fraction of protein kinase C molecules that have at least one bound ligand (phosphatidylserine) and do not indicate the fraction of phosphatidylserine binding sites that are occupied by ligand. Knowledge of the latter is required to determine the degree of cooperativity (21) in the interaction of phosphatidylserine with protein kinase C.

Figure 2 shows fractional saturation curves for the data in Figure 1 that were obtained by the transformation described in the Appendix and Experimental Procedures. The parameters of the functions used in the calculations of the derivative of K_{app} are listed in Table 1. Three points are noteworthy. First, fractional saturation levels off at 100% only when n is set to 8. Hence, protein kinase C interacts

Table 1: Parameters of the Functions Used in the Calculations of the Derivative of K_{app} for the Results Shown in Figure 2

	mol % PS	points used	formula used	$A \pm \Delta A$	$B \pm \Delta B$
1	20	1-3	F2	0.210 ± 0.001	0.059 ± 0.001
2	25	1-3	F2	0.210 ± 0.001	0.059 ± 0.001
3	30	2-4	F2	0.184 ± 0.00	0.13 ± 0.014
4	40	2-6	F3	6.19 ± 0.09	$(2.5 \pm 0.3) \times 10^{-8}$
5	50	2-6	F3	6.19 ± 0.09	$(2.5 \pm 0.3) \times 10^{-8}$
6	60	4-8	F2	0.125 ± 0.007	1.48 ± 0.19
7	70	4-8	F2	0.125 ± 0.007	1.48 ± 0.19
8	80	7-10	F2	0.077 ± 0.004	56 ± 5
9	90	7-10	F2	0.077 ± 0.004	56 ± 5
10	99.8	7-10	F2	0.077 ± 0.004	56 ± 5

with eight phosphatidylserine molecules. That is, the number of phosphatidylserine binding sites on protein kinase C is given by the number n in the definition of the fractional saturation (eq A9 in the Appendix): this number is solved such that the dependence of fractional saturation on the mole percent of lipid levels off asymptotically at 100% of site occupancy. If n is set too low, fractional saturation levels off above 100% occupancy, and if n is set too high, fractional saturation levels off below 100% occupancy. For the data in Figure 2, fractional saturation levels off at 100% only for $n = 8$. Second, Hill coefficients obtained from a fit of the Hill equation to the data in Figure 2 are lower than those obtained for the binding curves in Figure 1. This arises because the dependence in Figure 2 contains a derivative of that in Figure 1 and the Hill coefficient is proportional to the slope of each dependence at the midpoint. Third, the Hill coefficients obtained for the data in Figure 2 decrease substantially with increasing total lipid concentration. For example, a 2 orders of magnitude increase in the total lipid concentration, from 0.05 to 5 mM, reduced the Hill coefficient from 6.9 to 4.

The decrease in the Hill coefficient upon increasing lipid concentration noted in Figure 2 suggests that the apparent cooperativity in the interaction of protein kinase C with phosphatidylserine results from the difference in the concentration of this lipid as experienced by the enzyme when it translocates from a three-dimensional solution to a two-dimensional surface. In other words, this apparent cooperativity should disappear entirely if the concentration of phosphatidylserine experienced by protein kinase C could be identical for the first and subsequent binding events.

To achieve such conditions, we took advantage of the fact that the binding of protein kinase C to PMA is sufficiently strong to cause significant association with membranes composed entirely of the zwitterionic lipid, phosphatidylcholine, and PMA (19). Figure 3 presents the binding of protein kinase C to vesicles containing 2 mol % PMA and increasing amounts of phosphatidylserine in phosphatidylcholine vesicles. In Figure 3, approximately 20% of the protein kinase C was bound to vesicles when the total lipid concentration was 2 mM, in the absence of phosphatidylserine. Addition of phosphatidylserine increased the fraction of membrane-bound protein kinase C; however, the degree of apparent cooperativity was markedly reduced relative to that observed in Figure 1. Specifically, Hill coefficients ranged from 7 to 8 for membranes containing 0.2 mol % DG (Figure 1) and varied from 3.0 to 4.4 for membranes containing 2 mol % PMA (Figure 2), at similar

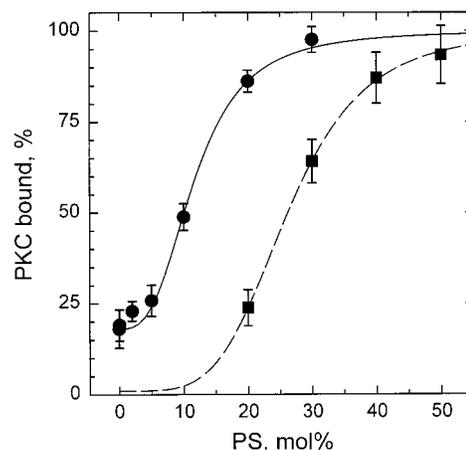


FIGURE 3: Dependence of protein kinase C binding on mol % phosphatidylserine to membranes containing 2 mol % PMA. The fraction of protein kinase C bound to sucrose-loaded vesicles was measured as described in Experimental Procedures. All vesicles contained 2 mol % PMA, the indicated mol % POPS, and POPC as the remaining lipid. Total lipid concentrations were 0.1 mM (■) or 2 mM (●). Protein kinase C (1 nM) and lipid vesicles were suspended in the solution described in the legend to Figure 1. Data represent an average of two independent experiments; error bars indicate the range. Curves represent the best fit of the data to the Hill equation, with Hill coefficients of 4.4 ± 0.4 (dashed line) and 3.1 ± 0.3 (solid line).

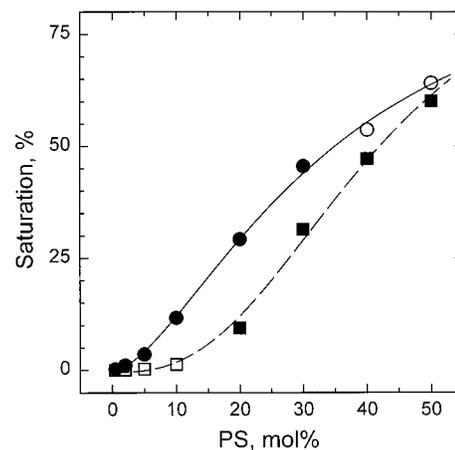


FIGURE 4: Saturation of phosphatidylserine-binding sites in protein kinase C by this lipid for vesicles containing 2 mol % PMA. The percentage of saturated phosphatidylserine binding sites was calculated as in Figure 2. Symbols are the same as in Figure 1. Closed symbols indicate the points for which the fraction of membrane-bound kinase was measured at the same lipid concentration as used for calculating the fractional saturation. Since the calculations of K_a for $<10\%$ or $>90\%$ bound protein are not accurate, we used K_a values obtained at higher or lower total lipid concentrations, respectively, to calculate fractional saturation under those conditions (open symbols). Curves are a graphic representation of the least-squares fit of the data to the Hill equation. Hill coefficients obtained from this fit are 2.7 ± 0.2 (dashed line) and 1.6 ± 0.1 (solid line).

total lipid concentrations. In the latter case, the smaller Hill coefficient was measured for the higher lipid concentration.

To determine the degree of cooperativity for the data in Figure 3, fractional saturation curves were calculated from these data (see Appendix and Experimental Procedures) and are presented in Figure 4. The parameters of the functions used in the calculations of the derivative of K_{app} are gathered in Table 2. Importantly, the Hill coefficient for the dependence of fractional saturation on the mole percent of

Table 2: Parameters of the Functions Used in the Calculations of the Derivative of K_{app} for the Results Shown in Figure 4

	mol % PS	points used	formula used	$A \pm \Delta A$	$B \pm \Delta B$
1	0	1-3	F1	40 ± 6	107 ± 5
2	0.5	1-3	F1	40 ± 6	107 ± 5
3	2	2-5	F2	0.169 ± 0.008	114 ± 22
4	5	2-5	F2	0.169 ± 0.008	114 ± 22
5	10	4-7	F2	0.172 ± 0.004	127 ± 7
6	20	4-7	F2	0.172 ± 0.004	127 ± 7
7	30	6-9	F3	3.93 ± 0.12	0.32 ± 0.01
8	40	6-9	F3	3.93 ± 0.12	0.32 ± 0.01
9	50	6-9	F3	3.93 ± 0.12	0.32 ± 0.01

phosphatidylserine dropped to 1.6 in the presence of PMA and 2 mM total lipid. This contrasts with the much higher Hill coefficient obtained for membranes containing the much weaker ligand, diacylglycerol (Figure 2). Thus, when all the phosphatidylserine-binding sites in protein kinase C experience the same concentration of phosphatidylserine, the apparent cooperativity in the protein-lipid interaction essentially vanishes.

Since K_{app} , by definition, does not depend on the total lipid concentration, the fractional saturation can be calculated from eq A10 (Appendix) for any lipid concentrations, including those that cannot be achieved experimentally. Subsequently, the degree of cooperativity can be determined with the classic Hill equation. Following the commonly accepted way of converting surface concentration into bulk concentration (22, 23) one can obtain the fractional saturation curve under hypothetical conditions for which the concentrations of ligand in the first and all subsequent binding steps are identical. Under such hypothetical conditions, the Hill coefficient for the dependence of fractional saturation on the mole percent of phosphatidylserine calculated from eq A10, based on the experimentally determined K_{app} , equals 1.1.

The apparent cooperativity in the interaction of protein kinase C with phosphatidylserine was first noted on the basis of the dependence of activity of this enzyme on the mole percent of this phospholipid in a Triton X-100 micelle (3). Thus, we were interested in measuring the apparent cooperativity in activation under conditions that maximally reduced the apparent cooperativity in binding. Figure 5 shows the dependence of enzymatic activity on the mole percent of phosphatidylserine under the conditions of the binding assay in Figure 3. Importantly, no significant cooperativity was observed in the dependence of protein kinase C activity on the mole percent of phosphatidylserine: the Hill coefficient obtained from the fit of the Hill equation to the data did not differ significantly from 1. Note that activity was measured in the presence of 0.5 mM EGTA, reducing the concentration of free Ca^{2+} to about 1 nM, as in Figure 1. Thus, as reported previously, there is no absolute requirement for Ca^{2+} in either membrane binding or enzymatic activity for protein kinase C β II (14). However, the rate of phosphorylation doubled in the presence of 0.2 mM Ca^{2+} compared to that in the presence of 0.5 mM EGTA and no exogenous Ca^{2+} (Figure 5). No cooperativity was apparent in the phosphatidylserine dependence in the presence of Ca^{2+} , either. Thus, the apparent cooperativity in the phosphatidylserine dependence for activation of protein kinase C can be abolished under conditions where membrane binding by a nonphosphatidylserine mechanism (namely via

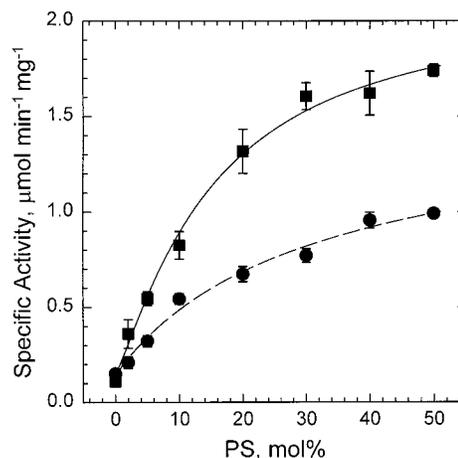


FIGURE 5: Dependence of protein kinase C activity on the mole percent of phosphatidylserine for vesicles containing 2 mol % PMA. Activity was quantified by measuring the amount of radiolabeled phosphate transferred from ATP on to a MARCKS-derived peptide. Both the lipid-independent activity (4% of maximal) and the PMA-induced increase of activity in the presence of POPC vesicles (5% maximal) were subtracted. Vesicles contained indicated mol % POPS, 2 mol % PMA, and POPC as the remaining lipid. Protein kinase C (2 nM) was assayed in the presence of large unilamellar vesicles (2 mM total lipid) as described in Experimental Procedures. Free Ca^{2+} concentration was approximately 1 nM (●) or 0.2 mM (■). The latter was achieved by addition of 0.7 mM $CaCl_2$. Measurements were performed in triplicate. Error bars indicate standard deviation. Curves represent the least-squares fit of the data to the Hill equation. Hill coefficients are 0.7 ± 0.2 (solid line) and 1.0 ± 0.3 (dashed line).

PMA) greatly increases the protein's membrane affinity.

The degree of apparent cooperativity observed in the interaction of protein kinase C with phosphatidylserine dispersed in Triton X-100 mixed micelles is typically much higher in the presence of diacylglycerol (6, 7). To test whether this results from allosteric effects of diacylglycerol upon the enzyme's interaction with phosphatidylserine, we measured the phosphatidylserine dependence of protein kinase C to vesicles containing 0 or 0.2 mol % DG. We adjusted the total lipid concentration of the two assays so that half-maximal binding would occur at approximately the same mole percent of phosphatidylserine and then asked whether the slope of the binding curves would differ. Figure 6 shows that the phosphatidylserine dependence is the same whether or not DG is included in the vesicles: Hill coefficients obtained from the fit of the Hill equation were 7.2 ± 0.6 and 6.6 ± 0.4 for vesicles with and without DG, respectively. To adjust for the tighter binding to the DG-containing vesicles, the total lipid concentration was 20 times lower when the binding to DG-containing vesicles was analyzed. Thus, DG does not influence the apparent cooperativity in the interaction of protein kinase C with phosphatidylserine.

To further address whether DG alters protein kinase C's interaction with phosphatidylserine, we compared how this ligand affected the sensitivity of the protein kinase C: membrane interaction to ionic strength. Figure 7 shows that 1 mol % DG (squares) caused protein kinase C to bind to membranes containing 25 mol % phosphatidylserine with 2 orders of magnitude higher affinity than in the absence of DG (circles). As the KCl concentration was raised from 100 to 400 mM, the membrane affinity dropped; however, the ratio of apparent binding constants measured in the presence

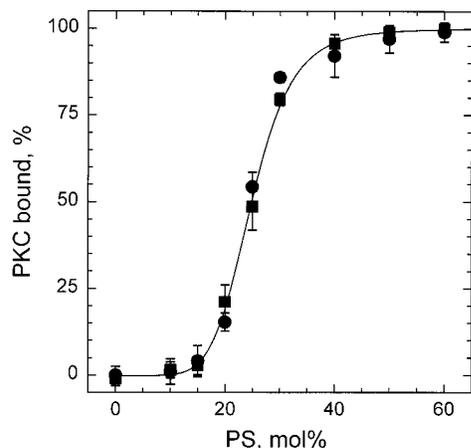


FIGURE 6: Effect of diacylglycerol on the apparent cooperativity in the binding of protein kinase C to phosphatidylserine. The percentage of protein kinase C associated with sucrose-loaded vesicles was measured as described in Experimental Procedures. Protein kinase C (1 nM) and large unilamellar vesicles (0.05 mM (●) or 1 (■) total lipid) were suspended in the solution described in the legend to Figure 1. Vesicles were composed of the indicated mol % POPS, 0 (■) or 0.2 (●) mol % DG, and POPC as the remaining lipid. Each point represents an average of two independent determinations; error bars show the range. Solid line illustrates the least-squares fit of Hill equation to the data obtained for vesicles without DG (■) with the Hill coefficient of 6.6 ± 0.4 . The Hill coefficient from the least-squares fit of the data to the Hill equation obtained for vesicles containing 0.2 mol % DG (●) was 7.1 ± 0.3 .

or absence of DG was unchanged. That is, a 4-fold increase in the concentration of KCl resulted in a 30-fold drop in membrane affinity of the enzyme whether or not DG was present. Thus, diacylglycerol does not change the nature of the interaction of protein kinase C with phosphatidylserine; rather it changes the magnitude of the membrane interaction through a direct interaction with the enzyme.

DISCUSSION

We have used a novel approach to address the mechanism of the apparent cooperativity in the activation of protein kinase C by phosphatidylserine. We show that (1) the high apparent cooperativity in the association of protein kinase C with anionic lipids does not reflect allosteric interactions between potential phosphatidylserine-binding sites but rather arises from effects specific to the interaction of this multivalent macromolecule with its membrane-associated ligands, and (2) diacylglycerol does not induce cooperativity between phosphatidylserine-binding sites.

Calculation of the Fractional Saturation of Phosphatidylserine-Binding Sites on Protein Kinase C. For this work, we first established that the level of saturation of a multivalent macromolecule by surface-restricted ligands can be determined from the dependence of the apparent membrane-association constant on the mole percent of the ligand in the membrane (see Appendix). This approach assumes that the macromolecule–ligand interaction follows the mass action law. Specifically, we show that the fractional saturation can be calculated from the first derivative of the experimentally measured dependence of the apparent membrane association constant of the protein on the mole percent of phosphatidylserine in the membrane. Plotting the fractional saturation as a function of the mole percent of phosphatidylserine can then reveal the maximal stoichiometry of the protein–ligand

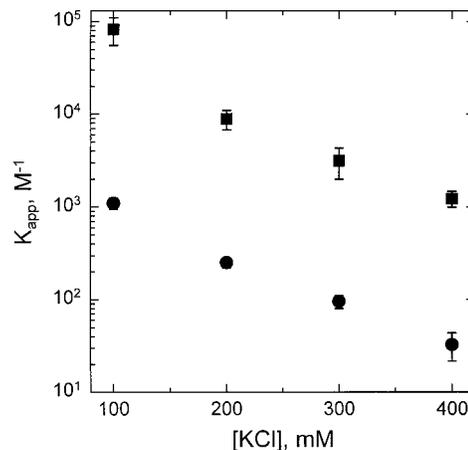


FIGURE 7: Effect of diacylglycerol on the ionic strength dependence of PKC association with vesicles containing phosphatidylserine. The binding of protein kinase C (1 nM) to sucrose-loaded vesicles was measured in the presence of 100, 200, 300, or 400 mM KCl. The apparent membrane-association constant, K_a , was determined from the ratio of vesicle-associated to free protein kinase C, as described in Experimental Procedures. Large unilamellar vesicles were composed of 25 mol % POPS, 0 (●) or 1 (■) mol % DODG, and POPC as the remaining lipid. Total lipid concentrations were 2.5 and 0.05 mM, respectively. Each point represents an average of two independent experiments; error bars indicate the range.

complex. Most importantly, this analysis allows one to dissect the apparent cooperativity arising from reduction of dimensionality (22) and/or entropic effects (24) from the classic cooperativity resulting from the interaction between binding sites.

Although the approach described here is ideally suited to the analysis of proteins such as protein kinase C, it is not free of limitations. First of all, although sequential mass action law is commonly assumed to be valid for the multivalent reactions taking place on surfaces such as membranes (see for example ref 23), it is true only for the limiting case of an empty lattice (25). Under our experimental conditions, the latter case was well-approximated because the protein:lipid molar ratio never exceeded 1:50 000, corresponding to two PKC molecules per lipid vesicle. This ratio assured that the apparent membrane association constant was a good approximation of an equilibrium constant. To test this, we measured the fraction of membrane bound protein kinase C as a function of varying total lipid concentration (no change in vesicle composition). As reported earlier (14, 19), for the range of lipid concentrations studied (0.01–5 mM), the ratio of membrane-bound to free PKC increased linearly with increasing total lipid concentration. A second concern is that the nonspecific accumulation of a positively charged molecule within the diffuse double layer, resulting from the electrostatic potential produced by anionic lipids (26), could mimic the specific binding of the multivalent macromolecule to these lipids (27, 28). This does not appear to be the case for protein kinase C: at very low Ca^{2+} levels, large modifications of the electrostatic potential of the membrane have little effect on the association of the enzyme with anionic lipids (14). Furthermore, the electrostatic potential of membranes containing phosphatidylserine increases rapidly at low mole percent and saturates at the higher mole percent of this lipid (28). The opposite is true for the dependence of K_a in the interaction of protein kinase C with such membranes. Although the interaction

of protein kinase C with phosphatidylserine is clearly electrostatic in nature (see Figure 7), it is likely to reflect a specific charge-charge interaction between the positively charged moieties of amino acids and anionic lipids rather than the less specific accumulation of charged molecules in the electrostatic field of the membrane, reported for other proteins and small basic peptides (27, 28). A third concern is that phosphatidylcholine was treated as an inert matrix in which phosphatidylserine was evenly distributed and free to diffuse. Although this is the most likely case for the mixture of lipids used in the experiments presented here (29), the interaction of protein kinase C with anionic membranes is sensitive to physical properties of the membrane (30).

Protein Kinase C β II Binds Eight Molecules of Phosphatidylserine. The approach described in the Appendix has allowed us to assess the dependence of the fractional saturation of protein kinase C binding sites with phosphatidylserine and consequently to determine that protein kinase C β II interacts with 8 molecules of phosphatidylserine. This number is in the range of earlier estimates of 4–12 based on Hill coefficients calculated for the dependence of either activity or association on the mole percent of phosphatidylserine in Triton X-100 mixed micelles (6, 9). As noted above, the Hill coefficient is only an estimate of the bottom limit for the number of binding sites for a ligand in a macromolecule; this coefficient can also be influenced by the electrostatic potential of the membrane (22). The number of binding sites determined here also agrees with the range of 8–13 that was suggested on the basis of using fluorescent probes to examine phospholipid clustering (31).

What is the mechanism of protein kinase C's interaction with such an unusually large number of ligands? One possibility is that size constraints of a basic surface of the protein allow it to interact electrostatically with approximately eight anionic lipids. This may, indeed, be the case in the absence of C1 domain ligands: under these conditions, the enzyme binds a variety of anionic lipids with equal affinity, with little selectivity beyond the requirement for negative charge (11). The most likely candidate for such an interaction domain would be the C2 module, with recent mutagenesis studies indicating that the anionic lipid-binding site is localized to the Ca^{2+} binding lobes of the domain (32, 33). However, the stoichiometry of the phosphatidylserine–C2 domain complex has not been established. In contrast, the presence of C1 domain ligands causes a selective and stereospecific interaction of protein kinase C with 1,2-*sn*-phosphatidyl-L-serine over other naturally occurring anionic lipids (11, 12). Studies with enantiomeric membranes have recently established that this selective recognition of 1,2-*sn*-phosphatidyl-L-serine arises from stereospecific recognition of this lipid, independently of membrane structural organization, suggesting the presence of a specific binding site for this phospholipid that becomes accessible or structured when the C1 domain is membrane bound (48). Whether this site accommodates one molecule of phosphatidylserine, with additional molecules interacting electrostatically, remains to be established. In this regard, the results shown in Figure 5 limit the number of diacylglycerol-dependent phosphatidylserine binding sites to one. Consistent with this, a number of anionic lipids can replace a fraction (but not all) of the phosphatidylserine molecules required to activate protein kinase C (6,7). Curiously, a mixture of Ca^{2+} -

dependent isozymes of protein kinase C has been shown to bind with equal affinity and apparent cooperativity to membranes containing either phosphatidylserine or the synthetic phospholipid, dansylphosphatidylethanolamine, both in the presence and absence of diacylglycerol (10, 30). One possibility is that determinants on protein kinase C are able to recognize determinants on this synthetic phospholipid that are absent in other nonphosphatidylserine anionic lipids.

Source of the Apparent Cooperativity in the Interaction of Protein Kinase C with Phosphatidylserine. The experiments illustrated in Figures 2 and 4 demonstrate that conditions which decrease the difference in the concentration of phosphatidylserine experienced by protein kinase C in the first and subsequent binding events also reduce the apparent cooperativity in the protein–lipid interaction. Specifically, an increase in the total lipid concentration reduces substantially the apparent cooperativity in the fractional saturation curves (Figures 2 and 4). The latter effect has also been reported for the phosphatidylserine dependence in membrane association of a mixture of Ca^{2+} -dependent isozymes of protein kinase C (10).

The reduction in the apparent cooperativity noted above is even more striking when the association of phosphatidylserine for the first and the subsequent binding sites in protein kinase C occur almost exclusively at the membrane interface. For example, the apparent cooperativity in the binding of phosphatidylserine to protein kinase C (Figure 2, $n_H = 6.9$) is almost completely abolished when the membrane affinity of the enzyme is increased by about 4 orders of magnitude (19) through its direct association with PMA (Figure 4, $n_H = 1.6$). A similar reduction in cooperativity is observed also in the dependence of activity of protein kinase C on the mole percent of phosphatidylserine when measured under conditions where membrane binding is strongly influenced by the PMA–C1 domain interaction (Figure 5).

These findings indicate that the apparent cooperativity in the binding of phosphatidylserine to protein kinase C arises primarily from changes in dimensionality of the phosphatidylserine concentration surrounding the enzyme following the first binding event. Thus, there is very little change in the affinity of protein kinase C for phosphatidylserine with progressing saturation of phosphatidylserine binding sites. This lack of interaction between binding sites is similar to that noted for the association of basic peptides, including one that mimicked the pseudosubstrate of PKC α , with membranes containing anionic lipids (34).

Reduction of dimensionality effects were also examined theoretically for other molecules (24). Theoretical consideration of binding of multivalent macromolecules to surface-restricted ligands suggested that the apparent cooperativity in such a process should arise due to entropy losses incurred in the first binding event (24). An experimental study demonstrated however that such losses are vanishingly small (35).

Diacylglycerol Increases Protein Kinase C's Membrane Affinity without Inducing Cooperativity in Protein Kinase C's Interaction with Phosphatidylserine. Diacylglycerol markedly increases the apparent cooperativity in the phosphatidylserine-dependent binding of protein kinase C to Triton X-100 mixed micelles (11). Here we show that this does not reflect the ability of diacylglycerol to induce

interactions between potential phosphatidylserine binding sites. Specifically, we measured the phosphatidylserine dependence for binding of protein kinase C to membranes under conditions where half-maximal binding was the same in the presence or absence of diacylglycerol. Binding curves in the presence or absence of this second messenger were superimposable, revealing that diacylglycerol increases protein kinase C's membrane affinity without inducing cooperativity. Rather, the effects of diacylglycerol appear to arise from its contribution to the binding energy of protein kinase C for membranes. Consistent with this, we show that the ionic strength of the solution exerts the same effect on the membrane affinity of the enzyme regardless of the presence or absence of DG (Figure 7). That is, a 4-fold increase in ionic strength resulted in a 30-fold decrease in membrane affinity both in the presence and absence of diacylglycerol. Thus, the effects of diacylglycerol on protein kinase C's membrane affinity appear to result from the direct binding of this second messenger to protein kinase C.

Concluding Remarks. The preceding 2 decades have brought to light a constantly growing group of amphitropic proteins (36) that transiently associate with cellular membranes in a ligand-dependent manner. Examples of this class of protein are best represented by large families of proteins such as: protein kinase C (2), proteases in the blood clotting cascade (e.g. refs 37, 38), annexins (e.g. refs 39–41), phospholipases A₂ (42), synaptotagmins (43), and, potentially, a multitude of other proteins containing the C2 domain (44). For these proteins, separation of protein-bound ligand from free ligand cannot be achieved by physical methods because the free and bound ligand (typically a specific lipid) are embedded in the same matrix. The method for determining the stoichiometry and degree of cooperativity presented here may be useful in the study of multivalent amphitropic proteins such as phospholipases A₂ and C, synaptotagmins, annexins, γ -carboxyl glutamic acid-containing proteins in the clotting cascade, and potentially hundreds of proteins with membrane-targeting modules such as myristoylated, pleckstrin homology, C2, and C1 domains. In addition, any other multivalent molecule that binds monovalent ligands restricted to a surface can be treated with a similar approach.

APPENDIX

Determination of Fractional Saturation of Ligand Binding Sites on an Amphitropic Multivalent Protein. In this derivation, we consider a macromolecule, P, with n binding sites for a monovalent ligand, L, that is incorporated in the membrane. The total concentration of the macromolecule, indicated by the index t, is a sum of concentrations of all forms in which this molecule exists, eq A1. Similarly, the

$$[P^t] = [P] + \sum_{i=1}^n [PL_i] \quad (\text{A1})$$

total concentration of the ligand associated with this macromolecule, indicated by the index b, is the sum of concentrations of all macromolecule/ligand complexes multiplied by the number of ligands in each complex (eq A2).

$$[L^b] = \sum_{i=1}^n i[PL_i] \quad (\text{A2})$$

The first binding event is between the ligand, L, and protein, P, and follows the mass-action eq A3. All the

$$K_1 = \frac{[PL]}{[P][L]} \quad (\text{A3})$$

$$K_i = \frac{\{PL_i\}}{\{PL_{i-1}\}\{L\}} \quad (\text{A4})$$

subsequent binding steps follow eq A4, where [] and { } denote bulk and surface concentrations, respectively. Substituting eqs A3 and A4 into eqs A1 and A2, as well as taking into account that $[X] = \{X\}[\text{lipid}]^i$, where X denotes L or PL_i, one arrives at

$$[P^t] = [P](1 + [\text{lipid}^t]) \sum_{i=1}^n \{L\}^i \prod_{j=1}^i K_j \quad (\text{A5})$$

$$[L^b] = [P][\text{lipid}^t] \sum_{i=1}^n i \{L\}^i \prod_{j=1}^i K_j \quad (\text{A6})$$

It follows that

$$[L^b] = \{L\} \frac{d[P^t]}{d\{L\}} \quad (\text{A7})$$

$$K_{\text{app}} = \sum_{i=1}^n \{L\}^i \prod_{j=1}^i K_j \quad (\text{A8})$$

By definition, the fractional saturation, Y , of the macromolecule P by the ligand L at any given concentration is

$$Y = [L^b]/n[P^t] \quad (\text{A9})$$

Combining eqs A5–A9

$$Y = \frac{[\text{lipid}^t]\{L\} \frac{dK_{\text{app}}}{d\{L\}}}{n(1 + [\text{lipid}^t]K_{\text{app}})} \quad (\text{A10})$$

Note, that eq A10 also holds if there are membrane binding modes other than the binding of P to L. In such cases, K_{app} is a sum of a constant and the polynomial containing variable $\{L\}$. However, the derivative of K_{app} is the same as that for the polynomial alone. Thus, for a macromolecule, P, with n binding sites for a ligand, L, that is embedded into a surface, the fractional saturation of this macromolecule can be calculated from the dependence of K_{app} on the surface concentration of the ligand, L.

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BI981344T