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Interaction of Protein Kinase C with Phosphatidylserine. 1. Cooperativity in Lipid Binding†

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Received July 12, 1991; Revised Manuscript Received January 31, 1992

ABSTRACT: The basis for the apparent cooperativity in the activation of protein kinase C by phosphatidylserine has been addressed using proteolytic sensitivity, resonance energy transfer, and enzymatic activity. We show that binding of protein kinase C to detergent–lipid mixed micelles and model membranes is cooperatively regulated by phosphatidylserine. The sigmoidal dependence on phosphatidylserine for binding is indistinguishable from that observed for the activation of the kinase by this lipid [Newton & Koshland (1989) *J. Biol. Chem. 264*, 14909–14915]. Thus, protein kinase C activity is linearly related to the amount of phosphatidylserine bound. Furthermore, under conditions where protein kinase C is bound to micelles at all lipid concentrations, activation of the enzyme continues to display a sigmoidal dependence on the phosphatidylserine content of the micelle. This indicates that the apparent cooperativity in binding does not arise because protein kinase C senses a higher concentration of phosphatidylserine once recruited to the micelle. Our results reveal that the affinity of protein kinase C for phosphatidylserine increases as more of this lipid binds, supporting the hypothesis that a domain of phosphatidylserine is cooperatively sequestered around the enzyme.

The Ca^{2+}/lipid-dependent protein kinase C transduces signals that promote phospholipid hydrolysis (Nishizuka, 1986, 1988; Bell & Burns, 1991). Binding of a wide variety of signals to cell surface receptors results in phospholipase C-catalyzed hydrolysis of phosphatidylinositol bisphosphate, producing the water-soluble head group inositol trisphosphate and the lipid backbone diacylglycerol. Both molecules are critical second messengers in the protein kinase C signaling pathway. Inositol trisphosphate mobilizes intracellular Ca^{2+}, thus causing protein kinase C, which is present in the cytosol under resting conditions, to bind to the plasma membrane. Diacylglycerol activates the enzyme to phosphorylate substrates as well as to autophosphorylate.

The "translocation" of protein kinase C from the cytosol to the plasma membrane is well documented for many stimuli and cell types (Kraft et al., 1982; Farrar & Anderson, 1985; TerBush & Holz, 1986). Insight into the mechanism of the kinase–membrane interaction has been obtained by studies with model membranes. Bazzi and Nelsestuen have shown

†This work was supported by National Institutes of Health Grant GM 43154 and by the Searle Scholars Program of the Chicago Community Trust (A.C.N.).
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0006-2960/92/0431-4661$03.00/0

that protein kinase C binds to acidic membranes in a Ca^{2+}-dependent manner (Bazzi & Nelsestuen, 1987a) and that, under the appropriate conditions, binding may be accompanied by insertion into the hydrophobic core of the membrane (Bazzi & Nelsestuen, 1988a,b). Recently, this group reported that soluble protein kinase C does not bind Ca^{2+} significantly, whereas membrane-bound protein kinase C binds at least eight calcium ions (Bazzi & Nelsestuen, 1990).

Protein kinase C requires the amino phospholipid phosphatidylserine (PS)[1] for optimal activity (Kaibuchi et al., 1981; Boni & Rando, 1985; Bazzi & Nelsestuen, 1987a). Bell and co-workers have developed a detergent–lipid mixed micelle assay to determine the specificity and stoichiometry of the lipid requirement for activity (Hannun et al., 1985). Systematic variation of the number and species of lipid interacting with one monomer of protein kinase C has revealed that one molecule of diacylglycerol, or one molecule of the functional analogue phorbol ester, per protein kinase C is sufficient to activate the enzyme (Hannun et al., 1985; Hannun & Bell, 1986). In contrast, optimal enzymatic activity occurs only at a PS:protein kinase C ratio that is ≥12 (Newton & Koshland, 1989). Extensive analyses by Lee and Bell have demonstrated remarkable specificity in the activity requirement for the serine head group: alterations in the stereochemistry of the serine head group, distance between carboxyl and amine groups, or removal of one or more functional groups result in phospholipids that are unable to activate protein kinase C (Lee & Bell, 1989).

The PS-stimulated activation of protein kinase C is highly cooperative both in detergent–lipid mixed micelles ($n \geq 8$) (Newton & Koshland, 1989) and in phospholipid bilayers compositionally similar to the plasma membrane (Newton & Koshland, 1990). Results from activity measurements led us to propose a model in which protein kinase C interacts with the membrane by cooperatively sequestering PS around a membrane-interacting domain (Newton & Koshland, 1989).

In this paper, we examine the binding of protein kinase C to PS-containing mixed micelles and model membranes. Our results indicate that the interaction of PS with protein kinase C is cooperative: binding of the first lipid increases the probability of binding subsequent PS molecules. In the following paper (Orr & Newton, 1992), we examine factors that affect the affinity of protein kinase C for PS.

Experimental Procedures

Materials

Bovine brain L-α-phosphatidylserine (PS), egg L-α-phosphatidylcholine (PC), transphosphatidylated egg L-α-phosphatidylethanolamine (PE), *sn*-1,2-dioleoylphosphatidic acid (PA), and *sn*-1,2-dioleoylglycerol (DG) were purchased from Avanti Polar Lipids, Inc. *N*-Dansylphosphatidylethanolamine was obtained from Molecular Probes, Inc. Triton X-100 [10%, w/v, aqueous solution low in carbonyl and peroxide content (Surfact-Amps)] was from Pierce Chemical Co. N^{α}-Benzoyl-L-arginine ethyl ester (BAEE), phenyl-Sepharose CL-4B, polylysine–agarose, histone H1 from calf thymus, and trypsin from bovine pancreas [L-1-(tosylamino)-2-phenylethyl chloromethyl ketone-treated, 1.22×10^4 BAEE units mg^{-1}] were supplied by Sigma Chemical Co. Hydroxylapatite (Bio-Gel HT) was from Bio-Rad, phenyl-Superose was from Pharmacia, and [γ-^{32}P]ATP (3000 Ci $mmol^{-1}$) was from Du Pont–New England Nuclear. All other chemicals were reagent grade. Unless otherwise noted, experiments were performed using 20 mM Tris, pH 7.5 at 30 °C (Tris buffer).

Methods

Protein Kinase C. Protein kinase C was purified from cytosolic extract of homogenized rat brain (Sprague-Dawley) by sequential DEAE, phenyl-Sepharose, and polylysine–agarose chromatography, similar to the procedure described by Huang et al. (1986a). Isozyme separation was achieved by hydroxylapatite chromatography of polylysine–agarose-purified protein kinase C, as described by Huang and coworkers (Huang et al., 1986b), followed by concentration on phenyl-Superose. Protein kinase C isozymes (type I, 0.1 mg mL^{-1}; type III, 0.02 mg mL^{-1}) were stored at −20 °C in buffer containing 50% glycerol, 0.5 mM dithiothreitol, 0.5 mM EDTA, 0.5 mM EGTA, and 10 mM Tris, pH 7.4. In the following experiments, qualitatively similar results were obtained using either isozyme.

Lipids. Triton X-100–lipid mixed micelles (1% aqueous solution of Triton X-100, corresponding to 14.7 mM) containing 5 mol % DG, 0–20 mol % PS, 0–10 mol % appropriate non-PS phospholipid (as noted), and 65–95 mol % Triton X-100 were prepared by a modified procedure (Newton & Koshland, 1989) of Hannun et al. (1985). The number of lipid molecules per micelle was calculated as described (Newton & Koshland, 1989), based on an average of 140 molecules of Triton X-100/micelle and on the assumption that micelle size (number of molecules in micelle) increases linearly with added lipid (Robson & Dennis, 1978). Small unilamellar vesicles, of the composition noted in the figure legends, were prepared by combining chloroform solutions of lipids, drying under N_2, adding 100 μL of absolute ethanol, drying under N_2, and hydrating in Tris buffer (Newton & Koshland, 1990). Suspensions (1 mM total lipid) were sonicated to clarity in a bath sonicator (Branson Model 3200, approximately 50 °C).

Protein Kinase C Assay. Protein kinase C activity (substrate phosphorylation) was assayed by measuring the initial rate of [^{32}P]phosphate incorporation from [γ-^{32}P]ATP into saturating amounts of histone (31 μM). Each sample consisted of protein kinase C (50 μL of a 50-fold dilution of stock enzyme into 2 mM dithiothreitol and 20 mM Tris, pH 7.5 at 30 °C), micelles (8 μL of 1% Triton X-100 mixed micelles), and $CaCl_2$ (5 μL of 20 mM solution). Substrate phosphorylation was initiated by addition of 16 μL of a solution containing histone H1 (157 μM), [γ-^{32}P]ATP (100 μM; 3000 Ci $mmol^{-1}$ ATP), 75 mM $MgCl_2$, and 20 mM Tris, pH 7.5 at 30 °C, and samples were incubated for 3 min at 30 °C. The reaction was quenched by addition of 25 μL of a solution containing 0.1 M ATP and 0.1 M EDTA, pH 7. Aliquots (75 μL) were spotted on Whatman P81 ion-exchange chromatography paper and washed 4 times with 0.4% (v/v) phosphoric acid, followed by a 95% ethanol rinse. Papers were added to 5 mL of scintillation fluid (Bio-Safe II, Research Products International Corp.), and ^{32}P was detected by liquid scintillation counting. Autophosphorylation was measured as described above, except that histone was omitted from the reaction mixture. Reactions were terminated after incubation for 3 min at 30 °C by addition of 25 μL of SDS–polyacrylamide gel electrophoresis sample buffer (0.25 M Tris, 8% SDS, 40% glycerol, 0.008% bromophenol blue, and 20% β-mercaptoethanol, pH 6.8). Samples were heated for 3 min at 100 °C and analyzed by SDS–polyacrylamide gel electrophoresis (10% polyacrylamide) followed by autoradiography (Kodak X-Omat film). Protein kinase C bands were excised from dried gels and added to 2.5 mL of scintillation fluid, and ^{32}P was detected by liquid scintillation counting.

[1] Abbreviations: BAEE, N^{α}-benzoyl-L-arginine ethyl ester; DG, dioleoylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine.

Resonance Energy Transfer. A 1-mL suspension of sonicated vesicles (10 mol % dansyl-PE, 5 mol % DG, 0–20 mol % PS, and 65–85 mol % PC) was equilibrated with 100 μM $CaCl_2$. Emission at 520 nm upon excitation at 275 nm was measured before and after the addition of approximately 0.5 μg of type I protein kinase C using an SLM 4800C spectrofluorometer (SLM-Aminco Instruments, Inc.).

Trypsin Digestion. Protein kinase C (50 μL of a 2-fold dilution of stock type III enzyme into 2 mM dithiothreitol and 20 mM Tris, pH 7.5 at 30 °C), micelles (8 μL of 1% Triton X-100 mixed micelles) or unilamellar vesicles (8 μL of 1 mM total lipid), and $CaCl_2$ (5 μL of 20 mM solution) were combined and incubated for 3 min at 30 °C. The reaction was initiated by addition of 16 μL of trypsin (2 μg mL^{-1}, approximately 5 units mL^{-1} in the assay) in Tris buffer, and samples were incubated at 30 °C for 5 min. Proteolysis was quenched by addition of 25 μL of SDS–polyacrylamide gel electrophoresis sample buffer. Samples were heated for 3 min at 100 °C and analyzed by SDS–polyacrylamide gel electrophoresis (10% polyacrylamide) followed by silver staining (Poehling & Neuhoff, 1981). Quantitative analysis of proteolysis was carried out by densitometric scanning of the intact protein kinase C bands with a Hoefer GS300 transmittance/reflectance scanning densitometer (Hoefer Scientific Instruments); under the conditions of these experiments, staining was linearly proportional to the amount of protein kinase C. Trypsin hydrolysis of the fluorogenic substrate N^{α}-benzoyl-L-arginine ethyl ester was unaffected by PS (Newton & Koshland, 1989) or PA. In addition, the rate of proteolysis of protein kinase C was qualitatively similar when the enzyme was bound to micelles containing PS or PA.

Data Analysis. The dependence of protein kinase C binding or activity on the PS content of micelles was analyzed by a nonlinear least-squares fit to a modified Hill equation (eq 1) (Newton & Koshland, 1989) where y is the measured binding

$$y = a + b\left(\frac{x^n}{k^n + x^n}\right) \quad (1)$$

(trypsin assay)/activity of protein kinase C, a is the binding/activity in the absence of phospholipid (background), b is the phospholipid-stimulated binding/activity, x is the concentration of PS, k is the concentration of PS resulting in half-maximal binding/activity, and n is the Hill coefficient. For resonance energy-transfer experiments, the background (a) represents the emission obtained in the absence of protein kinase C.

Results and Discussion

Proteolysis of Protein Kinase C as an Index of Lipid Binding. In order to examine the relation between PS-mediated binding and activity, a binding assay was developed based on the difference in proteolytic sensitivities of soluble and membrane-bound protein kinase C. Trypsin cleaves the native 80-kDa protein kinase C to yield a 45-kDa catalytic domain and a 35-kDa regulatory domain. Protein kinase C is approximately 10-fold more sensitive to trypsinolysis when it is membrane-bound (or micelle-bound) compared to when it is soluble (Newton & Koshland, 1989). Figure 1A shows the trypsin sensitivity of protein kinase C in the presence of Ca^{2+} and Triton X-100 mixed micelles containing 5 mol % DG and 0–8 mol % PS. Protein kinase C was relatively resistant to proteolysis when incubated with mixed micelles containing <4 mol % PS but became sensitive to trypsin when incubated with micelles containing ≥4 mol % PS. Densitometric scanning of the intact protein kinase C bands allowed quantification of micelle binding: the largest peak area was correlated with soluble protein kinase C and the smallest peak area with membrane-bound protein kinase C (Figure 1B).

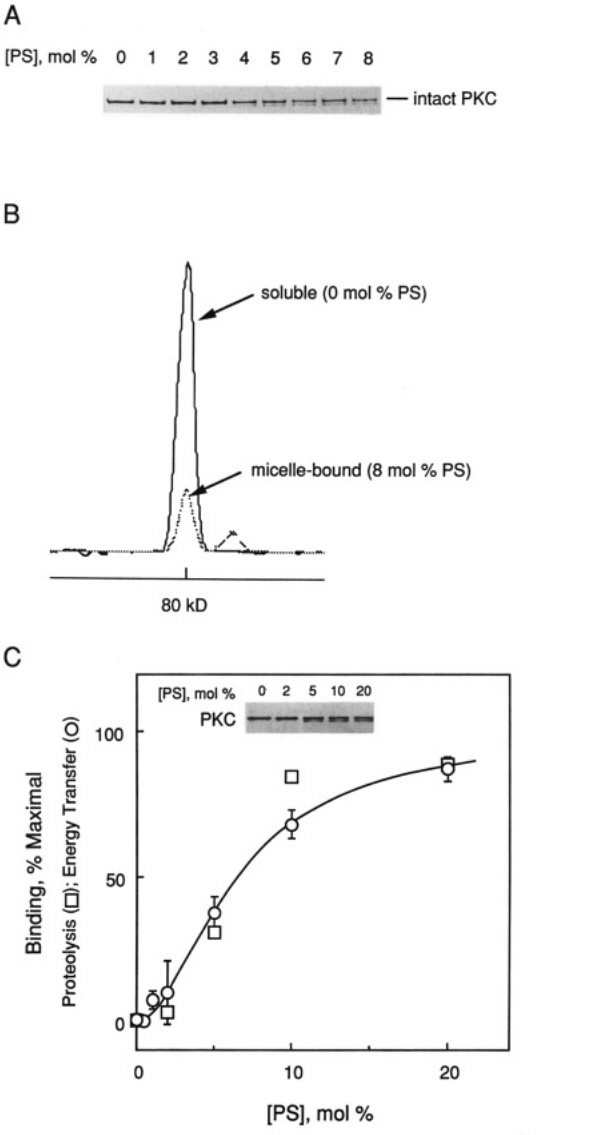

FIGURE 1: Protein kinase C–micelle binding assay. Binding of protein kinase C to PS-containing micelles was monitored by measuring the trypsin sensitivity of protein kinase C in the presence of Triton X-100 mixed micelles (0.1%, w/v) containing 5 mol % DG, the indicated amounts of PS, and $CaCl_2$ (1.3 mM). Samples were treated with trypsin (5 units mL^{-1}) for 5 min at 30 °C and analyzed by SDS–polyacrylamide gel electrophoresis (10%). (A) Silver-stained polyacrylamide gel of intact protein kinase C bands. (B) Superposition of densitometric scans of intact protein kinase C bands from (A) for 0 mol % PS and 8 mol % PS. Binding was quantified by correlating the largest integrated peak area for the intact protein kinase C band with 0% bound protein kinase C and the smallest peak area with 100% bound enzyme. (C) Comparison of resonance energy-transfer and proteolysis binding assays: protein kinase C binding to sonicated vesicles (0–20 mol % PS, 2 mol % DG, 10 mol % dansyl-PE, and 68–88 mol % PC; 1 μM total lipid) in the presence of 100 μM $CaCl_2$ measured by resonance energy transfer (○); binding to sonicated vesicles (0–20 mol % PS, 2 mol % DG, and 78–98 mol % PC; 100 μM total lipid) in the presence of 1.3 mM $CaCl_2$ measured by limited proteolysis assay (□). 100% binding was determined by nonlinear least-squares fit of the data to eq 1 described under Methods.

To confirm that the increased proteolytic sensitivity of protein kinase C reflects binding of the enzyme to PS, binding was assessed using resonance energy transfer from protein kinase C tryptophans to sonicated vesicles labeled with dansylphosphatidylethanolamine (Bazzi & Nelsestuen, 1987a). Figure 1C shows that the increase in resonance energy transfer that occurred upon membrane binding was coincident with the

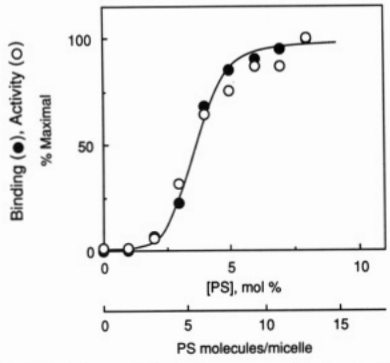

FIGURE 2: Protein kinase C binds cooperatively to PS-containing micelles. Binding (●) of protein kinase C to Triton X-100 (0.1%, w/v) mixed micelles containing 5 mol % DG and 0–8 mol % PS was quantified by densitometric scanning of the intact protein kinase C bands from Figure 1A. The curve is based on the binding data and is that predicted from eq 1 described under Methods. Activity (○) repesents the rate of protein kinase C-catalyzed histone phosphorylation in the presence of Triton X-100 (0.1%, w/v) mixed micelles containing 5 mol % DG, 0–8 mol % PS, and $CaCl_2$ (1.3 mM). 100% activity was defined as the maximum rate of phosphorylation measured.

enzyme's increase in proteolytic sensitivity: half-maximal binding was mediated by 6.1 ± 0.6 mol % PS and 5.5 ± 1.1 mol % PS as measured by resonance energy transfer and proteolysis, respectively. Therefore, the increased trypsin sensitivity of protein kinase C is a measure of its interaction with the membrane. The rates of trypsinolysis of micelle-bound and membrane-bound protein kinase C are indistinguishable (Newton & Koshland, 1989). Although binding to vesicles was measured at lipid concentrations that differed by 2 orders of magnitude in the two assays (1 μM for resonance energy transfer;[2] 100 μM for proteolysis), the PS dependencies for binding did not differ significantly. Bazzi and Nelsestuen have estimated that the dissociation constant for the protein kinase C–PS interaction is less than 5 nM (Bazzi & Nelsestuen, 1987a). Figure 1C also reveals that the binding of protein kinase C to model membranes displayed a sigmoidal dependence on PS content, similar to the activity dependence (Newton & Koshland, 1990). A Hill coefficient of 1.8 ± 0.2 (mean ± SEM) was calculated from the data from three separate experiments. [It is noteworthy that Hill coefficients calculated from membrane data are significantly lower than those obtained from micelle data (Newton & Koshland, 1990). This may reflect differences in the physical properties of the two lipid-presenting systems, differences in the accessibility of PS, or differences in the concentration of PS dispersed in detergent rather than lipid.]

Binding of Protein Kinase C to Phosphatidylserine-Containing Micelles. Figure 2 shows the dependence of protein kinase C binding (filled circles) and activity (open circles) on the PS content of mixed micelles containing 5 mol % DG. Activity was assessed by measuring the initial rate of histone phosphorylation; in the presence of 5 mol % DG, the PS dependencies of histone phosphorylation and autophosphorylation are the same (Newton & Koshland, 1989). Binding displayed the highly cooperative dependence (n = 6.2 ± 0.7) on PS characteristic of the enzyme's activity requirement for this lipid. Maximum activity and binding occurred when micelles contained ≥8 mol % PS. Bell and co-workers have established that protein kinase C binding does not alter significantly the size of Triton X-100–lipid mixed micelles, allowing the determination of the number of PS molecules in a micelle based on the mole percent (Hannun et al., 1985). Thus, 8 mol % PS corresponds to an average of 12 molecules of PS per micelle [see Newton and Koshland (1989)]. Binding and activity were measured in the presence of saturating Ca^{2+}; qualitatively similar results were obtained at lower concentrations of Ca^{2+} (Orr & Newton, 1992).

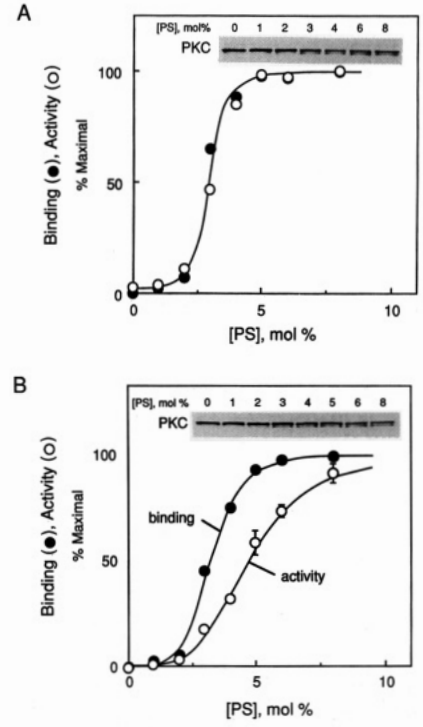

FIGURE 3: Protein kinase C activation is coincident with binding to micelles containing equimolar PS and PE. Binding (●) and activity (○) were measured for protein kinase C in the presence of $CaCl_2$ (1.3 mM) and Triton X-100 (0.1%, w/v) mixed micelles containing 5 mol % DG and (A) equimolar PS and PE (0–16 mol % phospholipid) and (B) equimolar PS and PA (0–16 mol % phospholipid). Percent maximal values were determined as in Figure 2; the standard deviation of triplicate measurements is shown for activity data. Curves are those predicted from eq 1 described under Methods. Insets show silver stains of polyacrylamide gel-separated 80-kDa protein kinase C bands that were scanned to yield the binding data. Concentrations of PS are indicated above the bands.

The possibility that protein kinase C recognizes the amine functionality of PS was investigated by measuring binding in the presence of Ca^{2+} and micelles containing DG and equimolar PS and PE. Figure 3A shows that the PS dependencies for binding (0–16 mol % phospholipid, filled circles) and activity (0–16 mol % phospholipid, open circles) were indistinguishable and highly cooperative (n = 7.4 ± 0.7). Maximal binding and activity occurred when micelles contained ≥5 mol % PS, corresponding to ≥8 molecules of PS per micelle. Earlier work showed that PE can replace 3 or 4 molecules of the ≥12 molecules required for the full activation of protein kinase C (Newton & Koshland, 1989). This result is consistent with the amine group of PE interacting with putative PS binding sites, although with a lower affinity than PS.

Figure 3B shows that binding (filled circles) of protein kinase C to mixed micelles containing DG and equimolar PS and PA precedes activation of the enzyme (open circles). PA caused a slight increase in the number of PS molecules necessary for full activity of protein kinase C and decreased the slope of the sigmoidal curve (n = 4.0 ± 0.5), as described previously (Newton & Koshland, 1989). This suggests that PA reduces the affinity of protein kinase C for PS. The ability

[2] In the fluorescence experiments, the ratio of phospholipid to protein kinase C was on the order of 200:1, so that the membrane surface area was in excess of that required for protein kinase C binding [approximately 100 lipids per protein kinase C; calculated from Bazzi and Nelsestuen (1987a)].

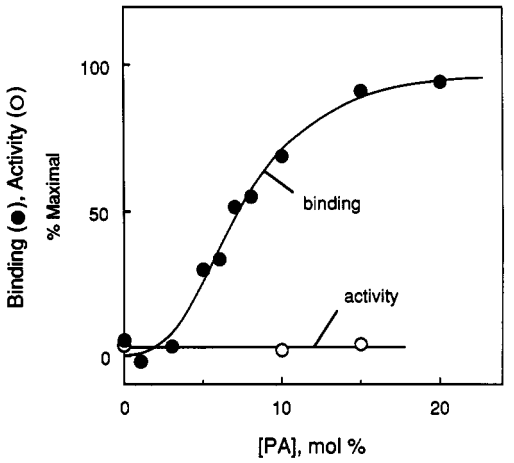

FIGURE 4: Increasing negative surface charge with PA increases protein kinase C binding, but not activity. Protein kinase C binding (●) and autophosphorylation (○) were measured in the presence of Triton X-100 (0.1%, w/v) mixed micelles containing 5 mol % DG, 0–20 mol % PA, and $CaCl_2$ (1.3 mM). The binding curve was obtained as in Figure 2. 100% activity was that stimulated by PS and DG as in Figure 2.

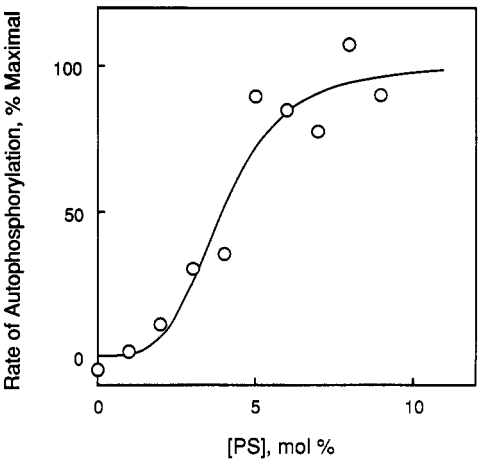

FIGURE 5: Rate of autophosphorylation of protein kinase C is cooperatively regulated by PS in micelles containing 15 mol % PA. The rate of protein kinase C autophosphorylation was measured in the presence of 1.3 mM $CaCl_2$ and Triton X-100 (0.1%, w/v) mixed micelles containing 5 mol % DG, 15 mol % PA, and 0–9 mol % PS. 100% activity was determined by nonlinear least-squares fit of the data to eq 1 described under Methods.

to bind PA-containing micelles at lower PS concentrations is consistent with protein kinase C binding to a negatively charged lipid surface (Bazzi & Nelsestuen, 1987a; Orr & Newton, 1992). However, since the PS requirement for activity is not reduced, it is unlikely that PA interacts productively with putative PS binding sites.

Binding of Protein Kinase C to Phosphatidic Acid-Containing Micelles. Figure 4 shows that protein kinase C bound to Triton X-100–DG mixed micelles containing phosphatidic acid but was not activated by this lipid. Compared with PS-mediated binding under similar conditions, the binding to PA was only slightly cooperative ($n = 2.9 \pm 0.5$). In the presence of saturating DG, maximal binding required approximately twice as much phosphatidic acid (15 mol %) as PS, despite the former having twice the negative charge of PS at neutral pH. Thus, protein kinase C can bind to negatively-charged, DG-containing micelles in the absence of PS, but with a lower affinity and with much less cooperativity compared with the binding to PS. [In the absence of DG, the binding to PA or to PS displays a similar dependence on the net charge of the lipid, despite the disparate sizes in the head group of the two lipids (Orr & Newton, 1992).]

Phosphatidylserine-Dependent Activation of Protein Kinase C Bound to Phosphatidic Acid-Containing Micelles. The highly sigmoidal curves observed for the PS dependence of protein kinase C binding to micelles can be accounted for by several models. Two of these are described below. First, binding to PS may display sigmoidal kinetics because the binding constant for the first PS molecule is lower than that for the second PS molecule, which in turn is lower than that for the third PS molecule etc. The increased probability of binding increasing numbers of PS molecules because of alterations in the affinity of binding sites for PS would represent true cooperativity [see Koshland (1970)]. Second, the sigmoidal dependence could arise because the concentration of PS sensed by soluble protein kinase C (on the order of 200 μM) is several orders of magnitude lower than that in the local environment surrounding micelle-bound protein kinase C. Thus, the probability of binding PS molecules after the first one has bound will increase because of an increase in the local concentration of the PS surrounding micelle-bound protein kinase C. This "reduction in dimensionality" accounts for the sigmoidal curves describing the binding of positively charged peptides to negatively charged membranes (Mosior & McLaughlin, 1991).

To distinguish between the above two models, the PS dependence of protein kinase C autophosphorylation was measured under conditions where the enzyme was bound to micelles at all concentrations of PS. Because protein kinase C autophosphorylates by an intrapeptide reaction, autophosphorylation serves as a measure of the intrinsic, lipid-regulated activity of protein kinase C (Newton & Koshland, 1987). This activity is not sensitive to PA[3] (Newton & Koshland, 1989), so that inclusion of this lipid in mixed micelles affords a mechanism to study the interaction of protein kinase C with PS under conditions where the enzyme is quantitatively bound to micelles. Figure 5 shows that in the presence of mixed micelles containing 15 mol % PA, the rate of autophosphorylation continued to be cooperatively regulated by PS. The cooperative dependence of protein kinase C autophosphorylation on the PS content of PA-containing mixed micelles, under conditions in which protein kinase C was bound to the micelles at all concentrations of PS, is consistent with an increase in binding affinity of protein kinase C for PS as more of this lipid binds. Activity plateaued when the negatively-charged micelles contained approximately 10 mol % PS (on the order of 20 molecules of PS), and the slope of the curve describing the data was lower ($n = 4$) relative to that obtained in the absence of PA (Figure 3; $n = 6$). The decrease in cooperativity suggests that PA reduces slightly the strength of the kinase–PS interaction, as described previously (Newton & Koshland, 1989).

Alternate explanations for the apparent cooperativity exist. For example, the first PS molecules may be inaccessible to protein kinase C. This could arise if the enzyme recognizes a domain of this lipid rather than individual PS molecules. Indeed, the phase-partitioning of PS by Ca^{2+}, present in the foregoing experiments, is well documented (McLaughlin et al., 1981; Ekerdt & Papahadjopoulos, 1982; Feigenson, 1986). While it is possible that protein kinase C is activated by a change in membrane structure, several observations make this

[3] Lee and Bell (1989) have reported that PA stimulates histone substrate phosphorylation, but only if histone is preincubated with protein kinase C and micelles. This activation may arise from histone-induced aggregation of the negatively charged micelles (Bazzi & Nelsestuen, 1987b).

unlikely. First, different isozymes of protein kinase C display different PS dependencies (Newton & Koshland, 1989; Burns et al., 1990), suggesting that the PS requirement is an intrinsic property of the enzyme rather than a property of the bilayer. Second, Ca^{2+}-independent isozymes of protein kinase C are cooperatively activated by PS in the absence of Ca^{2+} (Schaap & Parker, 1990). Third, the greatly reduced ability of lipids with a D-serine head group to activate the kinase (Lee & Bell, 1989) suggests that a property of the protein rather than of the bilayer is the important determinant in a productive protein–lipid interaction.

Conclusions

The foregoing results reveal that binding of PS to protein kinase C is sigmoidal and that activity is linearly proportional to the number of PS molecules bound. Thus, the probability of binding PS increases as more lipid binds to the protein. Taken together with the specificity for PS, the above data are most consistent with an allosteric interaction between protein kinase C and PS, as has been observed for the interaction of β-hydroxybutyrate dehydrogenase with bilayer phosphatidylcholine (Sandermann et al., 1986; Cortese et al., 1989). This is in marked contrast to enzymes such as the Na^+/K^+-ATPase that display apparent cooperativity in their activation by lipids because the enzymes are only active if all, or nearly all, the lipid binding sites are occupied (Sandermann, 1983).

The sigmoidal dependence of binding on PS is consistent with a domain of this lipid sequestered cooperatively around protein kinase C (Newton & Koshland, 1989). Using a fluorescence quenching assay, Bazzi and Nelsestuen have recently shown that protein kinase C clusters acidic lipids upon membrane binding (Bazzi & Nelsestuen, 1991). Additionally, a photoactivatable phorbol ester has been shown to label exclusively PS and PE in brain membranes (Blumberg et al., 1984), consistent with enrichment of these lipids around protein kinase C. Data from our studies with detergent–lipid mixed micelles indicate that the enzyme cooperatively binds ≥12 PS molecules. Furthermore, Hill coefficients ranging from 5 to 11 have been calculated for the PS-dependent activation of various protein kinase C isozymes, consistent with a requirement for at least this many interacting lipid sites (Newton & Koshland, 1989; Burns et al., 1990). Factors that influence the affinity of protein kinase C for PS are addressed in the following paper (Orr & Newton, 1992).

The cooperative interaction with PS, and attendant cooperative dependence of enzymatic activity, provides a sensitive mechanism for regulation of protein kinase C function. Local changes in the intrabilayer concentration of PS could fine-tune the diacylglycerol-mediated activation of the enzyme. Changes in PS synthesis have been reported in response to a number of stimuli. For instance, interleukin 1 stimulation of T cells has been shown to result in an increase in PS synthesis (Didier et al., 1988), whereas phorbol ester treatment of promyelocytic leukemia cells has been reported to inhibit PS synthesis (Kiss et al., 1987). Although changes in PS synthesis were measured several hours after cell stimulation, it is nonetheless interesting that extracellular signals can modulate the formation of the phospholipid that regulates protein kinase C, possibly altering the long-term function of the enzyme. In this regard, alterations in the phospholipid composition of dystrophic cells have been correlated with changes in the fraction of protein kinase C bound to the cell membrane (Cossu et al., 1986). Additionally, nonrandom distribution of lipids promoted by specific interactions with membrane proteins could alter the local concentration of PS in the plasma membrane (Boggs et al., 1977; Wang et al. 1988). Changes in the affinity of protein kinase C for PS may allow the enzyme to respond to subtle changes in the local concentration of this phospholipid in the plasma membrane.

Acknowledgments

We are grateful to Dr. Wayne Weaver for help in purifying protein kinase C and to Dr. David Daleke for helpful discussions.

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Interaction of Protein Kinase C with Phosphatidylserine. 2. Specificity and Regulation†

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Received July 12, 1991; Revised Manuscript Received January 31, 1992

ABSTRACT: The roles of specific and nonspecific interactions in the regulation of protein kinase C by lipid have been examined. Binding and activity measurements reveal two mechanisms by which protein kinase C interacts with membranes: (1) a specific binding to the activating lipid phosphatidylserine and (2) a nonspecific binding to nonactivating, acidic lipids. The specific interaction with phosphatidylserine is relatively insensitive to ionic strength, surface charge, and the presence of nonactivating lipids. The two second messengers of the kinase, diacylglycerol and Ca^{2+}, increase markedly the affinity of the kinase for phosphatidylserine. In contrast, the nonspecific interaction is sensitive to ionic strength and surface charge, and is unaffected by diacylglycerol. These results suggest that electrostatic interactions promote the binding of protein kinase C to membranes but the cooperative and selective binding of phosphatidylserine is the dominant driving force in a productive protein–lipid interaction.

Specificity in the interaction of proteins with lipid plays an essential role in regulating the structure and function of biological membranes. An increasing number of proteins whose function is regulated by specific lipids have been characterized, including transporters (Uratani et al., 1987; Carruthers & Melchior, 1988; Yeagle et al., 1988), receptors (Conforti et al., 1990; Arnold & Newton, 1991), and cytoskeletal proteins (Goldschmidt-Clermont et al., 1990; Maekawa & Sakai, 1990), as well as diverse enzymes such as phosphatases (Politino & King, 1990), oxidases (Tamura et al., 1989), and nucleases (Parks et al., 1990). For many proteins, the lipid requirement for function is met by any acidic lipid, indicating that the protein–lipid interaction is driven by electrostatic forces (Palatini et al., 1977; Gerads et al., 1990; Cornell, 1991). However, absolute specificity in the lipid requirement for at least one protein, β-hydroxybutyrate dehydrogenase, has been described (Sandermann et al., 1986), suggesting that the driving force for the interaction may be through specific binding sites on the protein.

The Ca^{2+}/lipid-dependent protein kinase C is an amphipathic protein that binds acidic membranes in a Ca^{2+}-dependent manner (Nishizuka, 1986). Activity displays a strict specificity for phosphatidyl-L-serine (PS)[1] (Bell & Burns, 1991), a lipid that is cooperatively sequestered around the enzyme (Orr & Newton, 1992). The specificity in the lipid requirement for activity, but relative lack of specificity in the lipid requirement for binding, poses the intriguing problem of how the enzyme interacts with the membrane.

In this paper, we examine the role of nonspecific and specific interactions in the binding of protein kinase C to membranes. The interaction is best described by two distinct mechanisms: a nonspecific binding to acidic, but nonactivating, lipids that is driven primarily by electrostatic forces and a highly specific binding to PS that is mediated by an interaction between the headgroup and putative binding sites specific for the L-serine headgroup.

Experimental Procedures

Materials

Bovine brain L-α-phosphatidylserine (PS), L-α-1,2-dioleoyl-*sn*-glycerol (DG), *sn*-1,2-dioleoylphosphatidic acid (PA), egg L-α-phosphatidylcholine (PC), and L-α-1,2-dioleoyl-*sn*-phosphatidylglycerol (PG) were obtained from Avanti Polar Lipids, Inc. *N*-Dansylphosphatidylethanolamine (dansyl-PE) was purchased from Molecular Probes, Inc. L-α-[1-^{14}C]Dipalmitoylphosphatidylcholine (114.0 mCi

† This work was supported by National Institutes of Health Grant GM 43154 and by the Searle Scholars Program of the Chicago Community Trust (A.C.N.).
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[1] Abbreviations: BAEE, N^{α}-benzoyl-L-arginine ethyl ester; DG, dioleoylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine.

0006-2960/92/0431-4667$03.00/0