Bode, W., Engh, R., Musil, D., Thiele, U., Huber, R., Karshikov, A., Brzin, J., Kos, J., & Turk, V. (1988) *EMBO J. 7*, 2593-2599.
Brocklehurst, K., Baines, B. S., & Malthouse, J. P. G. (1981) *Biochem. J. 197*, 739-746.
Brunger, A. T., Kuriyan, J., & Karplus, M. (1987) *Science 235*, 458-460.
Carne, A., & Moore, C. H. (1978) *Biochem. J. 173*, 73-83.
Hanada, K., Tamai, M., Yamagishi, S., Ohmura, S., Sawada, J., & Tanaka, I. (1978a) *Agric. Biol. Chem. 42*, 523-528.
Hanada, K., Tamai, M., Ohmura, S., Sawada, J., Seki, T., & Tanaka, I. (1978b) *Agric. Biol. Chem. 42*, 529-536.
Jones, T. A. (1975) *J. Appl. Crystallogr. 11*, 268-272.
Kamphuis, I. G., Drenth, J., & Baker, E. N. (1985) *J. Mol. Biol. 182*, 317-329.
Kar, N. C., & Pearson, C. M. (1976) *Clin. Chim. Acta 73*, 293-297.
Machleidt, W., Thiele, U., Laber, B., Assfalg-Machleidt, I., Esterl, A., Wiegand, G., Kos, J., Turk, V., & Bode, W. (1989) *FEBS Lett. 243*, 234-238.
Menard, R., Carriere, J., Laflamme, P., Plouffe, C., Khouri, H. E., Vernet, T., Tessier, D. C., Thomas, D. Y., & Storer, A. C. (1991) *Biochemistry 30*, 8924-8928.
Podivinsky, E., Forster, R. L. S., & Gardner, R. C. (1989) *Nucleic Acids Res. 17*, 8363-8363.
Rich, D. (1986) in *Proteinase Inhibitors* (Barrett & Salvesen, Eds.) pp 153-178, Elsevier, Amsterdam.
Schechter, I., & Berger, A. (1967) *Biochem. Biophys. Res. Commun. 27*, 157-162.
Stubbs, M. T., Laber, B., Bode, W., Huber, R., Jerala, R., Lenarcic, B., & Turk, V. (1990) *EMBO J. 9*, 1939-1947.
Sugita, H. (1988) *Medicina 22*, 282-295.
Turk, V., & Bode, W. (1991) *FEBS Lett. 285*, 213-219.
Varughese, K. I., Ahmed, F. R., Carey, P. R., Hasnain, S., Huber, C. P., & Storer, A. C. (1989) *Biochemistry 28*, 1330-1332.
Xuong, N. H., Sullivan, D., Neilson, C., & Hamlin, R. (1985) *Acta Crystallogr. B41*, 267-269.
Yamamoto, D., Ishida, T., & Inoue, M. (1990) *Biochem. Biophys. Res. Commun. 171*, 711-716.

Supplementation of the Phosphatidyl-L-serine Requirement of Protein Kinase C with Nonactivating Phospholipids†

Myung-Ho Lee and Robert M. Bell*
Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710
Received June 10, 1991; Revised Manuscript Received March 13, 1992

ABSTRACT: The mechanism of protein kinase C (PKC) activation by phosphatidyl-L-serine (PS) is highly specific and occurs with high cooperativity [Lee, M.-H., & Bell, R. M. (1989) *J. Biol. Chem. 264*, 14797-14805]. To further investigate the multiplicity and specificity of PS cofactor requirement, some of the PS molecules present in Triton X-100 mixed micelles were substituted with nonactivating phospholipids devoid of required amino or carboxyl functional groups. The ability of these phospholipids to spare or reduce the mole percent of PS required was determined. Addition of phosphatidyl-(3-hydroxypropionate) (PP) or phosphatidate (PA) reduced the mole percent of PS required for maximal activity from 10 to 4 mol %, and also reduced the cooperativity of activation with PS. In contrast, phosphatidylethanolamine did not alter the dependence on PS. Phosphatidylethanol (P-Et) reduced the PS requirement to 2-4 mol % and cooperativity less efficiently than PP or PA. Phosphatidylglycerol and phosphatidylinositol resemble P-Et in their ability to reduce PS requirements and cooperativity. Therefore, it appears that the ability of phospholipids to substitute for PS in PKC activation depends on the negative charge in the phospholipid head group and the efficiency of substitution appears to be directly related to the negative charge density. The presence of two acyl groups within the phospholipid cofactor proved important since lyso-PS and lyso-PA replaced a portion of PS molecules required less efficiently than P-Et. Sodium oleate and sodium dodecyl sulfate behaved like lyso-PS. When other anionic lipids are present, approximately four molecules of PS per micelle are required for maximal PKC activity. These data indicated that there are two distinct sets of lipid-protein interactions occurring when PKC activation occurs. One set of interactions displays high specificity for PS. The other set of interactions is far less specific and involves anionic lipids. These data raise the possibility that acidic lipids, notably PA, may regulate PKC activity in cells.

Protein kinase C (PKC),[1] a Ca^{2+}-, DAG-, and phospholipid-dependent serine/threonine-specific protein kinase, has a crucial role in cellular signal transduction [for a review, see Nishizuka (1984a,b)]. When cells are activated by hormones and growth factors which generate DAG in the inner leaflet of the plasma membrane, or treated with tumor-promoting phorbol esters, PKC associates with the membrane and becomes active (Kikkawa et al., 1983; Kraft & Anderson, 1983; Fearon & Tashjian, 1985; Hirota et al., 1985; Noar et al., 1985). Among the various phospholipids tested in vitro, PS is the most effective in activating PKC. Other acidic phos-

† This work was supported by National Institutes of Health Grant GM38737.
* To whom correspondence should be addressed.

[1] Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DAG, *sn*-1,2-diacylglycerol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PS, phosphatidylserine; PA, phosphatidate; PP, phosphatidyl-(3-hydroxypropionate); PG, phosphatidylglycerol; PI, phosphatidylinositol; P-Et, phosphatidylethanol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SM, sphingomyelin.

0006-2960/92/0431-5176$03.00/0

pholipids are less active, and cationic phospholipids are totally inactive (Takai et al., 1979; Hannun et al., 1986). Using the Triton X-100 mixed micellar assay, the structural features within phospholipid cofactors have been shown to be highly specific (Lee & Bell, 1989); in the presence of Ca^{2+} and DAG, anionic phospholipids such as PS, PA, and phosphatidyl-3-hydroxypropionate (PP) are sufficient to support the binding of PKC to mixed micelles; however, only PS, which contained both a free amino group and a free carboxyl group in the L configuration, activates PKC. These studies indicated that the amino group of PS is important for coupling the binding of the phospholipid to the activation of the enzyme. The phospholipid specificity and PS concentration dependence for activating the PKC isozymes α, βII, and γ were shown to be similar (Burns et al., 1990).

Important questions remained to be answered concerning whether the levels of PS present in cellular membranes ever regulate PKC activity and whether other phospholipids modulate activity. The dependence of PKC activity on PS in vitro was highly cooperative and occurred in the range from 4 to 10 mol % when histone was employed as the substrate (Hannun et al., 1985; Newton & Koshland, 1989). The PS level present in the inner leaflet of the plasma membrane is 15–20 mol % (Verleij et al., 1973; Zwaal & Bevers, 1983). The level of PS has not been observed to change dramatically upon cell activation (Soling et al., 1987; Lacal et al., 1987). Therefore, the PS level may always be sufficient to support PKC activation in cells. However, PS interacts with cytoskeletal components and Ca^{2+}-dependent phospholipid binding proteins in the plasma membrane (Geisow & Walker, 1986; Cohen et al., 1988). Moreover, PS was shown to be transported to the outer leaflet of the plasma membrane in thrombin- and collagen-activated platelets (Bevers et al., 1983). Thus, the level of PS available for PKC activation may not be saturating and thereby could control enzyme activity under some conditions. Although PKC activation depends on PS, previous workers have also observed that other phospholipids modulate PS-dependent activity. PE further stimulates the enzyme activity, whereas PC and SM inhibit it (Kaibuchi et al., 1981). In addition, lyso-PC affects PKC activity (Oishi et al., 1988). However, studies were conducted at a single concentration of PS, where the degree of saturation was not established. PE and PG were also shown to reduce the PS dependence of PKC activation (Newton & Koshland, 1989). However, the phospholipids were only tested at equimolar concentration with PS.

In this report, the multiplicity and specificity of the PS phospholipid cofactor requirement were systematically investigated by addition of nonactivating phospholipids at various concentrations of PS using the Triton X-100 mixed micellar assay. The ability of these nonactivating phospholipids to reduce the mole percent of PS required for maximal activation and to reduce the cooperativity of PS activation was quantitated. The studies revealed that anionic lipids spare the number of PS molecules required and reduce PS cooperativity. The data suggest that the cellular anionic lipids may exert a multiplicity of regulatory effects on PKC.

Experimental Procedures

Materials. Dioleoyl-PS, -PA, -PG, -PC, -PE, and PI (from bovine liver) were purchased from Avanti Polar Lipids. Deoxycholate (sodium), oleate (sodium), oleic acid methyl ester, oleyl alcohol, lyso-PA (oleoyl), and lyso-PC (oleoyl) were from Sigma. CHAPS was from Calbiochem. Sodium dodecyl sulfate was from Schwarz-Mann. *sn*-1,2-Dioleoylglycerol was prepared from dioleoyl-PC digestion with phospholipase C (type XIII from Sigma) (Mavis et al., 1972). All other lipids were synthesized as described before (Lee & Bell, 1989).

Purification of PKC. PKC was purified from frozen female Sprague-Dawley rat brain (Pel-Freez Biologicals, Rogers, AK) by DEAE-, threonine-, and phenyl-chromatography as described before (Kitano et al., 1986). The final specific activity was 2 μmol of phosphate incorporated min^{-1} (mg of protein)$^{-1}$. Since the final preparation has negligible Ca^{2+}-independent activity, it mainly consisted of Ca^{2+}-dependent PKCs: α, β, and γ isoforms. These have been shown to be highly similar with respect to phospholipid cofactor specificity and PS dependence for activation (Burns et al., 1990).

Preparations of Triton X-100 Mixed Micelles. Triton X-100 mixed micelles were prepared fresh just before the enzyme assays were conducted. Mixed micelles containing specific concentrations of lipids were prepared by pipetting the appropriate volume of lipids in chloroform, drying under a stream of nitrogen, adding an appropriate volume of 3% Triton X-100 solution in 10 mM HEPES buffer (pH 7.5), vortexing for 1 min, and incubating for 10 min at room temperature (22 °C).

PKC Assays. PKC activity was determined by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into histone type IIIS in the presence of 100 μM Ca^{2+}, 1 mol % DAG, and phospholipids as indicated in the text, using the Triton X-100 mixed micellar method (Hannun et al., 1986). Surprisingly, the ability to support PKC activation by PA depended on the enzyme reaction conditions. When PA in Triton X-100 micelles was incubated with Mg^{2+} and Ca^{2+} before addition of histone, it did not support PKC activation at all as a phospholipid cofactor. In contrast, when PA was incubated with histone before addition of Mg^{2+} and Ca^{2+}, it supported PKC activation greatly as previously described (Hannun et al., 1986; Lee & Bell, 1989). Therefore, we conducted the phospholipid-supplementing experiments under two different conditions. In method 1, HEPES buffer (pH 7.5), Ca^{2+}, Mg^{2+}, mixed micelles, and the enzyme were mixed in a final volume of 200 μL. After 10-min incubation, the enzyme reaction was started by adding 50 μL of a solution containing histone (1 mg/mL), 100 μM ATP, and 1 μCi of [γ-^{32}P]ATP. In method 2, HEPES buffer (pH 7.5), Ca^{2+}, mixed micelles, histone, and the enzyme were mixed in a final volume of 225 μL. After incubation for 10 min, the enzyme reaction was started by adding 25 μL of solution containing 100 mM Mg^{2+}, 100 μM ATP, and 1 μCi of [γ-^{32}P]ATP. The enzyme reaction was conducted for 10 min at room temperature (22 °C). No significant difference was found in the ability to supplement PS by lipids tested in this study between the two assay conditions, except that method 1 gave 20% higher activity than method 2. Unless otherwise specified, the results as shown in the text are those conducted with method 1.

Other Methods. The determination of the lipid concentration was made as described for DAG (Stern & Shapiro, 1953) and phospholipids (Ames & Dubin, 1960). All experiments were conducted twice in duplicates. All kinetic data were analyzed by a nonlinear regression fit to the standard Hill equation using the EZ-FIT computer program (Perrella, 1988). All enzyme kinase activities taken were subtracted by phospholipid-independent protein kinase activities; these generally were less than 5% of the total protein kinase activity.

Results and Discussion

When individual phospholipids were employed, the amino and carboxyl groups present in PS proved essential for maximal enzyme activation (Lee & Bell, 1989). Since several molecules of PS were required for activation, we further investigated the

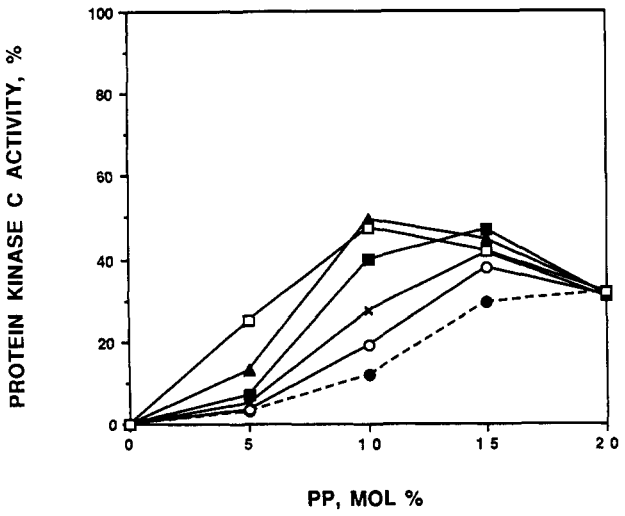

FIGURE 1: Effect of a mixture of PP and PE on PKC activation. In the presence of 100 μM Ca^{2+} and 1 mol % DAG, PKC activities were measured by method 1 (see Experimental Procedures) at various concentrations of PP and PE in the 0.3% Triton X-100 mixed micelles indicated: none (●), 1 (○), 2 (×), 4 (■), 6 (▲), and 8 mol % PE (□). The activity was shown as a percentage of the activity obtained in the presence of 100 μM Ca^{2+}, 1 mol % DAG, and 12 mol % PS, which was 35 pmol of phosphate incorporated per minute.

multiplicity and specificity of the phospholipid cofactor requirement of PKC by employing mixtures of phospholipids in mixed micelles. When phosphatidyl-(3-hydroxypropionate) (PP), which contains only the carboxyl group, was mixed with PE, which contains only the amino group, PKC activity was only 50% of that observed with PS (Figure 1). This indicates that the amino and carboxyl groups must reside within the same phospholipid to function most efficiently as a cofactor.

The multiplicity of the PS requirement was investigated by increasingly substituting molecules of PP for PS in Triton X-100 mixed micelles. As the PP concentration was increased at subsaturating concentrations of PS, enzyme activity increased. For maximal activation, at least 4 mol % PS was required (Figure 2A). Increasing the concentration of PP greatly reduced the PS dependence for enzyme activation (Figure 2B). Increasing the concentrations of PP to 4 mol % reduced linearly the half-maximal concentration of PS from 6.5 ± 0.1 to 2.5 ± 0.3 mol %; the Hill constant of the cooperative activation by PS was reduced from 7.7 ± 0.3 to 2.2 ± 0.5. These data suggest that only a small number of PS molecules are actually necessary for PKC activation. Under subsaturating concentrations of PS, the PP dependence of enzyme activation was highly cooperative; at 2 mol % PS, the Hill constant for PP was 3.6 ± 0.3 (Figure 2A). Clearly, PP can substitute for a portion of the PS molecules required for activation. Further, these results suggest an essential role for the carboxyl group within PS in cooperative activation of PKC.

The importance of the amino group within PS for PKC activation was investigated by substituting molecules of PS with PE. As the PE concentration was increased, the enzyme activity increased; however, for maximal activation, more than 6 mol % PS was required (Figure 3A). At saturating concentrations of PS, PE did not further activate the enzyme. In addition, under the same conditon, high concentrations of PE inhibited the PKC activity (Figure 3A). Increasing the concentration of PE caused a slight increase in the cooperativity of PS and a slight decrease in the half-maximal concentration of PS required for enzyme activation (Figure 3B). Therefore, PE neither inhibited the cooperativity of PS nor substituted effectively for PS in enzyme activation. These data with PE contrast sharply with those obtained with PP (Figure 2).

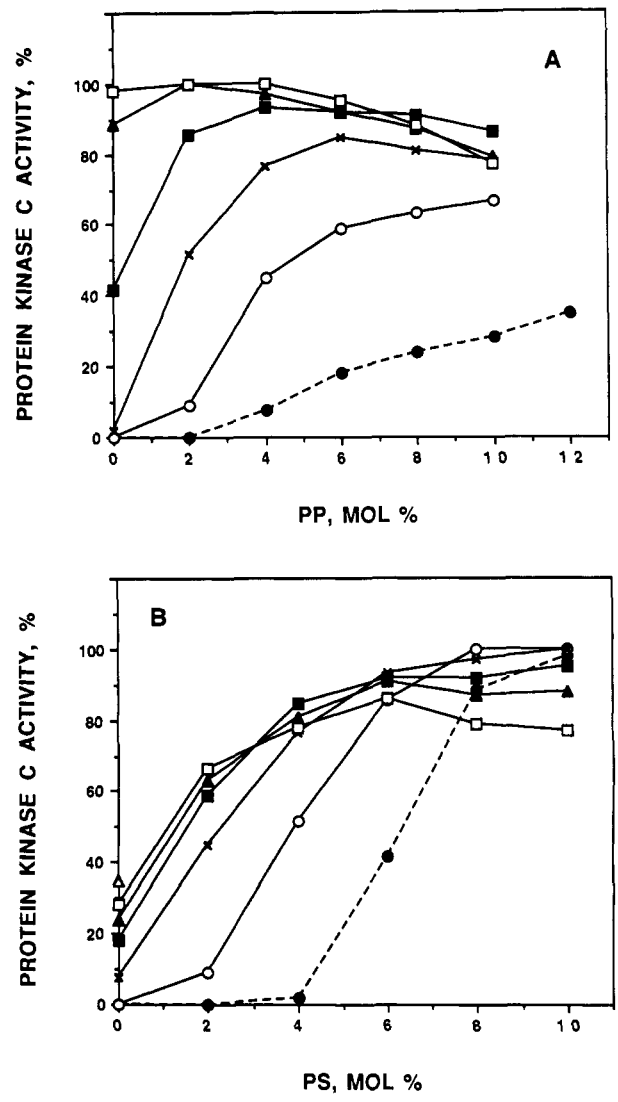

FIGURE 2: Effect of supplemented PP on the PS dependence of PKC activation. PKC activities were measured at various concentrations of PS and PP. (A) PP dependence of PKC activation at different concentrations of PS indicated: none (●), 2 (○), 4 (×), 6 (■), 8 (▲), and 10 mol % PS (□). (B) PS dependence of PKC activation at different concentrations of PP indicated: none (●), 2 (○), 4 (×), 6 (■), 8 (▲), and 10 mol % PP (□). All other conditions were the same as those used in Figure 1.

To investigate the importance of the chemical nature of the carboxyl head group for supplementing PS, PS was increasingly substituted by PA. When PA was allowed to interact with histone in the presence of Ca^{2+} and Mg^{2+} before the enzyme reaction is started (method 1, see Experimental Procedures), PA did not support PKC activity at all as a phospholipid cofactor (Figure 4A). When PS was present above 4 mol %, PA supported the enzyme activity to the level of the activity observed with saturating levels of PS (Figure 4A). As the concentration of PA was increased, the PS dependence for PKC activation concomitantly decreased greatly (Figure 4B). Increasing the concentration of PA to 2 mol % reduced linearly the half-maximal concentration of PS to 3.0 ± 0.2 mol % and the Hill constant for the cooperative activation by PS to 2.9 ± 0.3. Under limiting concentrations of PS, supplementation with PA was highly cooperative; at 2 mol % PS, the Hill constant for PA was 4.5 ± 0.3 (Figure 4A). At levels of PA up to 3 mol %, the combined concentrations of PS and PA in the mixed micelles required for PKC activation were lower than that for PS alone. These results with PA indicate that the anionic character of a phospholipid is a major determinant

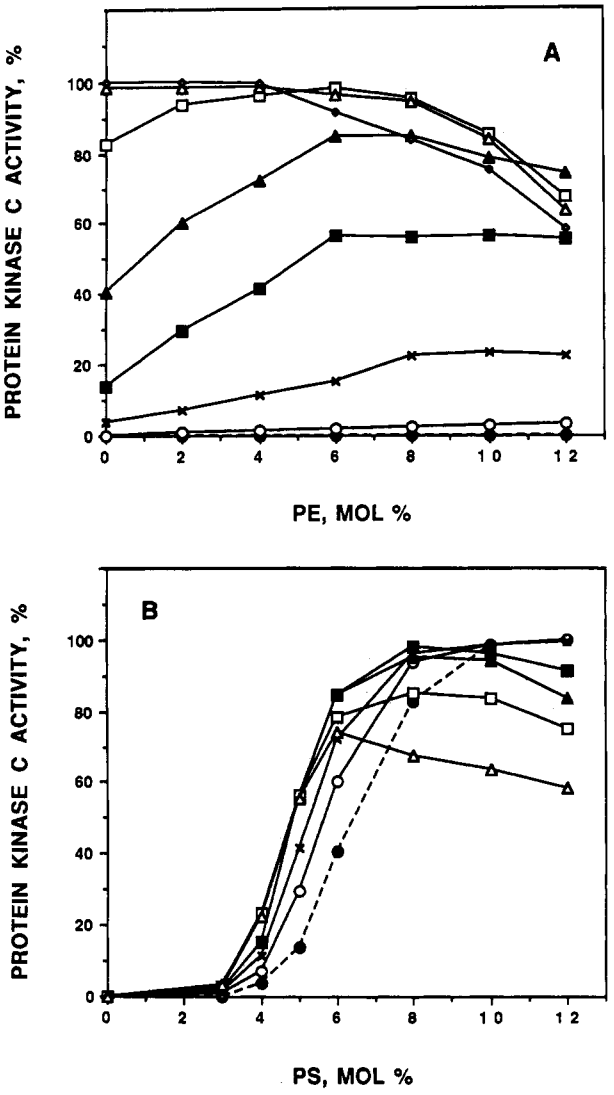

FIGURE 3: Effect of supplemented PE on the PS dependence of PKC activation. PKC activities were measured at various concentrations of PE and PS. (A) PE dependence of PKC activation at different concentrations of PS indicated: none (●), 2 (●), 3 (○), 4 (×), 5 (■), 6 (▲), 8 (□), 10 (△), and 12 mol % PS (◇). (B) PS dependence of PKC activation at different concentrations of PE indicated: none (●), 2 (○), 4 (×), 6 (■), 8 (▲), 10 (□), and 12 mol % PE (△). All other conditions were the same as those used in Figure 1.

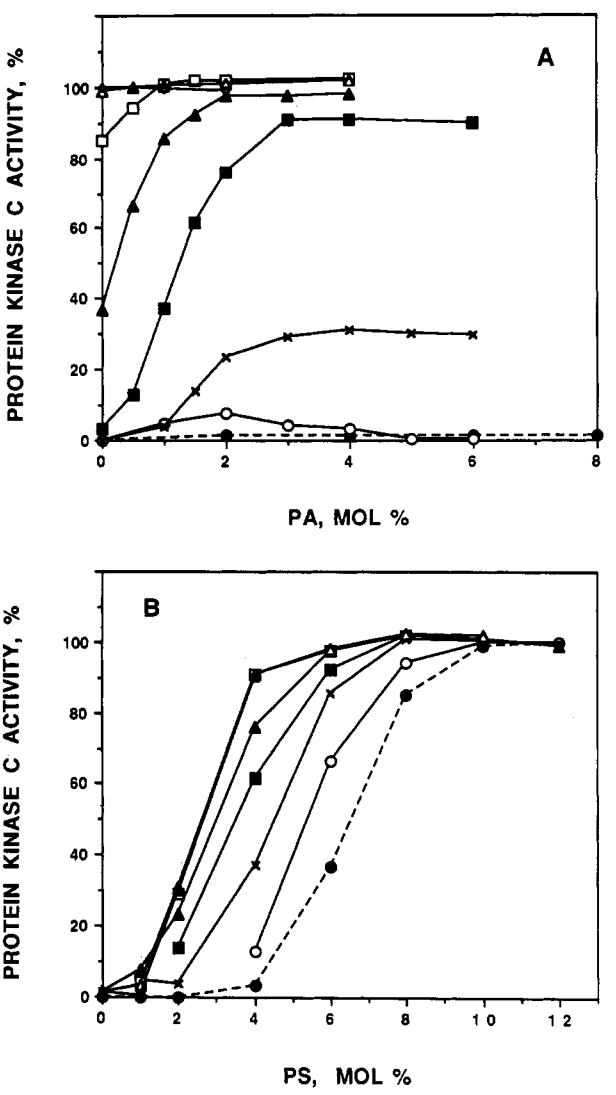

FIGURE 4: Effect of supplemented PA on the PS dependence of PKC activation. PKC activities were measured at various concentrations of PA and PS. (A) PA dependence of PKC activation at different concentrations of PS indicated: none (●), 1 (○), 2 (×), 4 (■), 6 (▲), 8 (□), 10 (△), and 12 mol % PA (◇). (B) PS dependence of PKC activation at different concentrations of PA indicated: none (●), 0.5 (○), 1 (×), 1.5 (■), 2 (▲), 3 (□), 4 (△), and 6 mol % PA (△). All other conditions were the same as those used in Figure 1.

of phospholipid's ability to cooperatively activate PKC.

When PA was allowed to interact with histone in the absence of Mg^{2+} before the enzyme reaction was started (method 2, see Experimental Procedures), PA supported PKC activation as a phospholipid cofactor to about 75% of the degree observed with PS, as described previously (Hannun et al., 1986; Lee & Bell, 1989). Under these conditions, however, PA reduced the PS requirement for enzyme activation in a manner similar to that obtained in method 1. As the concentration of PA was increased to 2 mol %, the half-maximal concentration of PS was linearly reduced from 6.1 ± 0.1 to 2.4 ± 0.2 mol %, and the Hill constant for the cooperative activation by PS was reduced from 5.4 ± 0.5 to 2.3 ± 0.3, respectively (Figure 5). Interaction of PA with the histone substrate during preincubation may account for the difference in supporting PKC activation between two assay conditions.[2] The ability of PA to supplement is of particular interest, since the level of PA is modulated in response to extracellular agents (Boccking et al., 1987).

The specificity of anionic phospholipids to supplement the PS requirement of PKC activation was further investigated using a phospholipid which contains only one acidic group, phosphatidylethanol (P-Et). At 2 mol % PS, 12 mol % P-Et supported the maximal PKC enzyme activity (Figure 6A). As the concentration of P-Et was increased, the PS dependence for enzyme activation was greatly reduced (Figure 6B). Increasing the concentrations of P-Et to 8 mol % decreased linearly the half-maximal concentration of PS to 2.0 ± 0.1 mol % and the Hill constant for the cooperative activation by PS to 2.6 ± 0.4. When the concentration of PS was limiting, activation by P-Et was highly cooperative. At 1 and 2 mol % PS, the Hill constants for P-Et were 6.9 ± 1.4 and 4.4 ± 0.7, respectively (Figure 6A). These values are similar to those of PA or PP. However, the concentration of P-Et required for activation was much higher than those of PA or PP.

The ability of other acidic phospholipids, PI and PG, to supplement PS activation was investigated. Both PG and PI

[2] The mechanism of PKC activation was proposed to be related to the phospholipid aggregation with protein substrate (Bazzi & Nelsestuen, 1987). Aggregation of PA, but not other lipids, with histone substrate was strongly inhibited by a Mg^{2+} concentration as low as 2 mM (unpublished observation).

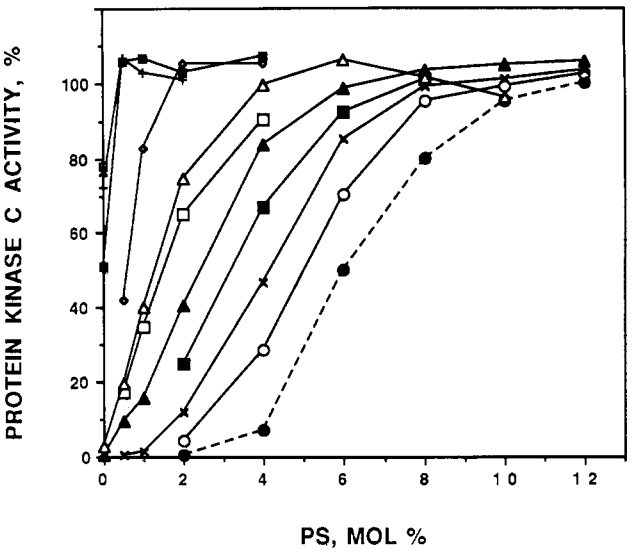

FIGURE 5: Effect of PA on PS dependence of PKC activation (method 2). The PS dependence of PKC activation was measured by method 2 (see Experimental Procedures) at various concentrations of PA in 0.3 % Triton X-100 mixed micelles indicated: none (●), 0.5 (○), 1 (×), 1.5 (■), 2 (▲), 3 (□), 4 (△), 5 (◊), 6 (■), and 8 mol % PA (+). The activity was shown as a percentage of the activity obtained in the presence of 100 μM Ca^{2+}, 1 mol % DAG, and 12 mol % PS, which was 28 pmol of phosphate incorporated per minute.

reduced the PS requirement for PKC activation with an efficiency similar to that of P-Et (data not shown). The efficiency of these phospholipids (P-Et, PI, and PG) to supplement the PS requirement of PKC was not affected by the alcohol head group.

To further investigate the importance of the acidic nature of the phosphate group moiety, phosphatidyldimethanol was tested for its ability to supplement PS. This compound did not alter PKC activity at any concentrations examined (2–12 mol %) (data not shown). The acidic phosphate moiety is an important determinant in the ability of phospholipids to supplement the PS requirement of PKC.

The necessity of two fatty acyl chains in PS for cooperative activation of PKC was investigated by increasingly substituting PS with lyso-PS. Lyso-PS reduced the PS dependence for enzyme activation less efficiently than P-Et. (Figure 7); increasing the concentration of lyso-PS to 10 mol % reduced linearly the half-maximal concentration of PS to 3.6 ± 0.1 mol %. The Hill constant of the cooperative activation by PS was reduced to 4,0 ± 0.3. Similar results were found with lyso-PA. These data indicate that neither lyso-PS nor lyso-PA substitutes efficiently for PS in enzyme activation, nor alters PS cooperativity. Therefore, the absence of the *sn*-2 fatty acyl chain in those phospholipids prevents their effective interaction with PKC.

PC and sphingomyelin inhibited PKC activity in vesicles (Kaibuchi et al., 1981), whereas lyso-PC increased activity (Oishi et al., 1988). In mixed micellar assays, PC, SM, and lyso-PC did not significantly affect PKC activity at 2–12 mol % PS. The PS dependence of activation was not reduced (data not shown).

Lipids other than phospholipids were also tested for their ability to supplement PS for PKC activation. Unsaturated fatty acids activate PKC (McPhail et al., 1984; Murakami et al., 1986; Verkest et al., 1988; Seifert et al., 1988). However, using the mixed micellar assays, oleate did not cause activation (Lee & Bell, 1989). When mixed with PS, oleate reduced the PS dependence of enzyme activation (Figure 8). Increasing the concentrations of oleate to 12 mol % reduced

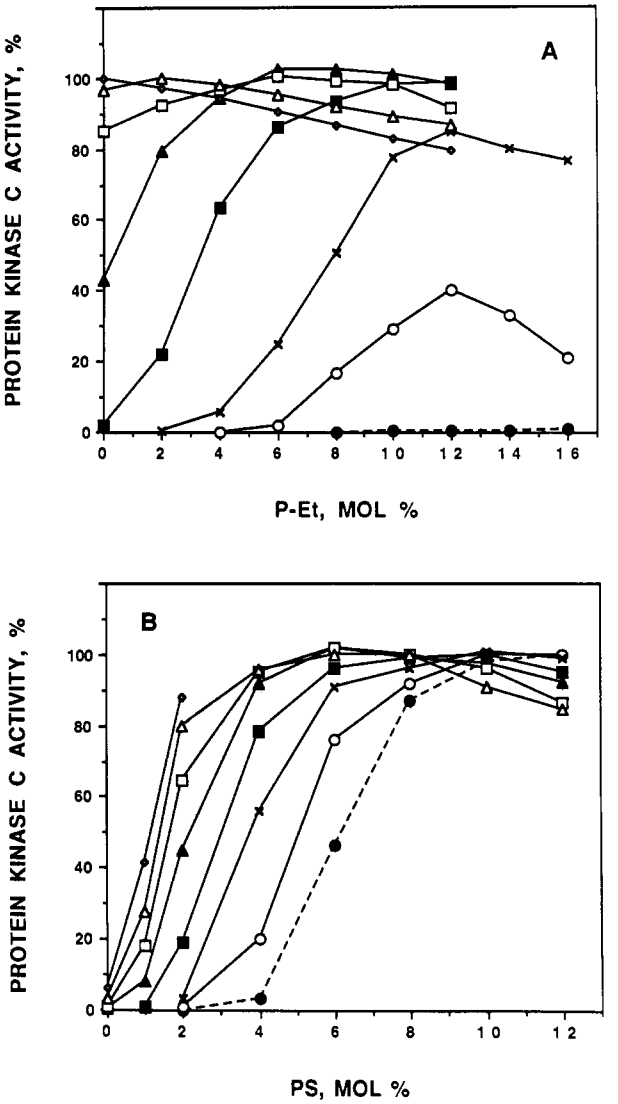

FIGURE 6: Effect of supplemented P-Et on the PS dependence of PKC activation. PKC activities were measured at various concentrations of P-Et and PS. (A) P-Et dependence of PKC activation at different concentrations of PS indicated: none (●), 1 (○), 2 (×), 4 (■), 6 (▲), 8 (□), 10 (△), and 12 mol % PS (◊). (B) PS dependence of PKC activation at different concentrations of P-Et indicated: none (●), 2 (○), 4 (×) 6 (■), 8 (▲), 10 (□), 12 (△), and 14 mol % P-Et (◊). All other conditions were the same as those used in Figure 1.

linearly the half-maximal concentration of PS to 3.5 ± 0.1 mol %. The Hill constant of the cooperative activation by PS was reduced to 2.8 ± 0.1. The efficiency of oleate supplementation was similar to that observed with lyso-PS. The free carboxyl group of oleate is required to supplement PS, since oleyl alcohol and oleoyl methyl ester, up to 20 mol %, did not affect the PS dependence of activation (data not shown). The chemical nature of the negative charge did not seem to be important, since sodium dodecyl sulfate, up to 12 mol %, could also reduce the PS dependence of PKC activation with an efficiency similar to oleate (data not shown). However, some structural features for anionic amphiphiles are apparent, because CHAPS and deoxycholate, which are ionic detergents, did not reduce the PS dependence of PKC activation (data not shown).

CONCLUSION

PKC has been shown to display a high degree of specificity for its lipid activator, *sn*-1,2-diacylglycerol, and for its phospholipid cofactor, PS. The high degree of specificity for the phospho-L-serine head group was characterized by stereospecificity and requirements for carboxyl and amino moieties

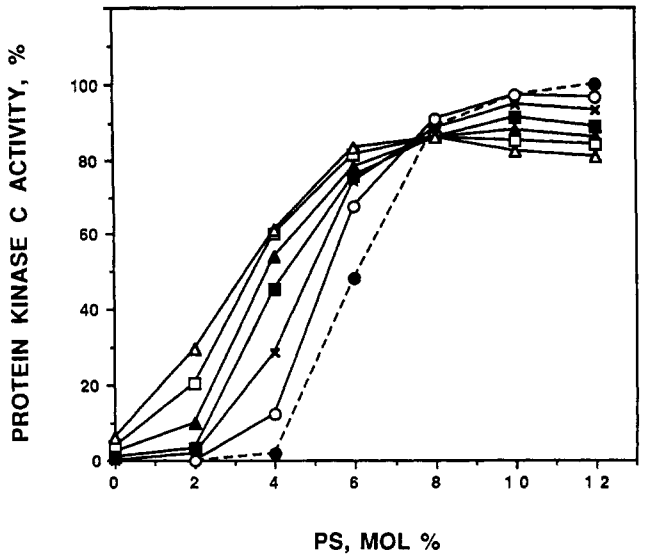

FIGURE 7: Effect of supplemented lyso-PS on the PS dependence of PKC activation. The PS dependence of PKC activation was measured at different concentrations of lyso-PS indicated: none (●), 2 (○), 4 (×), 6 (■), 8 (▲), 10 (□), and 12 mol % lyso-PS (△). All other conditions were the same as those used in Figure 1.

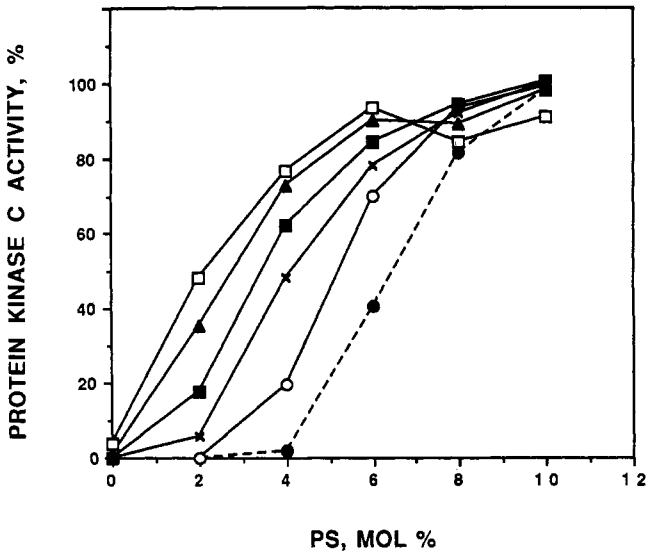

FIGURE 8: Effect of supplemented oleate on the PS dependence of PKC activation. The PS dependence of PKC activation was measured at different concentrations of oleate indicated: none (●), 4 (○), 8 (×), 12 (■), 16 (▲), and 20 mol % oleate (□). All other conditions were the same as those used in Figure 1.

(Lee & Bell, 1989). The data in this paper substantially extend knowledge of cofactor specificity and multiplicity (stoichiometry) by systematic investigation of the ability of nonactivating phospholipids and other lipids to spare or substitute for a portion of the PS molecules required. These lipids by themselves were incapable of maximally activating PKC, yet many of them substituted effectively for some of the PS molecules by reducing the mole percent of PS required for maximal activation and by reducing cooperativity. Our data agree, in part, with the data of Newton and Koshland (1989) showing that some phospholipid can replace some of the PS molecules required for activation. Equally important, at limiting PS, the lipids able to supplement PS did so in cooperative fashion. All of the data substantiated that numerous PS and anionic phospholipids are essential for activity. In light of the results from PI, PIP, and PIP_2 for supplementation of PS for PKC activation (Lee & Bell, 1991), it appears that the ability of phospholipids to substitute for PS in enzyme activation depends on the negative charge in the phospholipid head group and the efficiency of substitution appears to be directly related to the negative charge density. No specific structural feature in the head group other than negative charge was identified in those lipids able to supplement the PS requirement. The quantitative aspects of the work also establish that 2–4 mol % PS is essential for PKC activation in micelles containing anionic phospholipids. This corresponds to approximately three to five molecules of PS per micelle. The functional significance of the amino and carboxyl groups within PS to coordinate PKC binding to the micelle and activation is demonstrated. This multiplicity is consistent with approximately one molecule of PS interacting with each of the two flanking regions of two proposed zinc fingers or zinc II binuclear clusters in PKC (Bell & Burns, 1991). The present analysis shows that several of the PS sites are highly specific and that additional less specific sites exist which interact with anionic lipids.

An alternate interpretation of the data is that the increased surface potential caused by the acidic lipids promotes histone-induced micelle aggregation and thereby spares the PS requirement by making PS more available to PKC after micelle aggregation. Although this interpretation cannot be entirely ruled out since all substrates are basic and cause aggregation, none of the acidic lipids employed in this study appeared to aggregate histone to a greater degree than PS. Notably, PA did not appear to cause histone aggregation under the assay conditions employed,[2] although it was one of the most effective lipids in its ability to supplement PS. Thus, the present data do not support this alternate interpretation; however, insufficient evidence exists to rule it out entirely.

Finally, the possible physiological significance of the ability of phospholipids to supplement for PS should be considered in light of the complexity of the physical interaction of PS with other cellular components, its asymmetry, and organellar distribution. These data combined with the multiplicity of lipid effectors, DAG, PIP_2, sphingosine, lysosphingolipids, ether lipids, lyso-PC, and PA, begin to describe a set of complex in vitro interactions that may lead to a better physiological understanding; at present, such significance is uncertain.

ACKNOWLEDGMENTS

We thank John Bloomenthal for providing purified PKC enzyme and Drs. Roy Bochardt and David Burns for critical review of the manuscript.

REFERENCES

Ames, B., & Dubin, D. T. (1960) *J. Biol. Chem. 235*, 769–775.
Bazzi, M. D., & Nelsestuen, G. L. (1987) *Biochemistry 26*, 1974–1982.
Bell, R. M., & Burns, J. D. (1991) *J. Biol. Chem. 266*, 4661–4664.
Bevers, E. M., Comfurius, P., & Awaal, R. F. A. (1983) *Biochim. Biophys. Acta 736*, 57–66.
Bocckino, S. B., Blackmore, P. F., Wilson, P. B., & Exton, J. H. (1987) *J. Biol. Chem. 261*, 15309–15315.
Burns, J. D., Bloomenthal, I. J., Lee, M.-H., & Bell, R. M. (1990) *J. Biol. Chem. 265*, 12044–12051.
Cohen, A. M., Liu, S. C., Lawler, J., Derick, L., & Palek, J. (1988) *Biochemistry 27*, 614–619.
Fearon, C. F., & Tashjian, A. H., Jr. (1985) *J. Biol. Chem. 260*, 8366–8371.
Geisow, M. J., & Walker, J. H. (1986) *Trends Biochem. Sci. 11*, 420–423.
Hannun, Y. A., Loomis, C. R., & Bell, R. M. (1985) *J. Biol. Chem. 260*, 10039–10043.

Hannun, Y. A., Loomis, C. R., & Bell, R. M. (1986) *J. Biol. Chem. 261*, 7184–7190.
Hirota, K., Hirota, T., Aguilera, G., & Catt, K. J. (1985) *J. Biol. Chem. 260*, 3243–3246.
Kaibuchi, K., Takai, Y., & Nishizuka, Y. (1981) *J. Biol. Chem. 256*, 7146–7149.
Kikkawa, U., Takai, Y., Tanaka, Y., Miyake, R., & Nishizuka, Y. (1983) *J. Biol. Chem. 258*, 11442–11445.
Kitano, T., Kikkawa, U., & Nishizuka, Y. (1986) *Methods Enzymol. 124*, 349–352.
Kraft, A. S., & Anderson, W. B. (1983) *Nature 301*, 621–623.
Lacal, J. C., Moscat, J., & Aaronson, S. (1987) *Nature 330*, 269–272.
Lee, M.-H., & Bell, R. M. (1989) *J. Biol. Chem. 264*, 14797–14805.
Lee, M.-H., & Bell, R. M. (1991) *Biochemistry 30*, 1041–1049.
Mavis, R. D., Bell, R. M., & Vagelos, P. R. (1972) *J. Biol. Chem. 247*, 2835–2841.
McPhail, L. C., Clayton, C. C., & Snyderman, R. (1984) *Science 224*, 622–625.
Murakami, K., Chan, S. Y., & Routtenberg, A. (1986) *J. Biol. Chem. 261*, 15424–15429.
Naor, Z., Zer, J., Zakut, H., & Hermon, J. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 8203–8207.
Newton, A. C., & Koshland, D. E., Jr. (1989) *J. Biol. Chem. 264*, 14909–14915.
Nishizuka, Y. (1984a) *Science 225*, 1365–1370.
Nishizuka, Y. (1984b) *Nature 308*, 693–698.
Oishi, K., Raynor, R. L., Charp, P. A., & Kuo, J. F. (1988) *J. Biol. Chem. 263*, 6865–6871.
Perrella, F. W. (1988) *Anal. Biochem. 174*, 437–447.
Seifert, R., Schachtele, C., Rosenthal, W., & Schultz, G. (1988) *Biochem. Biophys. Res. Commun. 154*, 20–26.
Soling, H.-D., Machado-De Domenech, E., Kleineke, J., & Fest, W. (1987) *J. Biol. Chem. 262*, 16786–16792.
Stern, I., & Shapiro, B. (1953) *J. Clin. Pathol. (London) 6*, 158–160.
Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T., & Nishizuka, Y. (1979) *J. Biol. Chem. 254*, 3692–3695.
Verkest, V., McArthur, M., & Hamilton, S. (1988) *Biochem. Biophys. Res. Commun. 152*, 825–829.
Verkleij, A. J., Zwaal, R. F. A., Roelofsen, B., Comfurius, P., Kastelijin, D., & van Deenen, L. L. M. (1973) *Biochim. Biophys. Acta 323*, 178–193.
Zwaal, R. F. A., & Bevers, E. M. (1983) *Subcell. Biochem. 9*, 299–334.