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Mechanism of Protein Kinase C Activation by Phosphatidylinositol 4,5-Bisphosphate†

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ABSTRACT: The mechanism of protein kinase C (PKC) activation by phosphatidylinositol 4,5-bisphosphate (PIP_2), phosphatidylinositol 4-monophosphate (PIP), and phosphatidylinositol (PI) was investigated by using Triton X-100 mixed micellar methods. The activation of PKC by PIP_2, for which maximal activity was 60% of that elicited by *sn*-1,2-diacylglycerol (DAG), was similar to activation by DAG in several respects: (1) activation by PIP_2 and DAG required phosphatidylserine (PS) as a phospholipid cofactor, (2) PIP_2 and DAG reduced the concentration of Ca^{2+} and PS required for activation, (3) the concentration dependences of activation by PIP_2 and DAG depended on the concentration of PS, and (4) PIP_2 and DAG complemented one another to achieve maximal activation. On the other hand, PIP_2 activation of PKC differed from activation by DAG in several respects. With increasing concentrations of PIP_2, (1) the optimal concentration of PS required was constant at 12 mol %, (2) the maximal activity at 12 mol % PS increased, and (3) the cooperativity for PS decreased. PIP_2 did not inhibit [^{3}H]phorbol 12,13-dibutyrate (PDBu) binding of PKC at saturating levels of PS; however, at subsaturating levels of PS, PIP_2 enhanced [^{3}H]PDBu binding by acting as a phospholipid cofactor. PIP did not function as an activator but served as a phospholipid cofactor in the presence of PS. While PIP_2, PIP, and PI did not support DAG-dependent PKC activation as phospholipid cofactors, their presence reduced the amount of PS required for maximal activation to as low as 2 mol % from 8 mol %. These data establish that PIP_2, PIP, and PI can function to spare, in part, the PS phospholipid cofactor requirement of PKC, and they demonstrate that PIP_2 but not PIP and PI can function as a lipid activator of PKC by mechanisms distinct from those of DAG and phorbol esters.

Phosphatidylinositol 4,5-bisphosphate (PIP_2),[1] phosphatidylinositol 4-monophosphate (PIP), and phosphatidylinositol (PI) are components of the phosphatidylinositol cycle, which is stimulated by growth factors, neurotransmitters, and hormones. When cells are activated by these agents, PIP_2 is hydrolyzed by phospholipase C, generating two intracellular second messengers, inositol 1,4,5-trisphosphate (IP_3) and *sn*-1,2-diacylglycerol (DAG) (Nishizuka, 1984; Berridge, 1984). IP_3 stimulates the release of Ca^{2+} from intracellular storage sites, while DAG activates protein kinase C (PKC), a Ca^{2+}- and phospholipid-dependent protein kinase, by increasing its affinity for Ca^{2+} and its phospholipid cofactor, PS (Takai et al., 1979; Hannun et al., 1986). Tumor-promoting phorbol esters activate PKC by mechanisms similar to that of DAG (Castagna et al., 1982). DAG inhibits phorbol ester binding through a competitive manner (Sharkey et al., 1984), suggesting that their interaction sites are the same. Studies with DAG analogues (Ganong et al., 1986; Molleyres & Rando, 1988) demonstrated that certain structural features within DAG are required for PKC activation: (1) two fatty acyl chains, which allow interaction with the membrane surface, (2) the stereospecific *sn*-1 and *sn*-2 oxygen ester bonds, and (3) a free hydroxyl group in the *sn*-3 position. The phospholipid cofactor requirement of PKC was recently shown to be highly specific for structural features contained within phosphatidyl-L-serine (Lee & Bell, 1989).

O'Brian et al. (1987) showed that, in the presence of 1 mM Ca^{2+}, PI, PIP, or PIP_2 acted to some extent as a phospholipid cofactor for PKC. Chauhan and Brockerhoff (1988) reported that PIP_2, but not PIP, activated PKC directly in a Ca^{2+}- and

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[1] Abbreviations: PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP, phosphatidylinositol 4-monophosphate; PI, phosphatidylinositol; IP_3, inositol 1,4,5-triphosphate; DAG, *sn*-1,2-diacylglycerol; PKC, protein kinase C; PS, phosphatidyl-L-serine; PA, phosphatidate; PG, phosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PDBu, phorbol 12,13-dibutyrate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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PS-dependent manner. Since PIP_2 was also shown to inhibit [^{3}H]PDBu binding to PKC (Chauhan et al., 1989), it was suggested that PIP_2 interacted at the DAG/phorbol ester binding site.

Given the strict structure–activity relationships established for PKC activation by DAG and phorbol esters (Nakamura et al., 1989), the results cited above (Chauhan & Brockerhoff, 1988; Chauhan et al., 1989) raised questions pertaining to the mechanism by which PIP_2 activated PKC. These questions and the potential physiological significance of PKC regulation by PIP_2, PIP, and PI caused us to undertake an in-depth investigation into the in vitro mechanisms by which these compounds exert their effects on PKC activity and on [^{3}H]-PDBu binding. Our results indicate that PIP_2 activates PKC by mechanisms different from that of DAG and interacts at a site distinct from the phorbol ester binding site. PIP_2, PIP, and PI also function to spare, in part, the phospholipid cofactor requirement of PKC.

Experimental Procedures

Materials. Cholesteryl acetate, crude brain lipids (bovine brain, Folch fraction 1), EGTA, HEPES, histone type IIIS, neomycin sulfate, PDBu, phospholipase C (type XIII from *Bacillus cereus*), and D-sphingosine were purchased from Sigma. Dioleoyl-PA, dioleoyl-PC, dioleoyl-PS, PI (bovine liver), and cardiolipin (bovine heart) were from Avanti Polar Lipids. All other phospholipids used were synthesized as previously described (Lee & Bell, 1989). Triton X-100 (10%) and Glycophase C (DG/100) (employed for neomycin affinity chromatography) were from Pierce. ATP (trisodium salt) was from Pharmacia. $MgCl_2$ and $CaCl_2$ were from Aldrich. [γ-^{32}P]ATP, [^{3}H]PDBu, and liquid scintillation fluid (Biofluor) were obtained from NEN (Dupont). The purified PKC isozymes α, βII, and γ, which were expressed in insect cells by using a baculovirus expression system (Burns et al., 1990), were kindly provided by Dr. David Burns in our laboratory.

Purification of PIP and PIP_2. PIP and PIP_2 were purified from brain extract (Folch fraction 1) by neomycin affinity chromatography (Schacht, 1978, 1981). Each phospholipid was further purified by silica gel chromatography to remove minor unidentified lipids and possible degradation products including DAG. The sample was applied to silica gel in chloroform. The gel was washed with 2 column volumes of chloroform, followed by 5 column volumes of chloroform/methanol (98/2). PIP_2 and PIP were eluted with chloroform/methanol (60/40). After evaporation of solvent, the samples were dissolved in chloroform. Following thin-layer chromatography, the final sample gave a single spot as visualized by I_2 vapor and phosphomolybdate spray. Purified PIP_2 and PIP have the same R_f values on thin-layer chromatography as standards obtained from Sigma. However, PIP_2 and PIP from Sigma were contaminated slightly with each other and with some unidentified lipids. After determination of the concentration of PIP_2 and PIP by phosphate analysis (Ames & Dubin, 1960), the sample was divided into aliquots, dried under N_2, and stored at −20 °C. Whenever a sample was dissolved in chloroform (normally 0.2 mM), it was used within 3 weeks. After this time, DAG was detected with the *Escherichia coli* DAG kinase assay (Preiss et al., 1986). After 3 weeks, the content of DAG in the samples was approximately 0.1% on a molar basis. Neither the PKC preparation employed nor *E. coli* DAG kinase preparations contained detectable phospholipase C activity. During the assay of PKC activity, PIP_2 was not hydrolyzed to DAG.

Purification of PKC. PKC was purified from frozen female Sprague-Dawley rat brain (Pel-Freez Biologicals, Rogers, AR)

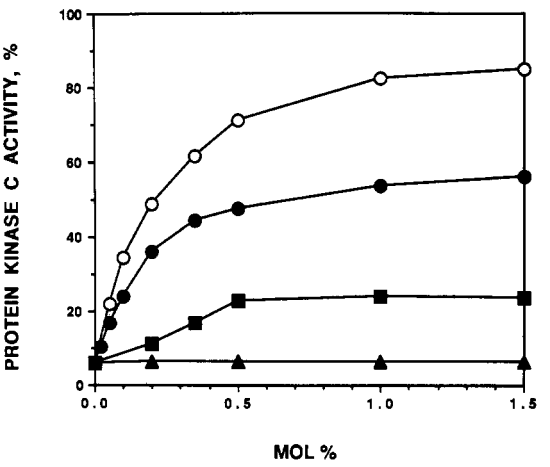

FIGURE 1: Activation of PKC by PIP_2, PIP, and PI. PKC activities were measured in the presence of 100 μM Ca^{2+} and 8 mol % PS at various concentrations of PIP_2 (●), PIP (■), PI (▲), and DAG (○). Each point represents the average of three determinations performed in duplicate. The error ranges of all data are within 10%. PKC activity is shown as a percentage of the activity obtained in the presence of 0.5 mol % DAG and 12 mol % PS, which was 35 picomoles of phosphate incorporated per minute.

by DEAE-Sepharose, threonine-Sepharose, and phenyl-Sepharose chromatography to near homogeneity as previously described (Kitano et al., 1986). The final specific activity was 2 μmol min^{-1} (mg of protein)$^{-1}$ when assayed under the conditions described below.

Assay of PKC Activity. PKC activity was determined by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into histone type IIIS for 10 min at 22 °C by using 0.3% Triton X-100 mixed micellar methods (Hannun et al., 1985). The enzyme reaction was started by addition of a premixed solution of [γ-^{32}P]ATP, ATP, and histone to prevent extensive aggregation between histone and phospholipid (especially PIP and PIP_2). This order of addition also prevents autophosphorylation of the enzyme prior to assay. One unit of enzyme activity was defined as 1 nanomole of P_i incorporated per minute. The assay was proportional with amount of enzyme and with time up to 20 min.

[^{3}H]PDBu Binding Assay. [^{3}H]PDBu binding activity of PKC was determined by using the Triton X-100 mixed micelle–gel filtration method (Hannun & Bell, 1986).

Analytical Methods. All phospholipids were quantitated by phosphorous determination using potassium phosphate as a standard (Ames & Dubin, 1960). The DAG standard was quantitated by ester determination using cholesteryl acetate as a standard (Stern & Shapiro, 1953). Ca^{2+} concentrations (up to 10 μM Ca^{2+}) were adjusted by a 2 mM EGTA–Ca^{2+} buffer system at pH 7.5, simultaneously accounting for the presence of 10 mM Mg^{2+} and 100 nM ATP (Fabiato & Fabiato, 1979). The kinetic parameters for enzyme activation were calculated by nonlinear regression analysis using the EZ-FIT computer program for the IBM PC (Perrella, 1988).

Results

Activation of PKC by PIP_2, PIP, and PI. Activation of PKC by PIP_2, PIP, and PI in the presence of 100 μM Ca^{2+} and 8 mol % PS was tested and compared with that of DAG. PIP_2 activated PKC to 60% of the maximal activity observed with DAG (Figure 1). The concentration of PIP_2 required for half-maximal activation was 0.18 ± 0.02 mol %, which is similar to that of DAG (0.21 ± 0.02 mol %). PIP increased activity to 20% of that seen when DAG stimulated maximally, while PI did not activate PKC. At 12 mol % PS, PIP_2 acti-

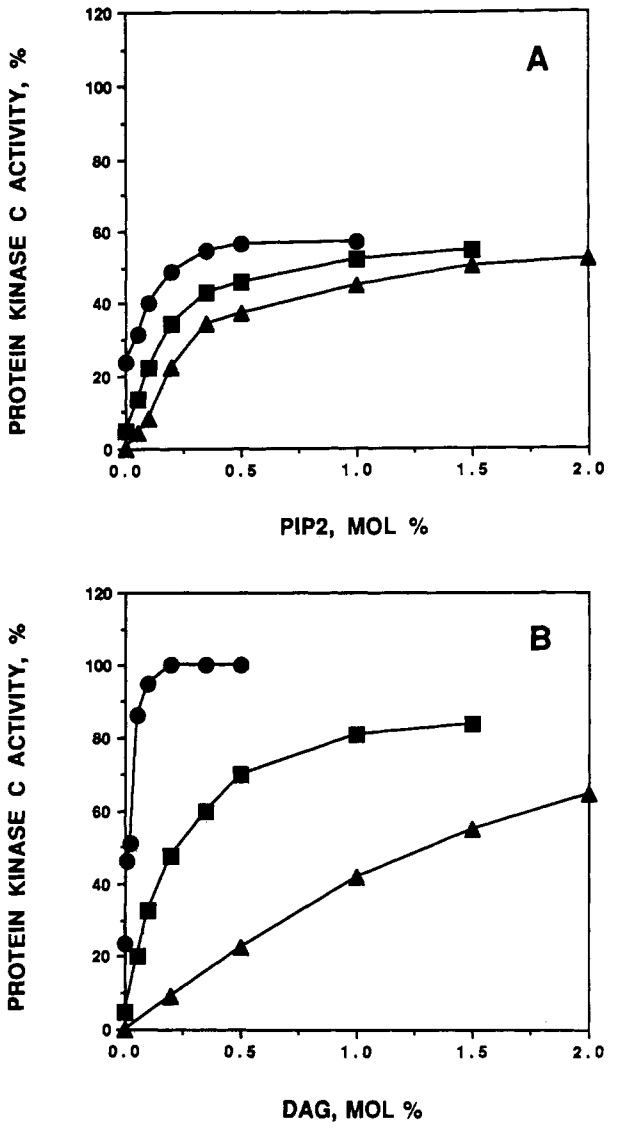

FIGURE 2: Dependencies of PIP_2 and DAG for PKC activation at different concentrations of PS. (A) PIP_2 and (B) DAG dependencies were measured in the presence of 100 μM Ca^{2+} at 6 mol % PS (▲), 8 mol % PS (■), and 12 mol % PS (●). The PKC activity is shown as a percentage of the activity obtained at 0.5 mol % DAG and 12 mol % PS.

vated PKC to 60% of the maximal activity observed with DAG. The concentration of PIP_2 required for half-maximal activation (0.14 ± 0.01 mol %) was not greatly changed from that observed at 8 mol % PS (Figure 2A), whereas that of DAG was greatly reduced to 0.025 ± 0.005 mol % (Figure 2B). At 12 mol % PS, PKC activity was not increased by PIP or PI. As shown in Figure 2, PIP_2 dependence was not strongly dependent on the PS concentrations employed. However, PIP_2, PIP, or PI, up to 10 mol %, did not activate the enzyme in the absence of PS (data not shown). PIP_2, unlike PIP and PI, is able to activate PKC in the presence of saturating PS. PIP_2-dependent activation was not isozyme specific; PIP_2 activated the α, βII, and γ isozymes with a similar concentration dependence but was less of an activator than DAG (Figure 3).

Ca^{2+} Dependence of PIP_2- and PIP-Dependent PKC Activation. Ca^{2+} dependence of PIP_2- and PIP-dependent PKC activation was investigated to determine whether both, like DAG, can reduce the amount of Ca^{2+} required for enzyme activation. At 12 mol % PS, 0.5 mol % of PIP_2 activated the enzyme at lower concentrations of Ca^{2+} than occurred with PS alone; the concentration of Ca^{2+} required for half-maximal activation by PIP_2 was 8.4 ± 0.8 μM, whereas that of 12 mol % PS was 73.5 ± 5.0 μM (Figure 4). In contrast, the presence of 1.0 mol % PIP did not reduce the Ca^{2+} requirement. PIP_2 activated the enzyme at slightly higher Ca^{2+} concentrations than DAG; the concentration of Ca^{2+} required for half-maximal activatino by DAG was 3.59 ± 0.02 μM. Therefore, PIP_2, like DAG, functions to reduce the concentration of Ca^{2+} required for activation.

Phospholipid Cofactor Specificity for PIP_2- and PIP-Dependent PKC Activation. As in the case of DAG and phorbol ester dependent activation of PKC, the phospholipid cofactor required for PIP_2-dependent activation was highly specific for structural features within phosphatidyl-L-serine (Figure 5). The other acidic phospholipids tested (PA, phosphatidyl-3-hydroxypropionate, PG, and lyso-PS), cationic phospholipids (PC and PE), or fatty acids (arachidonate and oleate) barely supported activity.

At 1 mol % PIP, only PS (at 8 mol %) supported activation. No phospholipid listed above, including PIP_2, PI, or fatty acids up to 10 mol %, supported activation by 1 mol % PIP (data not shown). Therefore, PIP_2- and PIP-dependent activation of PKC displays specificity for PS as a phospholipid cofactor.

PS Dependence of PIP_2- and PIP-Dependent Activation of PKC. We studied the PS dependence of PIP_2- and PIP-dependent PKC activation to determine whether these activators reduce the amount of phospholipid cofactor required for activation. Maximal enzyme activity at 0.5 mol % PIP_2, 1.0 mol % PIP, or 0.5 mol % DAG occurred at about 10 mol % PS (Figure 6). However, PIP_2 and PIP activated PKC at lower concentrations of PS than did DAG and PS alone. The concentrations of PS required for half-maximal activation by PIP_2 and PIP were 5.5 ± 0.4 and 5.3 ± 0.2 mol %, respectively, whereas those of DAG and PS alone were 7.0 ± 0.1 and 9.1 ± 0.1 mol %, respectively. Therefore, PIP_2, like DAG, can activate PKC by reducing the levels of PS and Ca^{2+} required for activation; in contrast, PIP only reduces the PS requirement. The maximal activity observed with PIP was similar to that observed with saturating PS. This result indicates that activation of PKC by PIP at 8 mol % PS is due to a PS supplementing effect. Thus, PIP appears to function as a phospholipid cofactor, not an activator like DAG or PIP_2.

In the presence of PIP_2 and PIP, the cooperativity observed with PS was low (Hill constants n = 2.5 ± 0.3 and 4.4 ± 0.3, respectively) compared to DAG (n = 7.9 ± 0.4) or PS alone (n = 10 ± 1). While DAG activation is dependent on the level of PS, PIP_2- and PIP-dependent activities are not affected as dramatically.

PIP_2 and DAG Activate PKC in a Complementary Manner. Since PIP_2 can activate PKC at saturating concentrations of Ca^{2+} and PS, PIP_2 functions as a lipid activator of the enzyme. We investigated whether DAG and PIP_2 activate PKC in an additive manner. At saturating concentrations of PS (12 mol %) and Ca^{2+} (100 μM), PIP_2-dependent activation was measured in the presence of subsaturating (0.02 mol %) and saturating concentrations (0.5 mol %) of DAG (Figure 7). At 0.02 mol % DAG, PIP_2 increased activity. At 0.5 mol % DAG, PIP_2 did not increase activity further. This indicates that DAG and PIP_2 can supplement one another to achieve maximal activity.

PIP_2 and PIP Do Not Inhibit [^{3}H]PDBu Binding of PKC. Since PIP_2 cooperates with DAG to activate PKC in the presence of saturating concentrations of Ca^{2+} and PS, PIP_2 could interact at the DAG/phorbol ester binding site of the enzyme. However, in contrast to DAG, PIP_2 (and PIP) did not inhibit but rather enhanced [^{3}H]PDBu binding of the

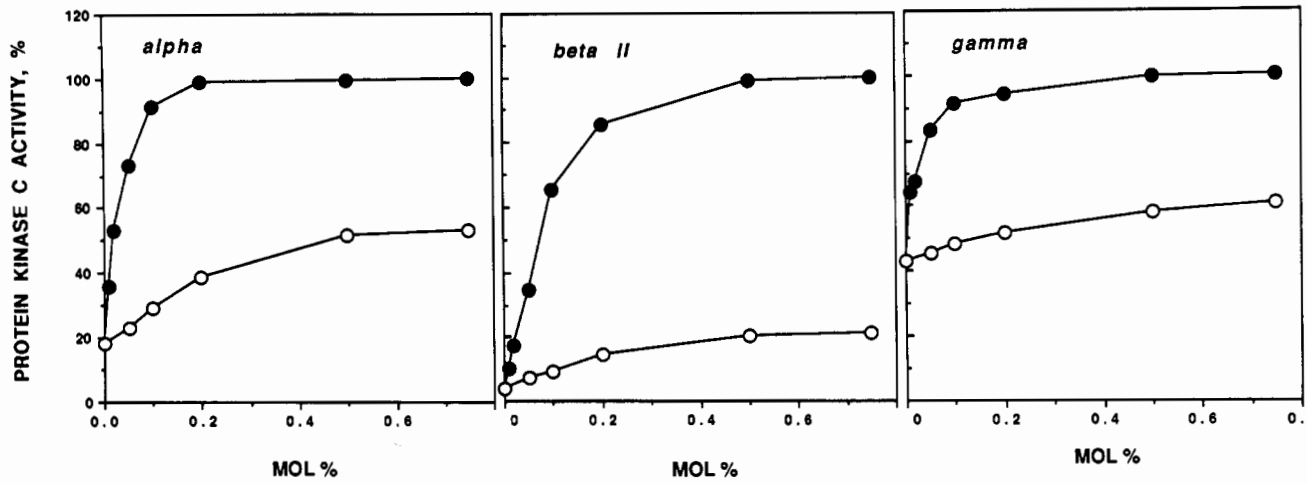

FIGURE 3: Activation of PKC isozymes by PIP_2. The activities of PKCα, PKCβII, and PKCγ were determined in the presence of 100 μM Ca^{2+} and 10 mol % PS with increasing concentrations of PIP_2 (○) and DAG (●). The specific activities for these isozymes were 1050 nmol min^{-1} (mg of protein)$^{-1}$ (PKCα), 660 nmol min^{-1} (mg of protein)$^{-1}$ (PKCβII), and 240 nmol min^{-1} (mg of protein)$^{-1}$ (PKCγ). In the absence of Ca^{2+} or PS, the activities of all isozymes were negligible. The activity of each PKC isozyme is shown as a percentage of the activity obtained at 100 μM Ca^{2+}, 0.5 mol % DAG, and 10 mol % PS.

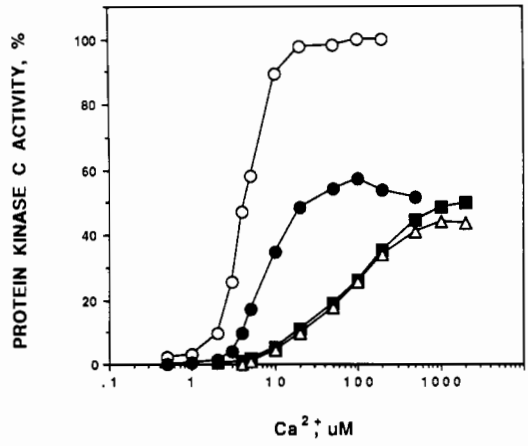

FIGURE 4: Ca^{2+} dependence of PIP_2- and PIP-dependent PKC activation. PKC activities were determined in the presence of 12 mol % PS and either 0.5 mol % PIP_2 (●), 1 mol % PIP (■), 0.5 mol % DAG (○), or PS alone (△) with increasing concentrations of Ca^{2+}. Data presented are from a typical experiment, performed in duplicate, which was repeated two times. PKC activity is shown as a percentage of the activity obtained at 100 μM Ca^{2+}, 0.5 mol % DAG, and 12 mol % PS.

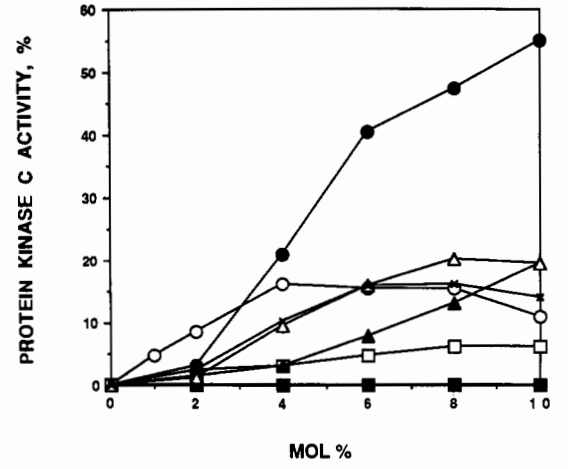

FIGURE 5: Specificity of phospholipid cofactors for PIP_2-dependent PKC activation. In the presence of 0.5 mol % PIP_2 and 100 μM Ca^{2+}, PKC activities were measured at various concentrations of the phospholipids indicated: phosphatidyl-L-serine (●), cardiolipin (○), PIP (■), PI (□), phosphatidylethanol (▲), phosphatidyl-L-homoserine (△), and phosphatidyl-D-serine (×). The fatty acyl chains of all phospholipids used were oleic acid except PI, PIP, PIP_2, and cardiolipin, which were purified from natural sources (see Experimental Procedures). Data presented are from a typical experiment, performed in duplicate, which was repeated one time. The results were normalized to the activity obtained in the presence of 0.5 mol % DAG and 12 mol % PS.

enzyme with 20 mol % PS (Figure 8A). The inability of PIP_2 to inhibit phorbol ester binding is not a function of the concentrations of PS (Figure 8B) and PDBu (Figure 8C). At 30 mol % PS, which is the saturating concentration of PS for [^{3}H]PDBu binding to PKC, neither PIP_2 nor PIP affected [^{3}H]PDBu binding to the enzyme (Figure 8D). Under all conditions, PIP_2 and PIP did not support PDBu binding to PKC in the absence of PS. Therefore, the PIP_2- and PIP-dependent increases in [^{3}H]PDBu binding of PKC observed at subsaturating concentrations of PS are due to PIP_2 and PIP serving as phospholipid cofactors. In addition, these data indicate that PIP_2 does not interact at the phorbol ester (DAG) binding site.

PS Dependence of PIP_2-Dependent PKC Activation at Varying Levels of PIP_2. Since PIP_2 can supplement PS as a phospholipid cofactor to attain maximal phorbol ester binding, the ability of PIP_2 to function simultaneously as a phospholipid cofactor for activation of PKC was investigated. The PS dependence of PIP_2-dependent activation was examined at varying levels of PIP_2. As the concentration of PIP_2 was increased up to 0.5 mol %, the optimal concentration of PS required for activation was not changed at 12 mol % (Figure 9A). However, increasing the concentration of PIP_2 reduced (1) the concentration of PS required for half-maximal activation from 9.1 ± 0.1 to 5.5 ± 0.4 mol % and (2) the Hill constant for activation by PS from 10 ± 1 to 2.5 ± 0.3. Therefore, PIP_2 can function as a phospholipid cofactor as well as a lipid activator.

Effect of PIP_2 on PS Dependence of DAG-Dependent PKC Activation. To further investigate the phospholipid-supplementing function of PIP_2, the PS dependence of PKC activation was investigated in the presence of a saturating concentration of DAG (2 mol %) at varying mol % of PIP_2 (Figure 9B). Under these conditions, PIP_2 alone did not support DAG-dependent enzyme activation as a phospholipid cofactor. The contribution of PIP_2 as an enzyme activator was minimal, as shown by comparison with Figure 9A. Increasing the concentration of PIP_2 to 0.75 mol % caused a reduction in (1)

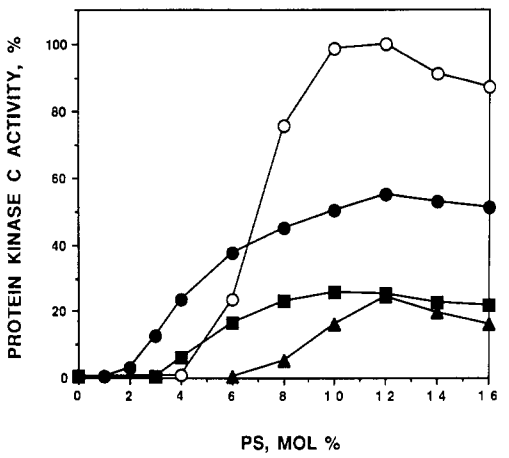

FIGURE 6: PS dependence of PIP_2- and PIP-dependent activation of PKC. The PS dependence of PKC activation by 0.5 mol % PIP_2 (●), 1 mol % PIP (■), or 0.5 mol % DAG (○) or without activator (▲) was measured in the presence of 100 μM Ca^{2+}. Each point represents the average of two determinations, performed in duplicate. The error ranges of all data are within 10%. PKC activity is shown as a percentage of the activity obtained in the presence of 0.5 mol % DAG and 12 mol % PS.

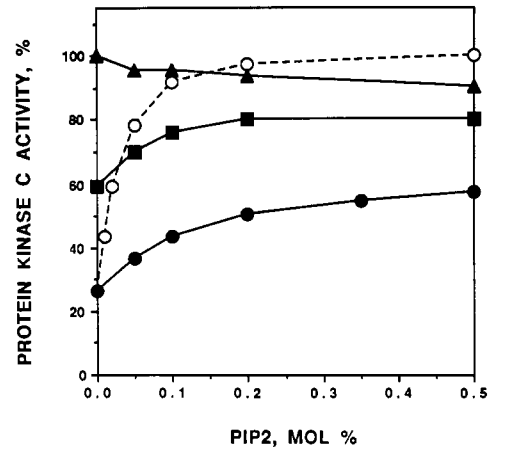

FIGURE 7: Effect of PIP_2 on DAG-dependent PKC activation. PKC activities were measured at 100 μM Ca^{2+} and 12 mol % PS with increasing concentrations of PIP_2 in the absence (●) or presence of 0.02 mol % DAG (■) and 0.5 mol % DAG (▲). DAG-dependent activities in the absence of PIP_2 are shown by open circles with the dotted line, with DAG concentrations equivalent to those shown for PIP_2. Each point represents the average of two determinations, performed in duplicate. The error ranges of all data are within 10%. PKC activity is shown as a percentage of the activity obtained in the presence of 0.5 mol % DAG and 12 mol % PS.

the optimal concentration of PS from 8 to 4 mol %, (2) the concentration of PS required for half-maximal activation of DAG from 5.2 ± 0.1 to 1.8 ± 0.1 mol %, and (3) the Hill constant for activation by PS from 7.4 ± 0.6 to 3.7 ± 0.4. These data indicate that, in the presence of 2 mol % DAG, PIP_2 supplements PS as a phospholipid cofactor, reducing the number of PS molecules per micelle required for enzyme activation to approximately 4.

Effect of PIP on PS Dependence of DAG-Dependent PKC Activation. Like PIP_2, PIP did not support DAG-dependent PKC activation as a phospholipid cofactor but dramatically reduced the PS concentration needed for maximal DAG-dependent enzyme activation (Figure 10). As the concentration of PIP was increased, reductions were observed in (1) the optimal PS concentration from 8 to 2 mol %, (2) the concentration of PS required for half-maximal activation from 5.2 ± 0.1 to 0.74 ± 0.04 mol %, and (3) the Hill constant for activation by PS from 7.4 ± 0.6 to 1.9 ± 0.2. PIP was less efficient than PIP_2 in replacing PS since higher concentrations were required. However, PIP reduced the minimum PS molecules required for PKC activation to a greater extent than PIP_2.

Effect of PI on PS Dependence of DAG-Dependent PKC Activation. Like PIP_2 and PIP, PI (up to 20 mol %) did not support DAG-dependent PKC activation as a phospholipid cofactor but supplemented PS as a phospholipid cofactor for enzyme activation. In the presence of 100 μM Ca^{2+} and 2 mol % DAG, increasing the concentration of PI reduced the requirement of PS for maximal activation of PKC to as low as 2 mol % (data not shown). However, its efficiency in supplementing PS is much lower than those of PIP_2 and PIP. At 2 and 4 mol % of PS, maximal activity of PKC required 16 and 10 mol % of PI, respectively. Therefore, the efficiency of phosphoinositides to supplement PS in PKC activation appears to be directly related to the number of phosphates present in the head group.

Discussion

PKC Activation by PIP_2. The results shown here confirm previous observations that PIP_2 directly activates PKC in a Ca^{2+}- and PS-dependent manner (Chauhan & Brockerhoff, 1988) and provide detailed new insights into the mechanism of PKC activation by PIP_2. The mixed micellar methods employed allowed a clear dissociation of the function of phospholipids as activators or as cofactors. Given the structural differences between PIP_2 and DAG, the mechanism of PKC activation by PIP_2 would be expected to be different from that by DAG. However, their activation mechanisms are quite similar, as supported by several lines of evidence: (1) PIP_2 and DAG activated PKC at reduced concentrations of Ca^{2+} and PS. (2) PIP_2 and DAG showed half-maximal activation of PKC with similar concentrations of ATP, Mg^{2+}, and histone type IIIS.[2] (3) PIP_2 and DAG specifically required PS as a phospholipid cofactor for enzyme activation. (4) PIP_2 and DAG concentration dependences of activation depended on the concentration of PS. (5) PIP_2 and DAG activated PKC in a complementary manner. (6) The activation by PIP_2 and DAG were similarly inhibited by D-sphingosine and neomycin[2] (see below). (7) The protein substrate specificities of PKC activated by PIP_2 and DAG were similar.[3]

However, PIP_2 and DAG also affected PKC activity with distinct characteristics. In contrast to DAG, (1) PIP_2 dependence was not strongly dependent on the PS concentration, (2) increasing the concentration of PIP_2 did not decrease the optimal concentration of PS required, (3) increasing the concentration of PIP_2 increased maximal activity at optimal PS concentration, (4) PIP_2 activated PKC with lower cooperativity for PS than DAG, (5) increasing the concentration of PIP_2 decreased the cooperativity for PS, (6) PIP_2 drastically decreased the requirement of PS for DAG-dependent activation, and (7) PIP_2 did not inhibit phorbol ester binding to PKC.

One point worth possibly discussing is that DAG used in this study was 1,2-dioleoylglycerol, whereas PIP and PIP_2 used were purified from bovine brain extract. The fatty acyl chains of these phospholipids were not chemically defined. A study showed that over 27 molecular species exist in material purified from bovine brain; a 1-stearoyl-2-arachidonyl derivative contributed more than 40% and a 1-stearoyl-2-eicosatrienoyl

[2] M.-H. Lee and R. M. Bell, unpublished observation.

[3] The relative activating abilities of PIP_2 and DAG were not significantly different regardless of the protein substrates used: histone type IIS, VS, VIS, VIIS, or VIIIS, myosin light chain, or poly(Lys,Arg) (3:1).

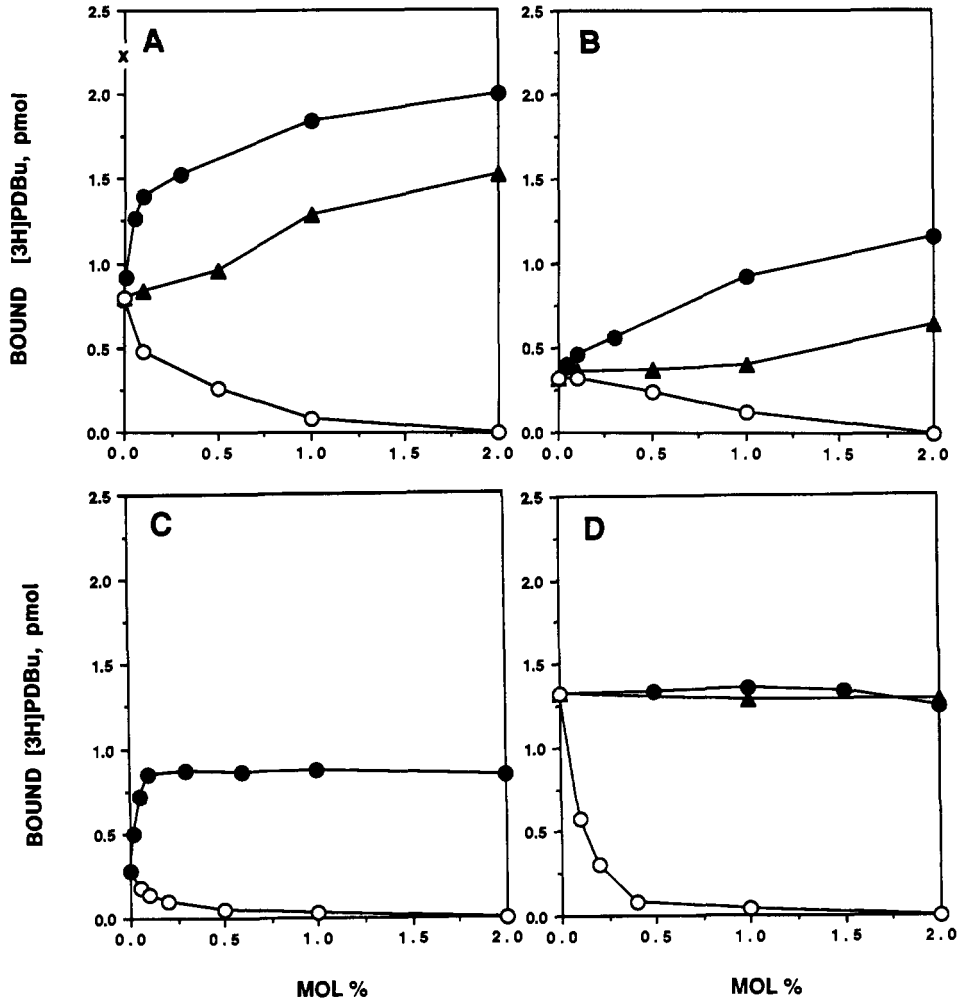

FIGURE 8: Effect of PIP_2 and PIP on the [^{3}H]PDBu binding of PKC. [^{3}H]PDBu binding activities of PKC were measured in the presence of 200 μM Ca^{2+} and various concentrations of PIP_2 (●), PIP (▲), or DAG (○) at the following concentrations of PS and PDBu: (A) 20 mol % PS and 50 nM [^{3}H]PDBu, (B) 14 mol % PS and 50 nM [^{3}H]PDBu, (C) 20 mol % PS and 25 nM [^{3}H]PDBu, and (D) 30 mol % PS and 25 nM [^{3}H]PDBu. After incubating 0.4 unit of PKC with the mixtures for 10 min at room temperature (22 °C), [^{3}H]PDBu-bound micelles were separated from free [^{3}H]PDBu by Ultro-Gel AcA 202 gel filtration. Nonspecific binding of [^{3}H]PDBu was determined in the presence of 10 μM unlabeled PDBu and found to be negligible, as was binding in the absence of PS. The [^{3}H]PDBu binding activity of PKC in the presence of 50 nM [^{3}H]PDBu and 30 mol % PS is indicated by × in panel A, which was 12 250 pmol bound/mg of protein. Each point represents the average of two determinations, performed in duplicate. The error ranges of all data are within 10%.

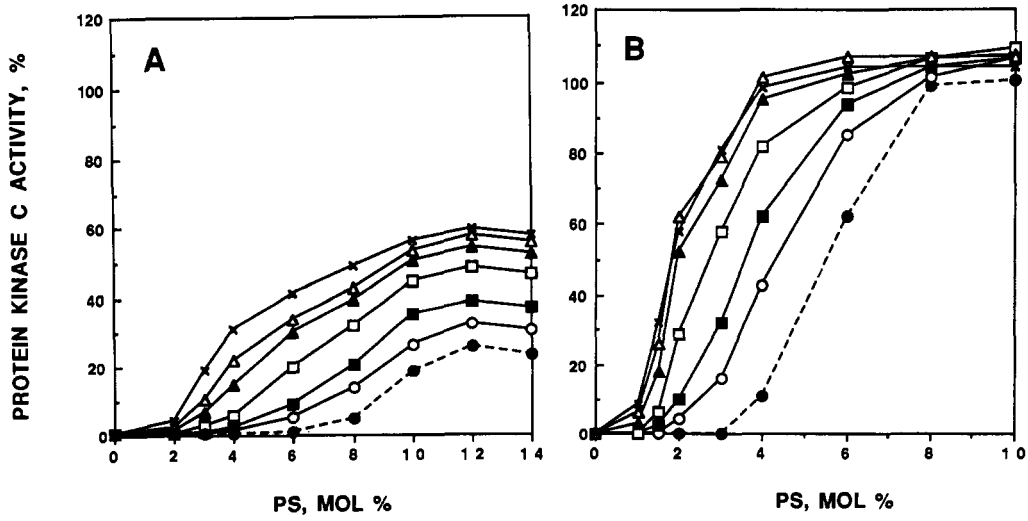

FIGURE 9: Effect of PIP_2 on PS dependence of PIP_2- and DAG-dependent PKC activation. PS dependence of PKC activation by PIP_2 (A) without or (B) with 2 mol % DAG was measured at the concentrations of PIP_2 indicated: none (●), 0.05 mol % (○), 0.1 mol % (■), 0.2 mol % (□), 0.35 mol % (▲), 0.5 mol % (△), and 1 mol % (×). Data presented are from a typical experiment, performed in duplicate, which was repeated two times. The activities are shown as a percentage of the activity obtained in the presence of 100 μM Ca^{2+}, 2 mol % DAG, and 10 mol % PS.

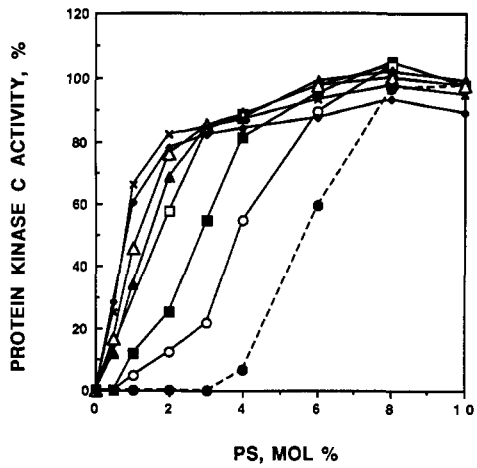

FIGURE 10: Effect of PIP on PS dependence of DAG-dependent activation of PKC. PS dependence of PKC activation by 2 mol % DAG was measured at the concentrations of PIP indicated: none (●), 0.25 mol % (○), 0.5 mol % (■), 0.75 mol % (□), 1 mol % (▲), 1.5 mol % (△), 2.0 mol % (×), and 2.5 mol % (◆). Data presented are from a typical experiment, performed in duplicate, which was repeated two times. Activities are shown as a percentage of activity obtained in the presence of 100 μM Ca^{2+}, 2 mol % DAG, and 10 mol % PS.

species was 20% of the total (Holub et al., 1970). It would be interesting to test the effect of fatty acyl chain composition of PIP_2 on PKC activation, but such species are not available at this time. However, the specificity of DAG molecular species on PKC activation has been investigated. Nishizuka's group showed that 1,2-dioleoylglycerol, 1-stearoyl-2-arachidonylglycerol, and many other naturally occurring DAGs have almost equal activity (Go et al., 1987).

Since PKC requires the precise structural features within DAG (Ganong et al., 1986; Molleyres & Rando, 1988) and PS (Lee & Bell, 1989) for activation, its activation by PIP_2 is intriguing. The activity of DAG for PKC activation is abolished by adding to the head group even one carbon molecule (methylene group) between C_3 of the glycerol backbone and the hydroxyl group or by adding one phosphate group (PA). In contrast, among phosphoinositides, only PIP_2, which has a bulky head group with three phosphate moieties, can activate PKC, while PIP and PI with fewer phosphates do not activate but can serve as cofactors. One possible explanation is that PIP_2 can activate PKC by interaction with Ca^{2+}, for which it is known to display high affinity (Hendrickson & Reinertsen, 1969). This possibility seems unlikely. First of all, PIP_2-dependent activation requires 2–100 μM Ca^{2+} in the presence of excess Mg^{2+} (10 mM); Mg^{2+} is known to have a similar affinity for the head-group phosphate moieties of PIP_2 as Ca^{2+} (Hendrickson & Reinertsen, 1969). Therefore, a minor portion of Ca^{2+} can actually interact with the head group of PIP_2 under assay conditions. Second, PIP, which is also known to avidly bind to Ca^{2+}, did not activate PKC or reduce the Ca^{2+} requirement for PKC activation. Third, neomycin, which was shown to bind the head group of PIP_2, thus displacing bound Ca^{2+} (Lodhi et al., 1976), did not selectively inhibit PIP_2-dependent PKC activation, compared to DAG-dependent or PS only dependent activation.[4] Finally, DAG, which does not by itself bind Ca^{2+}, actually reduces the Ca^{2+} requirement to a greater extent than PIP_2.

On the basis of these data and the dissimilarities in structure, PIP_2 and DAG may interact at distinct sites on PKC or form a different activation complex. This separate-site hypothesis is consistent with the observation that PIP_2 does not inhibit [^{3}H]PDBu binding. However, our data are opposite from those of a previous report (Chauhan et al., 1989) in which PIP_2 inhibited [^{3}H]PDBu binding to PKC. The reason for these different experimental results is not known at this time. One possibility is that the impure enzyme employed in the earlier study may hydrolyze PIP_2 into DAG during the binding assay, and samples of PIP_2 employed may have been of different purities. In our studies, the enzyme preparation employed was pure and did not contain any phospholipase C activity, and PIP_2 was purified and care was taken to establish and monitor purity.

Comparison of the inferred structures of the PKC isozymes reveals four regions of homology that are conserved (C region) and five regions of variable, unconserved sequences [for review, see Kikkawa et al. (1989)]. The C_1 region near the amino terminus is known to lie within the lipid-binding, regulatory domain and to be necessary for high-affinity PDBu binding (Kikkawa et al., 1989). The other sequence conserved in the regulatory domain, C_2, has been suggested to confer Ca^{2+} dependency to phorbol ester binding (Ono et al., 1989); other functions are unknown. Interestingly, there is a strong sequence homology between the C_2 region of PKC and a region of the phosphoinositide-specific phospholipase C (Baker, 1989). If this region in phospholipase C is the interaction site for PIP_2, the C_2 region of the regulatory domain of PKC may be the site of PIP_2 interaction. This possibility is presently being investigated by deletion analysis.

At 8 mol % PS, both PIP_2 and DAG activated PKC with similar efficiencies; the concentration of DAG and PIP_2 required for half-maximal activation was 0.2 mol %. At a saturating concentration of PS (12 mol %), PIP_2 activated PKC less efficiently than DAG did; the concentrations of PIP_2 and DAG required for half-maximal activation were 0.14 and 0.025 mol %, respectively. These results are inconsistent with those of a previous report (Chauhan & Brockerhoff, 1988), which showed that, at 9 mol % PS, PIP_2 activates the enzyme 50 times more efficiently than DAG; the concentrations of PIP_2 and DAG required for half-maximal activation were 0.04 and 2 mol %, respectively. A major discrepancy lies in the reported values for DAG. However, other reports (Hannun et al., 1985, 1986) support the value for DAG obtained from this study.

PIP_2 and PIP comprise 1–3% of total inositol lipids (Creba et al., 1983). PI is mainly located at the inner leaflet of the plasma membrane, where it comprises about 10 mol % of total phospholipid (White, 1973). If we assume that PIP_2 and PIP are mainly present in the inner leaflet of the membrane, and the level of PIP_2 is similar to that of PIP, the concentration of PIP_2 in the inner leaflet of the plasma membrane is approximately 0.05–0.25 mol %. This level is in the suboptimal concentration range for PKC activation in vitro. Interestingly, an increase in the steady-state level of PIP_2 has been reported in agonist-stimulated thymocytes (Taylor et al., 1984), platelets (Imai et al., 1983; Perret et al., 1983; de Chaffoy de Courcelles et al., 1984), and kidney (Farese et al., 1979), in *src*- (Sugimoto et al., 1984), *ros*- (Macara et al., 1984), and *ras*- (Huang et al., 1988) transformed fibroblasts, and in nuclei of DMSO-induced differentiated erythroleukemia cells (Cocco et al., 1987). Therefore, increasing PIP_2 might activate PKC in some cells, at least in conjunction with a subsaturating

[4] Neomycin inhibited 0.5 mol % DAG- and 0.5 mol % PIP_2-dependent PKC activation (at 12 mol % PS and 100 μM Ca^{2+}) in the range of 0.05–5 mM. Half-maximal inhibition occurred at 0.48 ± 0.09 and 0.84 ± 0.08 mM, respectively. Neomycin also inhibited the enzyme activation supported by 12 mol % PS alone with half-maximal inhibition at 0.71 ± 0.07 mM.

concentration of DAG. The physiological significance, however, will require additional lines of study.

PS Sparing Effect of PIP_2, PIP, and PI. PS supports PKC activation as a phospholipid cofactor in a highly cooperative manner with a Hill constant between 8 and 11 (Newton & Koshland, 1989). Since PS is present in the inner leaflet of the plasma membrane at about 15–20 mol %, PS may not be a limiting factor for regulation of PKC at that location. However, PS is a structural element for cytoskeletal components (Cohen et al., 1988; Geisow & Walker, 1986) and Ca^{2+}-dependent phospholipid binding proteins (Geisow & Walker, 1986). Therefore, the actual concentration of PS available for PKC activation may be limiting. When cells are stimulated, PKC is also located on cytoskeletons (Papadopoulos & Hall, 1989) and on the nuclear envelope (Leach et al., 1989), where the PS mol % might be limiting (Getz et al., 1968). The cooperative activation of PKC by PS was proposed to be a mechanism to regulate PKC physiologically (Newton & Koshland, 1989). However, from the data presented, the presence of PI, PIP, and PIP_2 could reduce greatly the requirement of PS for PKC activation; for instance, the presence of 0.5 mol % PIP reduced the concentration of PS for half-maximal activation to 0.74 ± 0.04 mol % with a Hill constant of 1.9 ± 0.2. This level of PIP was reported in platelets (Perret et al., 1983). PS may not function as the only phospholipid cofactor for PKC activation in cells.

The ability of the phosphoinositides to replace, in part, PS is important for a mechanistic understanding of PKC activation. Both amino and carboxyl moieties within the PS head group are stereospecifically required for PKC activation (Lee & Bell, 1989). The present study suggests that some of these phospholipid molecules may be replaced by PIP_2, PIP, and PI. In the presence of essential PS molecules (2–4 mol %), PI, PIP, and PIP_2 can function as phospholipid cofactors. The presence of PIP_2 and PIP reduced not only the PS concentration but also the total phospholipid concentration (mol %) required for PKC activation. When PS was supplemented by PIP_2 and PIP, the phospholipid concentrations required for optimal activation by 2 mol % DAG were 4.5 mol % (Figure 9B) and 3 mol % (Figure 10), respectively. Since the efficiency for sparing PS is positively related to the number of phosphates in the head group of phosphoinositides, the acidic group in supplementing phospholipids seems to be an important factor in supporting enzyme activation. However, the structural features of phospholipids able to supplement PS remains to be established. Nonetheless, the data are consistent with four or more molecules of PS or other phospholipid cofactors participating in forming a PKC–phospholipid cofactor–Ca^{2+}–DAG(PIP_2) complex.

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Sulfuryl Transfer Catalyzed by Phosphokinases†

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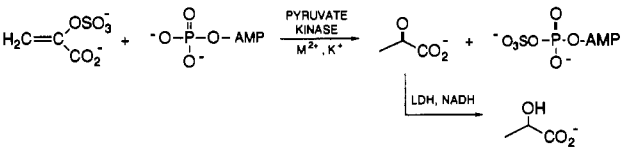
ABSTRACT: Adenosine 5′-sulfatopyrophosphate is a substrate for nucleoside diphosphate kinase. The reaction appears to proceed through a ping-pong mechanism analogous to the physiological reaction involving ATP, presumably by way of a sulfohistidine intermediate. Unlike the phosphoryl transfer reactions, the corresponding sulfuryl transfers catalyzed by nucleoside diphosphate kinase do not have a strict divalent metal requirement. The estimated rate constants for the metal- and nonmetal-catalyzed sulfuryl transfers differ by less than an order of magnitude and are approximately 1000-fold slower than the corresponding phosphate transfers. These results suggest that the role of the metal ion in nucleoside diphosphate kinase is to coordinate the α,β-phosphates of the substrate. Sulfuryl and phosphoryl transfer probably occur through dissociative transition states.

We recently showed that pyruvate kinase catalyzes the reaction between sulfoenolpyruvate, the sulfate analogue of PEP,[1] and ADP to form $ADPSO_3$ and pyruvate (Scheme I) (Peliska & O'Leary, 1989). This represents the first demonstrated case of a sulfuryl transfer catalyzed by a phosphokinase. We were interested in determining whether other phosphokinases can catalyze similar reactions and, if so, what factors might govern sulfuryl transfer mechanisms. For this reason, we have investigated the substrate and inhibition characteristics of $ADPSO_3$ with a variety of other phosphokinases.

The synthesis of $ADPSO_3$ was originally performed by Ikehara et al. (1961). Yount et al. (1966) determined that snake venom phosphodiesterase cleaves $ADPSO_3$ between the α- and β-phosphates. The compound is not a substrate for myosin or alkaline phosphatase. Perhaps these negative results prevented further investigation of $ADPSO_3$ as an analogue of ATP in enzyme-catalyzed reactions.

A large number of other ATP analogues have been studied as substrates for various enzymes (Yount, 1975). Enzymes can often tolerate modifications in the base portion of the nucleotide but not in the transferring phosphate. The thiophosphate analogues, used in studying the stereochemistry and mechanism of phosphoryl transfer (Eckstein, 1983), seem to be the only useful, catalytically active derivatives of the phosphate moiety.

Scheme I



In the present study, we examined the chemistry and enzymatic activity of $ADPSO_3$ with nucleoside diphosphate kinase and the inhibitory properties of this same substrate with various other kinases.

Materials and Methods

Materials. Sulfur trioxide pyridine complex (Aldrich), sodium pyruvate (Sigma), sodium formate (Mallinckrodt), sodium acetate (Mallinckrodt), disodium NADH (Sigma), disodium NAD (Sigma), dicyclohexylammonium ADP (Sigma), dicyclohexylammonium ATP (Sigma), sodium adenosine 5′-phosphosulfate, glucose (MC&B), and disodium fructose

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[1] Abbreviations: HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PEP, phosphoenolpyruvate; $ADPSO_3$, ADP sulfate; LDH, lactate dehydrogenase; APS, adenosine phosphosulfate; NDP kinase, nucleoside diphosphate kinase; NDP, nucleoside diphosphate.

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