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

Jeffrey W. Orr and Alexandra C. Newton*
Department of Chemistry, Indiana University, Bloomington, Indiana 47405
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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

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* To whom correspondence should be addressed.

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

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mmol^{-1}) and [γ-^{32}P]ATP (3000 Ci mmol^{-1}) were from Du Pont–New England Nuclear. Triton X-100 [10%, w/v, aqueous solution (Sufact-Amps)] was obtained from Pierce Chemical Co. Histone H1 from calf thymus, L-(1-tosylamino)-2-phenylethyl chloromethyl ketone-treated trypsin from bovine pancreas (1.22×10^4 BAEE units, mg^{-1}), phenyl-Sepharose CL-4B, and poly(L-lysine)–agarose were purchased from Sigma Chemical Co. Q-Sepharose Fast Flow and phenyl-Superose were supplied by Pharmacia, and hydroxylapatite (Bio-Gel HT) was from Bio-Rad. All other chemicals were reagent grade. Reactions were carried out using 20 mM Tris, pH 7.5 at 30 °C (Tris buffer).

Methods

Protein Kinase C. Purification of protein kinase C from cytosolic extract of rat brain (Sprague-Dawley) was carried out by a modified procedure of Huang and co-workers (Huang et al., 1986a) involving Q-Sepharose, phenyl-Sepharose, and poly(L-lysine)–agarose chromatography, followed by isozyme separation on hydroxylapatite (Huang et al., 1986b) and concentration on phenyl-Superose. Isozymes were stored at −20 °C in buffer consisting of 50% glycerol, 10 mM Tris, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, and 0.5 mM dithiothreitol. The following experiments employed either separated isozymes (I or III) or a mixture of type I, II, and III isozymes; results obtained using all protein kinases C were qualitatively similar (Newton & Koshland, 1989; Burns et al., 1991).

Lipids. Triton X-100–lipid mixed micelles were prepared as described in the preceding paper (Orr & Newton, 1992). All phospholipid concentrations in chloroform were determined by phosphate assay of acid-hydrolyzed sample (Bartlett, 1959). Sonicated vesicle suspensions were prepared by drying 1 μmol of total lipid, including trace L-α-[1-^{14}C]dipalmitoylphosphatidylcholine (0.1–0.3 μCi), in chloroform under N_2, adding 100 μL of absolute ethanol, drying under N_2, and resuspending in 1 mL of Tris buffer. Suspensions were sonicated to clarity at approximately 50 °C in a bath sonicator and then centrifuged at 150000g for 30 min to pellet multilamellar structures. Total lipid concentration was adjusted to 0.2 mM in Tris buffer, based on the amount of ^{14}C label recovered in the supernatant (40–70% of total ^{14}C).

Protein Kinase C Activity Assay. Initial rates of autophosphorylation and [^{32}P]phosphate incorporation into histone were measured as described in the preceding paper (Orr & Newton, 1992). Phosphorylation assays were performed in the presence of Triton X-100–lipid mixed micelles or sonicated vesicles; compositions and concentrations are noted in the Figure legends. At the concentrations employed, vesicle-supported activity depended on the mole percent PS and was independent of the absolute micelle or vesicle concentration.

Resonance Energy Transfer. Protein kinase C (type I, approximately 0.5 μg mL^{-1}), small unilamellar vesicles (1 μM total lipid, 10 mol % dansyl-PE), and $CaCl_2$ (100 μM) were equilibrated in 1 mL of Tris buffer containing 0 or 150 mM NaCl, corresponding to ionic strengths of 21 and 171 mM, respectively. Resonance energy transfer was measured from the tryptophan emission to the dansyl-PE probe with an Aminco Instruments, Inc., SLM 4800C spectrofluorometer. Excitation was at 280 nm, and emission was monitored using an open channel with a 418-nm cut-off filter.

Trypsin Digestion. A solution of protein kinase C (approximately 0.2 μg mL^{-1} either of a mixture of isozymes or of type III protein kinase C) in 50 μL of Tris buffer containing 2 mM dithiothreitol was incubated with 8 μL of 1% (w/v) Triton X-100–lipid mixed micelles and 5 μL of $CaCl_2$ (2 or 20 mM) for 2 min at 30 °C. Trypsinolysis was initiated by addition of 16 μL of trypsin in Tris buffer (see concentrations noted in figure legends), and the reaction mixture was incubated at 30 °C for the indicated times. Separated isozymes or a mixture of isozymes yielded qualitatively similar results upon mild treatment with trypsin, undergoing a 10-fold increase in trypsin sensitivity after membrane or micelle binding.

Data Analysis. The dependence of protein kinase C activity or binding on the lipid composition of micelles or vesicles was analyzed by a nonlinear least-squares fit to the modified Hill equation (eq 1) described in the preceding paper (Orr & Newton, 1992).

Results

Diacylglycerol. The effect of diacylglycerol on the interaction of protein kinase C with Triton X-100–lipid mixed micelles was measured by monitoring the change in proteolytic sensitivity that accompanies micelle binding (Orr & Newton, 1992). Figure 1A shows that diacylglycerol markedly enhanced the affinity of protein kinase C for phosphatidylserine, in the presence of saturating Ca^{2+}. In the absence of diacylglycerol, binding displayed a cooperative dependence on PS ($n = 2.5 \pm 0.8$), with half-maximal binding occurring at 11 mol % PS. In the presence of 2 mol % diacylglycerol, half-maximal binding was observed at 5 mol % PS, and n increased to 6.8 ± 1.7. Higher concentrations of DG (5 mol %) caused only a slight additional decrease in the concentration of PS necessary for half-maximal binding and no significant effect on n. At these saturating diacylglycerol concentrations, the dependence of binding on PS concentration is indistinguishable from the sigmoidal dependence for activity (Orr & Newton, 1992).

In marked contrast to its effect on the interaction of protein kinase C with PS, diacylglycerol had no significant effect on the affinity of protein kinase C for PA. Figure 1B shows that half-maximal binding to micelles required approximately 6 mol % PA, in the presence or absence of diacylglycerol (5 mol %). PA has twice the negative charge of PS at neutral pH, and this may account for why protein kinase C binding required half as much PA as PS in the absence of diacylglycerol.

Calcium. Figure 2 shows that the PS-dependent binding of protein kinase C was sensitive to Ca^{2+} concentration, with less PS required for binding at saturating concentrations of the cation. Half-maximal binding required 7.5 mol % PS in the presence of 0.13 mM Ca^{2+}, compared with 4.4 mol % in the presence of 1.3 mM Ca^{2+}. Binding to PA displayed the same dependence on PA concentration at both these concentrations of Ca^{2+}: half-maximal binding required approximately 6 mol % PA in the presence of 1.3 mM Ca^{2+} [see Figure 4 in Orr and Newton (1992)] or 0.13 mM Ca^{2+} (Figure 1B).

Ionic Strength. The PS-dependent interaction of protein kinase C with sonicated vesicles, containing DG, displayed only small differences in the presence of 150 mM NaCl compared with no NaCl. Figure 3A shows that approximately an additional 5 mol % PS was needed to attain half-maximal binding of protein kinase C in the presence of 150 mM NaCl (ionic strength = 171 mM) as in its absence (ionic strength = 21 mM). At saturating PS, NaCl had no significant effect on the binding of protein kinase C to vesicles, as reported previously (Bazzi & Nelsestuen, 1987b).

The interaction of protein kinase C with PS was also measured by autophosphorylation. Protein kinase C autophosphorylates by an intrapeptide reaction, so that its autophosphorylation activity depends on the enzyme's interaction with its cofactors and is independent of protein–protein interactions or substrate–micelle interactions (Newton & Koshland, 1987). Moreover, because activity is linearly pro-

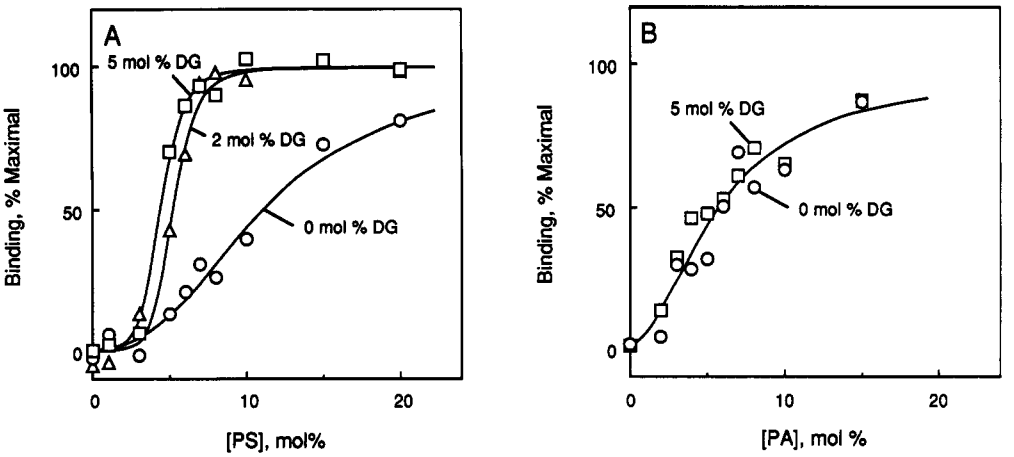

FIGURE 1: Diacylglycerol affects the affinity of protein kinase C for phosphatidylserine-, but not phosphatidic acid-, containing mixed micelles. Protein kinase C binding to PS- or PA-containing micelles was monitored by measuring the trypsin sensitivity of protein kinase C. (A) Binding in the presence of Triton X-100 mixed micelles (0.1%, w/v) containing 0 (○), 2 (△), or 5 mol % DG (□), and 0–20 mol % PS, and $CaCl_2$ (1.3 mM). Samples containing a mixture of isozymes were treated with trypsin (0.4 $\mu g\ mL^{-1}$, corresponding to 5 units mL^{-1}) for 2 min at 30 °C, and the fraction of intact protein kinase C was analyzed by SDS–polyacrylamide gel electrophoresis (10%), as described (Orr & Newton, 1992). (B) Binding in the presence of Triton X-100 mixed micelles (0.1%, w/v) containing 0 (○) or 5 mol % DG (□), 0–15 mol % PA, and $CaCl_2$ (0.13 mM). Samples containing type III enzyme were treated with trypsin (0.02 $\mu g\ mL^{-1}$, corresponding to 0.25 unit mL^{-1}) for 3 min at 30 °C and analyzed by SDS–polyacrylamide electrophoresis (10%). Data were analyzed by nonlinear least-squares fit to the modified Hill equation (eq 1) in the preceding paper (Orr & Newton, 1992). All curves are those predicted from the equation.

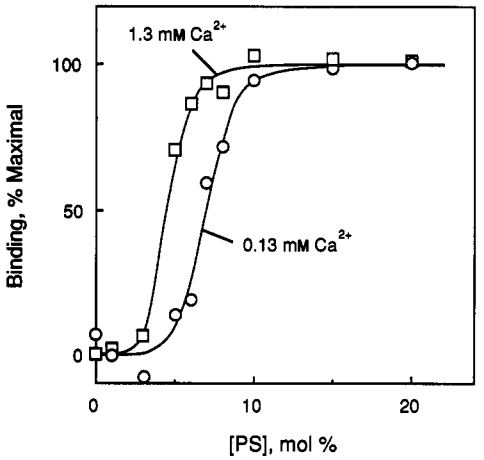

FIGURE 2: Affinity of protein kinase C for phosphatidylserine-containing mixed micelles is affected by calcium concentration. Binding of protein kinase C (mixture of isozymes) to Triton X-100 mixed micelles (0.1%, w/v) containing 5 mol % DG and 0–20 mol % PS, in the presence of 0.13 (○) or 1.3 mM $CaCl_2$ (□), was measured by limited trypsinolysis (5 units mL^{-1} for 2 min at 30 °C). Data were analyzed as described in Figure 1. Curves are those predicted from eq 1 of Orr and Newton (1992).

portional to the amount of PS bound (Orr & Newton, 1992), this activity provides a useful measure of the enzyme's interaction with PS.

Figure 3B shows that the PS dependence for autophosphorylation, at subsaturating Ca^{2+} concentrations, was relatively insensitive to ionic strength: half-maximal activity required 14 mol % PS in the presence of 0 or 150 mM NaCl, corresponding to ionic strengths of 81 and 231 mM, respectively. The absolute rate of autophosphorylation was inhibited by 56% in the presence of NaCl (Figure 3B, inset), consistent with inhibition of the catalytic activity of the enzyme by salt (Hannun & Bell, 1990).

The effect of increasing ionic strength on protein kinase C activity in the presence of Triton X-100 micelles was examined in the experiment described in Figure 4. NaCl increased slightly the concentration of PS eliciting half-maximal activity, which required 7 mol % PS in the absence of NaCl and 9 mol % in the presence of 150 mM NaCl (Figure 4A). Because NaCl alters the size of micelles (Robson & Dennis, 1977), this difference does not necessarily reflect a change in the interaction of the enzyme with this lipid. In contrast, NaCl (150 mM) increased by over 3-fold the concentration of PS required for half-maximal activity toward phosphorylation of the basic substrate histone (Figure 4B). This is consistent with electrostatic interactions playing a major role in targeting this basic substrate to the micelle surface and hence to protein kinase C.

Figure 5 shows that binding of protein kinase C to sonicated vesicles was sensitive to membrane surface charge. Protein kinase C bound to vesicles containing 20 mol % PA in the absence of PS. In contrast, activity was dependent on PS, as described in the preceding paper (Orr & Newton, 1992).

Effects of Other Phospholipids on Phosphatidylserine Requirement. The effect of non-PS phospholipids on the interaction of protein kinase C with PS was examined. Figure 6A shows that inclusion of the neutral lipid phosphatidylcholine (PC) (10 mol %) in Triton X-100 mixed micelles had little effect on the PS requirement for autophosphorylation: half-maximal activity required 4.7 ± 0.3 mol % PS in the presence of PC compared with 4.4 ± 0.2 mol % in the absence of PC. As reported previously, a slight decrease in cooperativity was observed ($n = 4.7 \pm 0.3$) in the presence of PC, indicating that the lipid may perturb the strength of PS–PS or PS–protein kinase C interactions (Newton & Koshland, 1989). The negatively charged phosphatidylglycerol (PG) decreased the concentration of PS required for activity: full enzymatic activity required 10 mol % PS (average of 18 molecules/micelle) in the presence of 10 mol % PG, but required 15 mol % PS (average of 30 molecules/micelle) in the presence of 10 mol % PC. This result is consistent with PG binding productively to protein kinase C. In contrast, the negatively charged PA did not substitute for PS in activating protein kinase C. This lipid markedly decreased the affinity of protein kinase C for PS, consistent with PA's effects on the PS requirement for activity in vesicles (Figure 5). This suggests that PA may compete with PS for binding to protein kinase C but the binding would not contribute to activity.

The ability of PG to substitute for PS was sensitive to ionic strength (Figure 6B). In the presence of 150 mM NaCl, the PS requirement for autophosphorylation was similar whether 10 mol % PG or 10 mol % PC was included in the micelles.

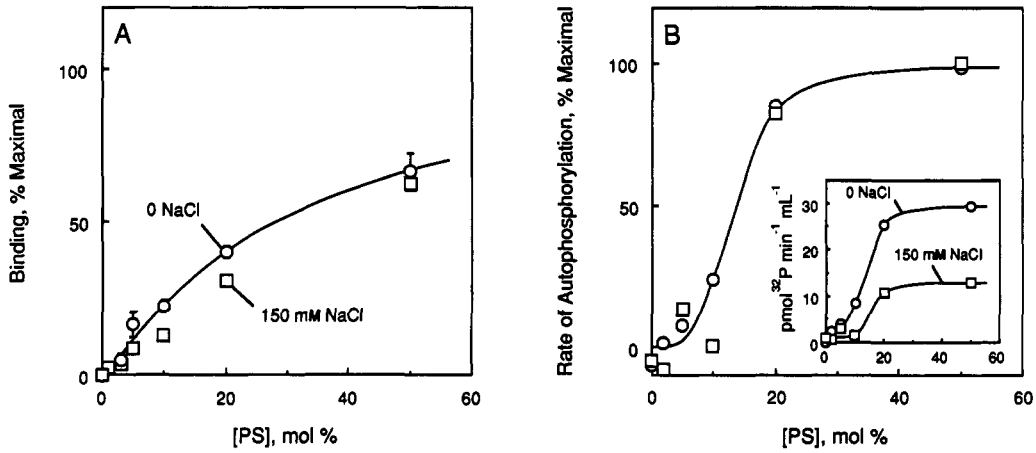

FIGURE 3: Increased ionic strength does not affect the affinity of protein kinase C for phosphatidylserine-containing small unilamellar vesicles. (A) Binding of protein kinase C (type I) to small unilamellar vesicles (1 μM total lipid) containing 2 mol % DG, 10 mol % dansyl-PE, 0–50 mol % PS, and 38–88 mol % PC, with 100 μM $CaCl_2$ and 0 (○; mean ± standard deviation of three separate experiments) or 150 mM NaCl (□; average of two separate experiments), was quantified by resonance energy transfer. Corresponding ionic strengths were 21 and 171 mM. (B) Rate of protein kinase C (type I) autophosphorylation supported by small unilamellar vesicles (70 μM total lipid) containing 5 mol % DG, 0–50 mol % PS, and 45–95 mol % PC, in the presence of 130 μM $CaCl_2$ and 0 (○) or 150 mM NaCl (□). Rate is expressed as percent maximal, determined by nonlinear least-squares fit of the data, as described in Figure 1. The inset shows the absolute rate of autophosphorylation (picomoles of [^{32}P]phosphate incorporated per minute per milliliter) in the presence of 0 (○) or 150 mM NaCl (□). The ionic strength in the absence and presence of NaCl was 81 mM and 231 mM, respectively. Curves in (A) and (B) are those predicted from nonlinear least-squares analysis of the data in the absence of NaCl [see eq 1 in Orr and Newton (1992)].

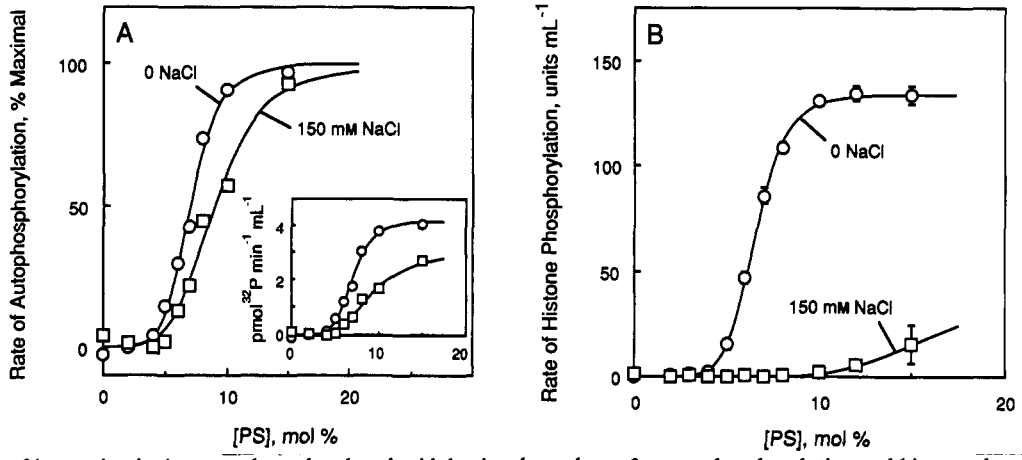

FIGURE 4: Effect of increasing ionic strength on the phosphatidylserine dependence for autophosphorylation and histone phosphorylation. (A) The rate of protein kinase C autophosphorylation (expressed as percent maximal) was measured in the presence of Triton X-100 (0.1%, w/v) mixed micelles containing 5 mol % DG, 0–15 mol % PS, 1.3 mM $CaCl_2$, and 0 (○) or 150 mM NaCl (□). Corresponding ionic strengths were 68 and 218 mM. The inset shows the absolute rate of autophosphorylation (picomoles of [^{32}P]phosphate incorporated per minute per milliliter) in the presence of 0 (○) or 150 mM NaCl (□). (B) Rate of protein kinase C catalyzed histone phosphorylation (average ± standard deviation of triplicate measurements) supported by Triton X-100 (0.1 %, w/v) mixed micelles containing 5 mol % DG and 0–15 mol % PS, with 0.13 mM $CaCl_2$ and 0 (○) or 150 mM NaCl (□). One unit is defined as 1 nmol of phosphate incorporated into a saturating concentration of histone in 1 min. A mixture of isozymes was used in these experiments. Curves are predicted from eq 1 of Orr and Newton (1992).

Thus, the interactions of protein kinase C with PG can be screened with increasing ionic strength, indicating the interaction is primarily electrostatic. In contrast, the interaction with PS is considerably less sensitive to ionic strength.

Discussion

Specific Interaction with Phosphatidylserine. Binding measurements support high specificity, multiplicity, and cooperativity in the interaction of protein kinase C with PS. This interaction is regulated by two effectors of the kinase, diacylglycerol and Ca^{2+}. Both these second messengers cause a marked increase in the affinity of protein kinase C for PS, as evidenced by a sharp increase in the degree of cooperativity in binding the lipid and a concomitant decrease in the concentration of lipid eliciting half-maximal activity. Studies with Triton X-100 mixed micelles reveal that, at saturating diacylglycerol and Ca^{2+} concentrations, protein kinase C cooperatively binds multiple PS molecules (Orr & Newton, 1992). Compared with binding to nonactivating acidic lipids, the binding to PS is relatively insensitive to increasing ionic strength, indicating specificity beyond a simple electrostatic interaction. Consistent with this, the binding of protein kinase C to vesicles containing 30 mol % PS is unaffected by up to 300 mM NaCl (Bazzi & Nelsestuen, 1987b). Similarly, Hannun and Bell have reported that NaCl does not affect the binding of phorbol esters to protein kinase C (Hannun & Bell, 1990). The ability of the second messengers to alter the affinity of protein kinase C for PS, the relative insensitivity of the interaction to ionic strength and surface charge, and the specificity for the L-serine headgroup (Lee & Bell, 1989) are consistent with protein kinase C having at least one binding site that is specific for PS. Furthermore, lipids sharing some of the functional groups of PS are not effective at reducing the large number of PS molecules required for activity, suggesting that protein kinase C has multiple binding sites specific for PS.

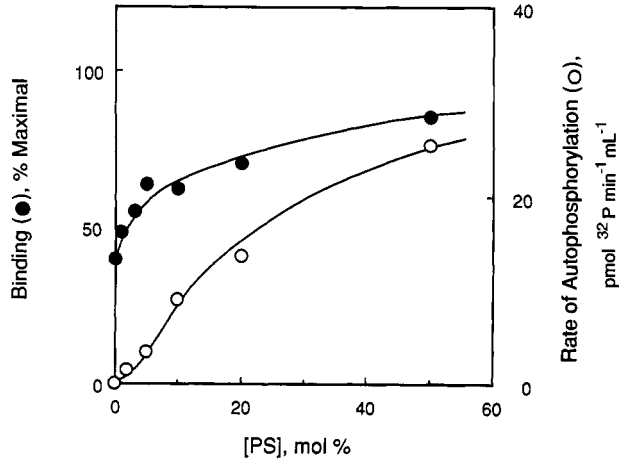

FIGURE 5: Phosphatidylserine dependence of binding and activation of protein kinase C in the presence of vesicles containing 20 mol % phosphatidic acid. Protein kinase C (type I) binding (●) and autophosphorylation (○) were measured in the presence of small unilamellar vesicles composed of 2 mol % DG, 20 mol % PA, 10 mol % dansyl-PE, 0–50 mol % PS, and 18–68 mol % PC. Binding was measured in the presence of 1 μM lipid and 100 μM $CaCl_2$; activity was measured in the presence of 70 μM lipid and 1.3 mM $CaCl_2$. Binding data represent the average of two separate experiments; the value for 100% maximal binding was that obtained from nonlinear least-squares analysis of the data in Figure 3A (no PA).

The existence of multiple, highly specific, interacting lipid binding sites would place protein kinase C in a new class of lipid binding enzymes (Newton & Koshland, 1989). Enzymes such as the Na^+/K^+-ATPase have multiple low-specificity binding sites, others such as β-hydroxybutyrate dehydrogenase have multiple low-specificity sites plus a few specific binding sites, and others have a few high-affinity sites that have low specificity (Sandermann, 1983). Whether other membrane proteins cooperatively bind multiple, specific lipids remains to be determined. In this regard, two proteins have been identified recently that contain domains homologous to the regulatory region of protein kinase C. A synaptic vesicle protein, p65 (Perin et al., 1990), and *n*-chimaerin (Ahmed et al., 1990) interact with PS, although it is not yet clear whether this is through multiple, specific, interacting lipid binding sites that appear to characterize the protein kinase C–PS interaction.

Nonspecific Interaction with Other Acidic Lipids. Protein kinase C binds to membranes containing the monovalent acidic lipids PG and phosphatidylinositol (PI) (Bazzi & Nelsestuen, 1987a) and, as described above, the divalent acidic lipid PA. The enzyme has also been shown to bind Triton X-100 mixed micelles containing a variety of carboxylic phospholipids and PA (Lee & Bell, 1989; Orr & Newton, 1992). Similar to the interaction with PS, binding to acidic membranes requires Ca^{2+} (Bazzi & Nelsestuen, 1987a). However, unlike its effects on PS, Ca^{2+} does not alter the PA dependence for binding (i.e., lipid concentration required for half-maximal binding) at the concentrations examined. Similarly, diacylglycerol does not increase the affinity of the kinase for these lipids. Thus, the binding to nonactivating acidic lipids is unlikely to be through the putative PS binding sites whose affinity for lipid is regulated by the two second messengers.

The binding of protein kinase C to non-PS acidic membranes may be mediated by electrostatic interactions between basic residues on the protein and the negatively charged lipids. Similar to the binding of basic proteins (Benfenati et al., 1989; Cornell, 1991) and basic peptides (Kim et al., 1991; Mosior & McLaughlin, 1991) to acidic membranes, the binding of protein kinase C to the negatively charged lipid, PG, is sensitive to ionic strength.

It has been reported recently that the highly negatively charged phosphoinositides are able to reduce the PS requirement for activity in a manner proportional to the number of phosphates, and hence charge, on the inositol headgroup (Lee & Bell, 1991). Whether this reflects an increased affinity of protein kinase C for the membranes resulting from the increased surface charge remains to be explored.

Ionic interactions are important in the phosphorylation of basic substrates by protein kinase C. Increasing ionic strength markedly inhibits the phosphorylation of histone without significantly affecting autophosphorylation. These data are consistent with membrane surface charge playing a critical role in targeting protein kinase C substrates (Bazzi & Nelsestuen, 1987b; Newton & Koshland, 1990).

Conclusion

Figure 7 illustrates a possible model for the binding of protein kinase C to PS or to nonactivating acidic membranes that is consistent with the present results. In this model, the binding to nonactivating acidic lipids is driven by electrostatic interactions (lower part of figure). In contrast, the binding to PS is mediated by multiple, interacting binding sites that

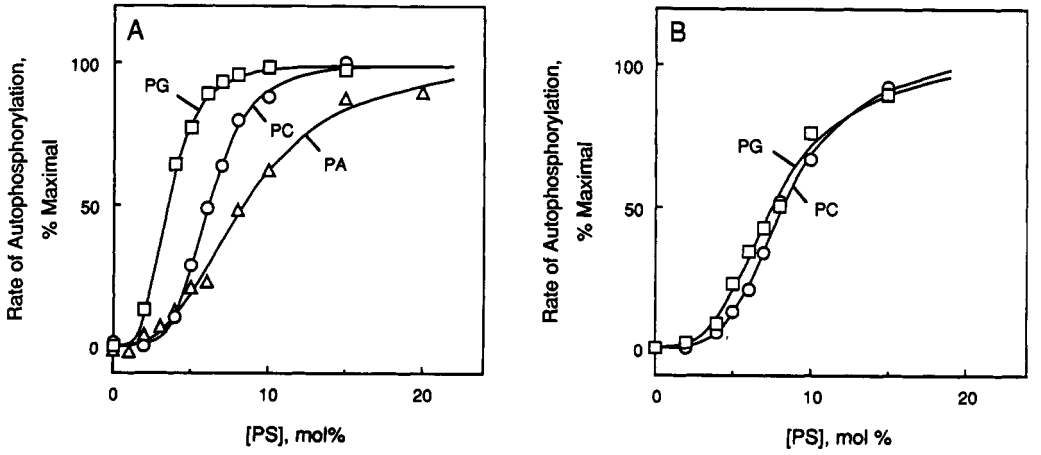

FIGURE 6: Effects of other phospholipids on the phosphatidylserine requirement for protein kinase C autophosphorylation at different ionic strengths. (A) The rate of protein kinase C autophosphorylation supported by Triton X-100 (0.1%, w/v) mixed micelles containing 5 mol % DG, 0–20 mol % PS, and 10 mol % PG (□), PC (○), or PA (△), in the presence of 1.3 mM $CaCl_2$, was determined as in Figure 4A. (B) The rate of protein kinase C autophosphorylation supported by Triton X-100 (0.1%, w/v) mixed micelles containing 5 mol % DG, 0–15 mol % PS, and 10 mol % PG (□) or PC (○), measured in the presence of 1.3 mM $CaCl_2$ and 150 mM NaCl. The ionic strength was 70 mM in (A) and 235 mM in (B). A mixture of isozymes was used in these experiments. Curves are predicted from eq 1 of Orr and Newton (1992).

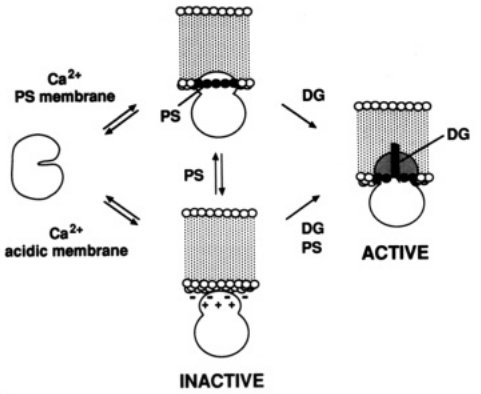

FIGURE 7: Possible model for protein kinase C–membrane interaction. In the absence of PS, electrostatic interactions drive the binding of protein kinase C to acidic membranes: this interaction is sensitive to ionic strength and surface charge and is insensitive to diacylglycerol. In the presence of PS, specific interactions with multiple, interacting PS binding sites are proposed to mediate the binding of protein kinase C to the membrane: this interaction is relatively insensitive to ionic strength and surface charge. Ca^{2+} and DG, allosteric effectors of the kinase, cause a marked increase in the affinity of protein kinase C for PS. DG activates the membrane-bound kinase, an event that may be accompanied by insertion of the protein into the hydrophobic core of the membrane.

are specific for the L-serine headgroup (upper part of figure). The role of diacylglycerol remains to be elucidated, although several lines of evidence suggest that it may promote insertion of the regulatory domain of the protein into the hydrophobic core of the membrane (Bazzi & Nelsestuen, 1988b; Snoek et al., 1988; Orr & Newton, 1990).

Other models can account for the cooperative interaction of protein kinase C with PS: most notably, protein kinase C could have one or two specific binding sites for PS and multiple nonspecific sites for acidic lipids, but cooperativity in activation could arise because the enzyme recognizes a preformed domain of PS in the membrane. Formation of such a domain could be mediated by Ca^{2+} (McLaughlin et al., 1981; Ekerdt & Papahadjopoulos, 1982; Feigenson, 1986). However, the ability of PS alone, in the absence of Ca^{2+}, to cooperatively activate isozymes of protein kinase C that are Ca^{2+}-independent is inconsistent with this possibility (Schaap & Parker, 1990). Furthermore, the amount of Ca^{2+} required for protein kinase C to bind to phosphatidylserines that have different affinities for Ca^{2+} is similar (Keranen et al., 1992), suggesting that the enzyme does not recognize a preformed Ca^{2+}–$(PS)_n$ complex. In addition, the concentrations of bilayer PS that activate protein kinase C are below the concentrations needed for phase separation of PS by Ca^{2+} (Hui et al., 1983; Boni & Rando, 1985). Nonetheless, for Ca^{2+}-dependent isozymes of protein kinase C, the interaction with Ca^{2+} is likely involved with the PS interaction. The membrane-bound enzyme binds approximately eight Ca^{2+} molecules, whereas the soluble protein kinase C interacts with at most one molecule of Ca^{2+} (Bazzi & Nelsestuen, 1990). Our studies indicate that protein kinase C interacts with at least 12 molecules of PS (Newton & Koshland, 1989; Orr & Newton, 1992), suggesting a possible stoichiometry of 1 or 2 PS per Ca^{2+}.

The foregoing results are consistent with two physically distinct mechanisms describing the protein kinase C–lipid interaction: a nonspecific electrostatic interaction occurring in the presence of acidic, non-PS lipids and a highly specific interaction occurring in the presence of PS. The binding to acidic, non-PS lipids is sensitive to ionic strength and surface charge but insensitive to diacylglycerol and Ca^{2+}. The specific interaction with PS contrasts in several aspects: first, under the conditions employed, the interaction with PS is relatively insensitive to ionic strength and surface charge. Second, the binding to PS is regulated by the two second messengers of the kinase, diacylglycerol and Ca^{2+}, both of which cause a marked increase in the affinity of the enzyme for PS. The interaction of protein kinase C with PS is most consistent with the existence of specific binding sites whose affinity for PS is regulated by diacylglycerol and Ca^{2+}.

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Comparison of Hydrogen Exchange Rates for Bovine Pancreatic Trypsin Inhibitor in Crystals and in Solution†

Warren Gallagher,‡ Feng Tao, and Clare Woodward*
Department of Biochemistry, University of Minnesota, St. Paul, Minnesota 55108
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ABSTRACT: Hydrogen exchange rate constants for the 17 slowest exchanging amide NH groups in bovine pancreatic trypsin inhibitor (BPTI) were measured in solution and in form II and form III crystals. All 17 amide hydrogens are buried and intramolecularly hydrogen bonded in the crystal structure, except Lys 41 which is buried and hydrogen bonded to a buried water. Large-scale crystallization procedures were developed for these experiments, and rate constants for both crystal and solution exchange were measured by ^{1}H NMR spectroscopy of exchange-quenched samples in solution. Two conditions of pH and temperature, pH 9.8 and 35 °C, and pH 9.4 and 25 °C, bring two groups of hydrogens into the experimental time window (minutes to weeks). One consists of the 10 slowest exchanging hydrogens, all of which are associated with the central β-sheet of BPTI. The second group consists of seven more rapidly exchanging hydrogens, which are distributed throughout the molecule, primarily in a loop or turn. In both groups, most hydrogens exchange more slowly in crystals, but there is considerable variation in the degree to which the exchange is depressed in crystals. Many differences observed for the more rapidly exchanging hydrogens can be attributed to local surface effects arising from intermolecular contacts in the crystal lattice. Within the slower group, however, a very large effect on exchange of Ile 18 and Tyr 35 appears to be selectively transmitted through the matrix of the molecule. Backbone atoms of Ile 18 and Tyr 35 are mutually hydrogen bonded to each other at the open end of a pair of twisted antiparallel β-sheet strands, which are closed at the other end in a hairpin-like arrangement by a short turn. Ile 18 and Tyr 35 exchange rates are slowed by 4–5 orders of magnitude in crystals. Their location in the protein suggests that crystallization selectively damps motions of the open end of the β-sheet, which connects the flexible loops with the more rigid central core of the molecule.

Nitrogen-bound hydrogens in the peptide and side chain amide groups of proteins are labile and exchange with solvent hydrogens. Their exchange kinetics can be measured by the rate of disappearance of amide resonances in ^{1}H NMR[1] spectra, as ^{1}H isotope is replaced by ^{2}H. Isotope exchange rate constants for amide groups in native globular proteins typically vary over many orders of magnitude. Surface amide hydrogens exchange most rapidly, while buried ones exchange with rates that are 3–10 orders of magnitude slower. The fact that buried amide hydrogens in folded proteins eventually exchange implies there are structural fluctuations which provide transient access of interior regions of a protein to water

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‡ Present address: Department of Chemistry, University of Wisconsin-Eau Claire, Eau Claire, WI 54702

[1] Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; NMR, nuclear magnetic resonance; FID, free induction decay.

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