Role of Substrate in Determining the Phospholipid Specificity of Protein Kinase C Activation†

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ABSTRACT: The phospholipid selectivity of protein kinase C (PKC) activation was examined by using two substrates, histone and a random copolymer of lysine and serine [poly(lysine, serine)] (PLS), plus phospholipids provided as vesicles or as Triton-mixed micelle preparations. The results indicated that substrate–phospholipid interaction was an essential component of PKC activation and that many in vitro properties of PKC activation are attributable to this interaction. The substrate histone interacted with phospholipid–Triton mixed micelles containing phosphatidylserine (PS), but not with those containing phosphatidylinositol (PI) or phosphatidylglycerol (PG). In direct correlation, only PS–Triton mixed micelles were effective in supporting PKC activity. Also, the minimum PS composition (4 mol % in Triton) required to induce significant histone–PS interaction coincided with the minimum composition required for phosphorylation of histones. Moreover, the PS composition required for maximum activity varied with the histone concentration of the reaction. In contrast to histone, PLS interacted with phospholipid–Triton mixed micelles containing either PS, PI, or PG, and all these mixed micelles supported the phosphorylation of PLS. In fact, by selection of appropriate experimental conditions (e.g., concentration of substrate and phospholipid), any of the three mixed micelles could appear the most effective in supporting PKC activity. Phospholipid vesicles containing PS, PG, or PI were found to interact with both histone and PLS and to support the activity of PKC. Physical properties of the solution and conditions used for preparation of phospholipid vesicles had considerable influence on PKC activation. At high phospholipid concentrations, vesicles containing PS, PI, or PG supported the activity of PKC to essentially the same level, provided that the physical differences among the phospholipid vesicles were minimized. In agreement with this general property, maximum binding of phorbol dibutyrate to PKC was not influenced by the head group of the phospholipids. Three major implications for the activation of PKC were apparent: (1) the phospholipid selectivity of PKC activation was related to properties of substrate–phospholipid interaction; (2) the best PKC substrates are membrane-associated proteins; and (3) selection of experimental conditions could render opposing relationships and conclusions for the influence of the same component(s) measured under different conditions.

Protein phosphorylation and dephosphorylation are major mechanisms for signal transductions in biological systems (Nishizuka, 1986a; Nestler et al., 1984; Cohen, 1982). Recently, protein kinase C (PKC)[1] has been the focus of many investigations because of the potential role of this enzyme in many cellular functions (Nishizuka, 1986b; Takia et al., 1985; Kuo et al., 1984). Activation of PKC by diacylglycerol has linked the activity of this enzyme to the function of many stimuli via phosphatidylinositol turnover (Nishizuka, 1984a; Berridge, 1984). Biologically active phorbol esters also activate PKC and are thereby presumed to exert their function (Ashendel, 1985; Nishizuka, 1984b, 1986a). A full understanding of the mechanism of PKC activation is needed to better understand how PKC can influence cellular events.

Activation of PKC requires the presence of Ca^{2+} and phospholipid when tested with histone as a substrate. However, the mechanism of PKC activation by phospholipid is not well understood. For example, activation of PKC displays strong selectivity for PS over other acidic phospholipids (Kaibuchi et al., 1981), whereas the binding of PKC to membrane does not display such selectivity (Bazzi & Nelsestuen, 1987a). In addition, the activity of PKC displayed selectivity for PS under conditions where enzyme–phospholipid binding could not occur (Bazzi & Nelsestuen, 1987b). The basis of this phospholipid selectivity is unknown. Recently, a number of common in vitro PKC substrates have been shown to interact strongly with phospholipid (Bazzi & Nelsestuen, 1987b). Since substrate interaction with the phospholipid appeared essential for phosphorylation by PKC, it is possible that substrate is responsible for producing the observed selectivity of PKC activity. This property may also provide an opportunity to demonstrate the most probable types of substrates under in vivo circumstances.

A major difficulty in examining the role of phospholipid in activation of PKC concerns the physical status of the phospholipid component. PS has been shown to activate PKC whether this phospholipid is provided in the form of vesicles or phospholipid–Triton mixed micelles. However, the activity of PKC showed significant differences in the two systems. For example, in the presence of phospholipid vesicles, PKC activity was influenced by the phospholipid concentration as well as by the vesicle's lamellar structure and size (Boni & Rando, 1985). With PS–Triton mixed micelles, the activity of PKC was sensitive only to the composition of the micelles (Hannun

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[1] Abbreviations: PKC, protein kinase C; PLS, a random copolymer of lysine and serine [poly(lysine, serine)] (3:1); DAG, diacylglycerol or diolein; PS, phosphatidylserine; PI, phosphatidylinositol; PG, phosphatidylglycerol; PC, phosphatidylcholine; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; BSA, bovine serum albumin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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et al., 1985). In addition, the appearance of PKC activity was highly cooperative with respect to PS in the presence of PS–Triton mixed micelles, but not in the presence of phospholipid vesicles. This may indicate that different factors influence the activity of PKC in the two different phospholipid systems. Identification of these possible factors is essential for better understanding of this conditional cooperativity.

In this study, we examined the phospholipid selectivity of PKC activation for two categorically different substrates, histone and the random copolymer poly(lysine, serine) (PLS). The measurements were performed with phospholipid vesicles and phospholipid–Triton mixed micelles. In all of the cases tested, the results indicated that substrate–phospholipid interaction was an essential component of PKC activity and was largely responsible for the apparent phospholipid selectivity and the observed cooperativity of PKC activation.

Experimental Procedures

Materials

Phosphatidylserine (egg yolk), phosphatidylinositol (soybean), phosphatidylglycerol (egg yolk), phosphatidylcholine (egg yolk), and diolein were purchased from Sigma Chemical Co. and were of the highest purity available (>98% purity reported). [γ-^{32}P]ATP was from Amersham Corp. [^{3}H]-Phorbol dibutyrate (PDBu) was from New England Nuclear Products. Glass fiber filters (GF/F) were purchased from Whatman. Nitrocellulose filters (pore size 0.45 μm) were from Millipore Corp. Triton X-100, histone (type III-S), protamine sulfate, and poly(lysine, serine) (3:1) were purchased from Sigma Chemical Co.

Methods

PKC Purification. PKC was purified to apparent homogeneity from bovine brain as described previously (Bazzi & Nelsestuen, 1987a). Protein concentration was determined by the method of Bradford (1976) with bovine serum albumin as a protein standard. Purified PKC was used in all of the experiments reported here.

Phospholipid Preparations. Small single-bilayer phospholipid vesicles were prepared by direct probe sonication of phospholipid suspensions, followed by gel filtration on a Sepharose 4B column (50 × 2.5 cm) as described by Huang (1969). The phospholipids were mixed at the desired composition, dried from the organic solvent under a stream of nitrogen, and then suspended in 40 mM HEPES buffer (pH 7.5). Phospholipid concentrations were determined by an organic phosphate assay (Chen et al., 1956) using a phospholipid/phosphorus weight ratio of 25.

Phospholipid–Triton mixed micelles were prepared as described by Hannun et al. (1985). In order to assure the quality of micelles containing more than 8 mol % phospholipid, micelles were subjected to brief direct probe sonication (Lichtenberg et al., 1983). In all experiments performed with micelles, the final concentration of Triton was 0.3% (w/w), and the phospholipid concentration was expressed as mole percentage of the Triton concentration.

Light Scattering Intensity Measurements. Light scattering intensity measurements were used to estimate membrane–protein binding as described previously (Bazzi & Nelsestuen, 1987b). Due to extensive aggregation of the phospholipid components by PKC substrates, light scattering intensity measurements were used only to qualitatively assess the binding of protein to phospholipid. In the case of substrate–phospholipid interaction, the dimensions of phospholipid–substrate aggregates exceed the wavelength of the light. In such cases, the light scattering intensity of the aggregates may increase or decrease depending upon particle size, shape, and scattering properties [for reviews see Tanford (1961) and Doty and Edsall (1951)].

Light scattering intensity was measured on a Perkin-Elmer spectrofluorometer (Model MPF 44A). The excitation and the emission wavelengths were set at 320 nm. The temperature was maintained at 25 °C.

Activity Measurements. PKC activity measurements were performed by using either phospholipid vesicles or phospholipid–Triton mixed micelles. The activity measurements in the presence of phospholipid vesicles were performed as described by Kikkawa et al. (1982). The assay mixture (0.25 mL) contained 40 mM HEPES (pH 7.5), 5 mM Mg^{2+}, 0.2 mM Ca^{2+}, 200 μg/mL histone, 15 μM ATP, ~25 ng of PKC, and 160 μg/mL phospholipid. Unless otherwise indicated, the phospholipid vesicle composition was 30% PS, 10% DAG, and 60% PC.

The activity measurements in the presence of phospholipid–Triton mixed micelles were performed as described by Hannun et al. (1985).

PDBu Binding. The binding of PDBu to PKC was measured essentially as described by Tanaka et al. (1986). The reaction mixture (0.25 mL) contained 40 mM HEPES (pH 7.5), 5 mM Mg^{2+}, 0.2 mM Ca^{2+}, 1 mg/mL BSA, 320 μg/mL phospholipid vesicles, 1.5 μg/mL PKC, and 0–60 nM [^{3}H]-PDBu (specific activity 15.8 Ci/mmol). Nonspecific binding of PDBu was determined by omitting PKC from the reaction mixture. After 30-min incubation, 100 μg of histone was added, and the reaction mixture was incubated for an additional 5 min. PKC was then collected and washed on glass fiber filters previously treated with poly(ethylenimine) as described by Tanaka et al. (1986). Counting efficiency on the glass fiber filters was not determined but was assumed to be similar for all experiments. Consequently, the amount of bound PDBu was only used for purposes of comparison.

Results

Phospholipid Selectivity of PKC in Phospholipid–Triton Mixed Micelles. PKC is known to display preference for PS over other acidic phospholipids such as PG or PI when the phospholipids are provided in the form of sonicated vesicles (Kaibuchi et al., 1981; Wise et al., 1982; Schatzman et al., 1983). PS–Triton mixed micelles were introduced by Hannun et al. (1985) as an alternative approach for examining the phospholipid requirements of PKC. The results shown in Figure 1 indicated that activation of PKC was highly specific for PS when phospholipids were provided in the form of phospholipid–Triton mixed micelles. This was true at different histone concentrations (Figure 1A), or at different phospholipid compositions (Figure 1B). In agreement with the reports of Hannun et al. (1985, 1986a), titration of PKC activity with PS–Triton mixed micelles indicated that a minimum of 4 mol % PS was needed for significant activation of PKC (Figure 1B).

Previously, we reported that many PKC substrates interacted with phospholipid in a calcium-independent manner and that this interaction was critical to activation of PKC in vesicles as well as in PS–Triton mixed micelles (Bazzi & Nelsestuen, 1987b). Consequently, the phospholipid specificity for interaction of the substrate, histone, with phospholipid–Triton mixed micelles was studied. The results shown in Figure 2A indicated that histone interacted with PS–Triton mixed micelles but not with PI- or PG–Triton mixed micelles. Thus, the high degree of selectivity for PS in the Triton-mixed micelles correlated with the specificity of substrate–micelle binding.

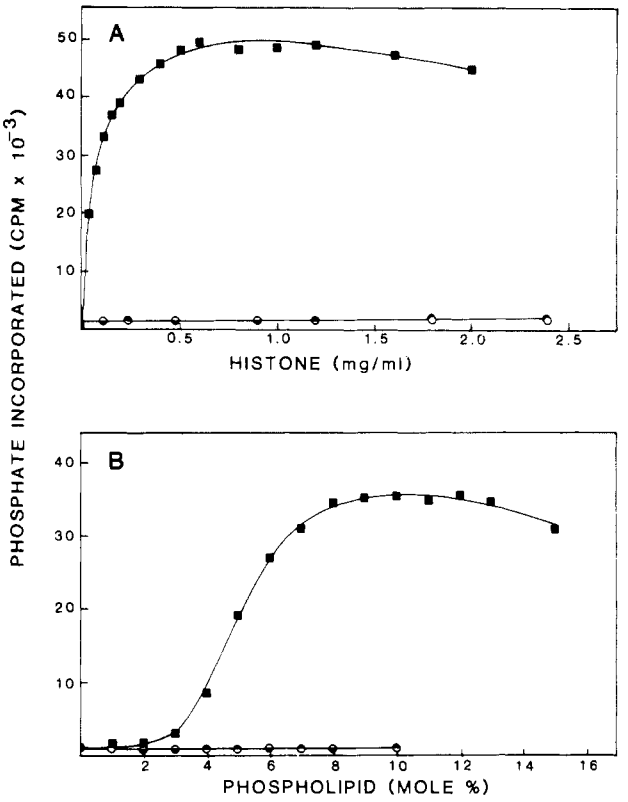

FIGURE 1: PKC activity in the presence of phospholipid–Triton mixed micelles. Panel A shows the activity of PKC as a function of histone concentration using micelles containing 10 mol % acidic phospholipid and 2.5 mol % DAG. Panel B shows the activity of PKC as a function of phospholipid concentration using 0.2 mg/mL histone. The micelles contained 2.5 mol % DAG and the indicated concentration of either PS (■), PI (●), or PG (○). The same symbols for acidic phospholipids applied for both panels.

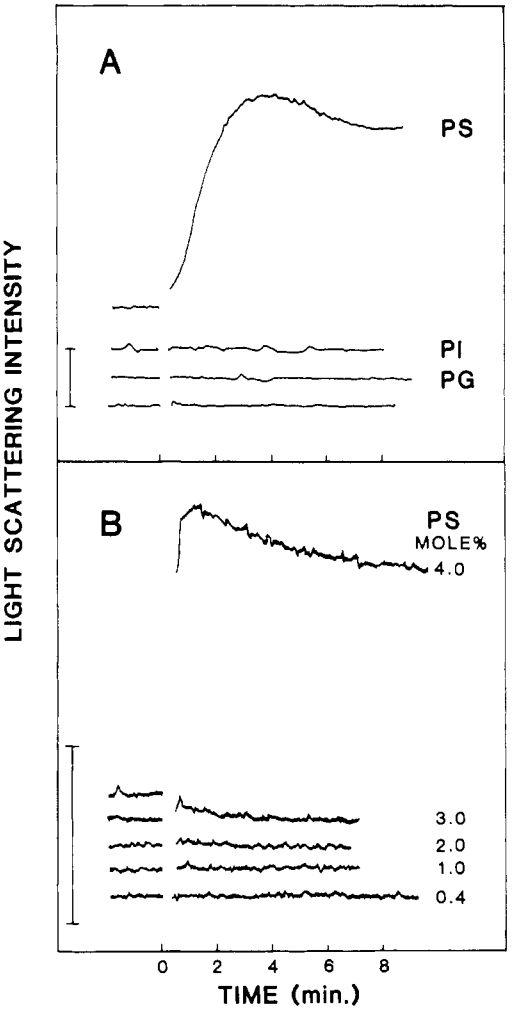

FIGURE 2: Interaction of histone with phospholipid–Triton mixed micelles. The interaction of histone with phospholipid–Triton mixed micelles was monitored by light scattering intensity measurements as a function of time. The measurements were performed in 1.6 mL of buffer (40 mM HEPES, pH 7.5). The light scattering intensity of the micelles was recorded, and 100 μg of histone was added at 0 time. Panel A shows the interaction of histone with micelles containing 2.5 mol % DAG either with 10 mol % of the indicated phospholipid or without any phospholipid (lower tracing). Panel B shows the interaction of histone with micelles containing 2.5 mol % DAG and the indicated concentration of PS. The tracings of various micelles in both panels were offset for clarity. The magnitude of light scattering intensity of either Triton micelles or mixed micelles containing PI or PG was the same and is given by the bar at the left of the figure. The light scattering intensity of PS–Triton mixed micelles was slightly larger than those of Triton alone. The final concentration of Triton was 0.3% (w/w).

The ability of histone to associate with PS–Triton mixed micelles was also examined as a function of the PS content of the micelles. The results (Figure 2B), obtained under the same conditions used for activity measurements, showed that no interaction occurred between histone and PS–Triton mixed micelles containing 0.4, 1.0, or 2.0 mol % PS. Slight interaction with micelles containing 3.0 mol % PS was evinced by a small change in the light scattering intensity when histone was added to the micelles. However, at 4 mol % PS, dramatic changes in the light scattering intensity occurred upon addition of histone, suggesting an aggregation event. Comparison of these results with the appearance of PKC activity (Figure 1B) showed a direct correlation between activity and substrate–phospholipid interaction. Therefore, the quantitative estimation of the phospholipid requirement of PKC activation with phospholipid–Triton mixed micelles appeared to represent substrate–phospholipid interaction.

The influence of the substrate–phospholipid interaction on PKC activation was examined further with PLS. This substrate typified substrates (designated category B substrates; Bazzi & Nelsestuen, 1987b) that required only phospholipid cofactor but not Ca^{2+} or diacylglycerol. Light scattering intensity measurements indicated that PLS interacted with and aggregated PS–Triton mixed micelles as well as those of PI and PG (Figure 3A). Consequently, the interpretations given above would predict that all of these acidic phospholipids should support PKC activity. Indeed, all three micelles supported the phosphorylation by PKC (Figure 3B). Furthermore, PKC activity was dependent on the concentration of PLS. At low concentrations, PI–Triton mixed micelles were actually more effective than micelles containing PG or PS. At higher substrate concentrations, preference for PS–Triton mixed micelles was observed. These measurements were performed in the presence of 2.0 mM EGTA which prevented direct interaction of PKC with micelles (Hannun et al., 1985). The different selectivities for phospholipid were therefore due to substrate–phospholipid interaction rather than to PKC–phospholipid interaction.

Examination of the phospholipid requirements of PLS phosphorylation revealed other aspects of PKC activation that were dependent on substrate concentration or micelle composition. At 80 μg of PLS/mL, comparable maximum rates of phosphorylation were obtained with phospholipid–Triton mixed micelles containing PI, PG, or PS (Figure 4A). However, the different micelles showed different optimum phospholipid concentrations. PS–Triton mixed micelles were most effective at 5–6 mol %, while PI–Triton mixed micelles

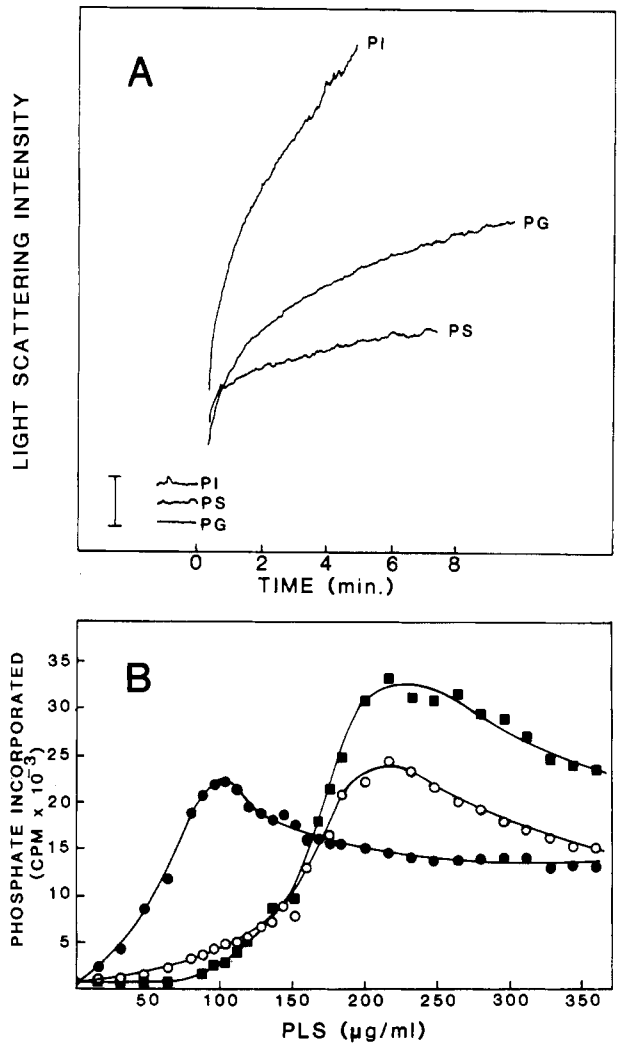

FIGURE 3: Interaction of PLS with phospholipid–Triton mixed micelles. Panel A shows the interaction of PLS with phospholipid–Triton mixed micelles as monitored by light scattering intensity. The light scattering intensity of the micelles was recorded, and 20 μg of PLS was added at 0 time. The phospholipid–Triton mixed micelles contained 2.5 mol % DAG and 10 mol % indicated phospholipid. Other experimental conditions were the same as in Figure 2. Panel B shows the phosphorylation of PLS by PKC in the presence of phospholipid–Triton mixed micelles. The activity measurements were performed in the presence of 2.0 mM EGTA by using phospholipid–Triton mixed micelles containing 2.5 mol % DAG and 10 mol % either PS (■), PI (●), or PG (○).

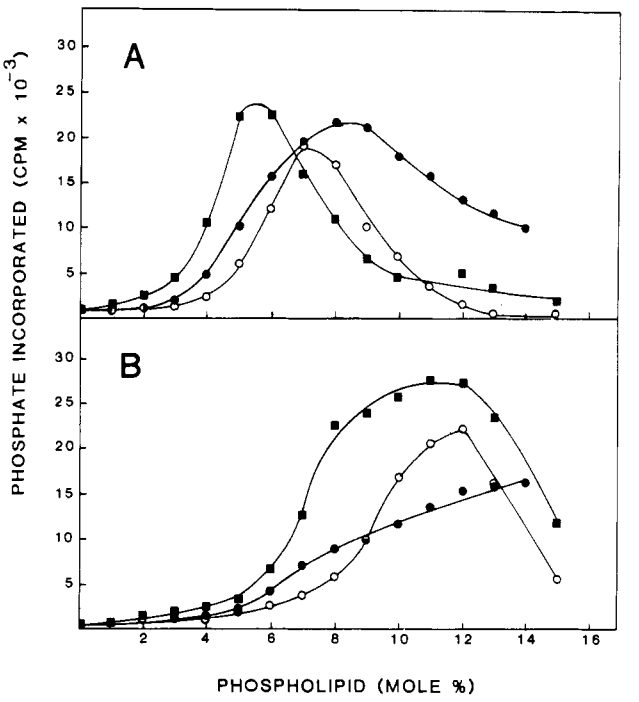

FIGURE 4: Phosphorylation of PLS by PKC in the presence of phospholipid–Triton mixed micelles. The activity of PKC was measured in the presence of 2.0 mM EGTA and either 80 (panel A) or 160 μg/mL PLS (panel B). Phospholipid–Triton mixed micelles contained 2.5 mol % DAG with either PS (■), PI (●), or PG (○).

were most effective at 8–9 mol %. Increasing the substrate concentration to 160 μg/mL resulted in apparent preference for PS–Triton mixed micelles over those containing PI or PG (Figure 4B). Also, the substrate concentration altered the minimum requirement for phospholipids. For example, the minimum requirement for PS was about 4 and 6 mol % at substrate concentrations of 80 and 160 μg of PLS/mL, respectively (compare panels A and B of Figure 4). Mixed micelles containing PI or PG showed similar behavior when substrate concentration was altered. It therefore appeared that, as in the case of histone, the phospholipid selectivity of PKC with PLS substrate was derived from the interaction of PLS with phospholipids and that selection of conditions could render almost any relative activity relationship.

A possible explanation for correlation of phospholipid selectivity with substrate concentration is that phosphorylation was dependent on the density of substrate in the phospholipid–substrate mixture. That is, there may be an optimum composition of substrate–phospholipid–Triton that gives maximum activity. Higher substrate concentrations require more phospholipid to achieve this density, and vice versa. The results in Figures 3 and 4 indicated that this was the case and that the optimum density was different for different acidic phospholipids. The order of optimum density of substrate on phospholipid–Triton mixed micelles, beginning with the one requiring the least phospholipid per mass of PLS, was PS > PG > PI.

There are differences between histone and PLS as substrates for PKC. Unlike PLS, the minimum PS content of PS–Triton mixed micelles required for PKC activation (about 4 mol %) was independent of the histone concentration in the range studied (0.2–2.0 mg/mL). However, to achieve the maximum rate of phosphorylation, the required PS content shifted from 8 mol % in the presence of 0.2 mg/mL histone (Figure 1B) to 14 mol % in the presence of 2.0 mg/mL histone (data not shown). In addition, PLS is a category B substrate which binds PKC directly whereas histone is a category C substrate which does not bind to PKC (Bazzi & Nelsestuen, 1987b). While both substrates showed a decline in their phosphorylation at high substrate concentration (Figures 1 and 4), PLS showed a sharper decline. This behavior is anticipated because PKC could bind to free PLS as the latter became in excess over the amount aggregated with phospholipid, thereby reducing the amount of enzyme in the substrate–phospholipid aggregate.

PKC Selectivity for Phospholipid Vesicles. The results shown above suggested that apparent selectivities could be varied by selection of experimental conditions. Further studies were conducted to determine if this behavior also extended to phospholipid vesicles. The composition of vesicles chosen for these comparisons consisted of 30% acidic phospholipid, 10% DAG, and 60% PC. The interaction of histone with these various phospholipid vesicles was examined by light scattering intensity measurements. The results presented in Figure 5A showed that histone interacted with and aggregated phospholipid vesicles containing PS, PG, and PI. While aggregation can only be detected in a qualitative sense (see Ex-

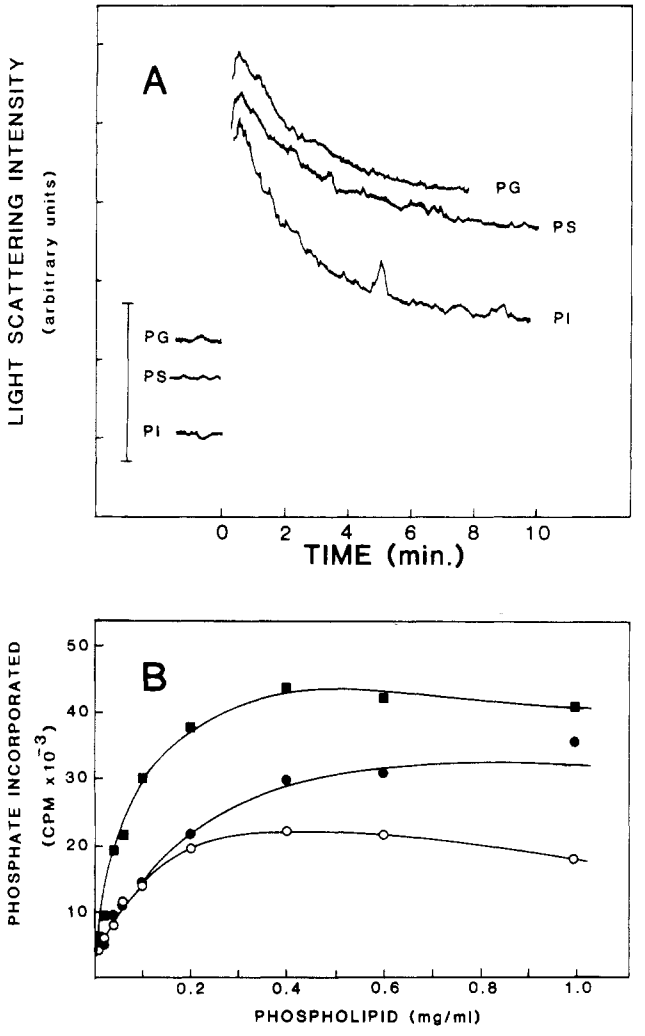

FIGURE 5: Interaction of histone with phospholipid vesicles. Panel A shows the interaction of histone with phospholipid vesicles as monitored by light scattering intensity. The phospholipid composition of the vesicles was 30% acidic phospholipid, 10% DAG, and 60% PC. The light scattering intensity of 40 μg of phospholipid vesicles was recorded, and 100 μg of histone was added at 0 time. The tracings of various vesicles were offset for clarity, but the magnitude of light scattering intensity of various vesicles was essentially the same and is given by the bar at the left of the panel. Panel B shows the phosphorylation of histone by PKC in the presence of phospholipid vesicles. The activity measurements were performed as described under Experimental Procedures. The phospholipid vesicles contained 10% DAG, 60% PC, and 30% either PS (■), PI (●), or PG (○).

perimental Procedures), it was nevertheless clear that interaction of histone with certain acidic phospholipids depended on whether the phospholipids were in vesicular or micellular form (compare Figure 5A with Figure 2A). In agreement with the general correlation between substrate–phospholipid interaction and PKC activity, all three types of acidic phospholipid vesicles supported the activity of PKC, although to somewhat different levels (Figure 5B).

In agreement with many previous reports (Kaibuchi et al., 1981; Wise et al., 1982; Schatzman et al., 1983), the results in Figure 5B suggested that PS was the most effective phospholipid in supporting the activity of PKC. However, this selectivity has been observed under conditions where PKC was not membrane bound (Bazzi & Nelsestuen, 1987b) and could arise from many sources. For example, selectivity could arise from DAG or phorbol ester binding. Kaibuchi et al. (1981) reported that in the presence of DAG and at low Ca^{2+} concentrations, PS was essential; raising the Ca^{2+} concentration rendered other acidic phospholipids effective in supporting

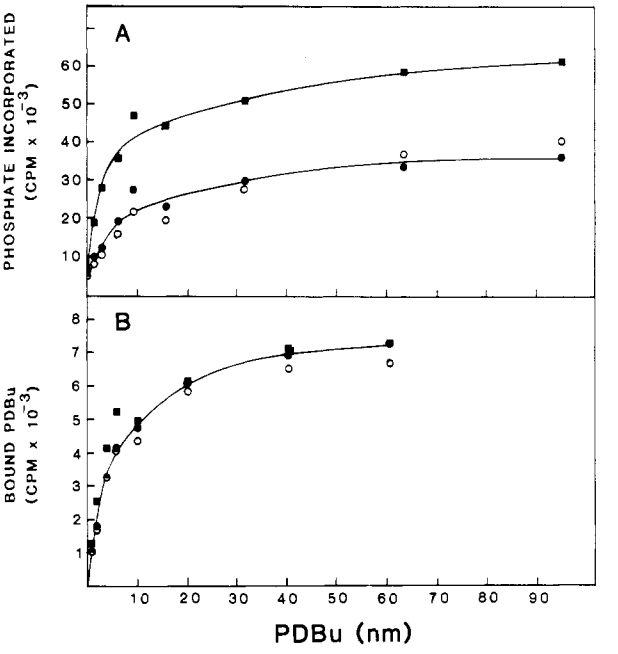

FIGURE 6: PDBu activation of, and binding to, PKC. Panel A shows the activity of PKC in the presence of various phospholipid vesicles. The measurements were performed in the presence of 0.2 mg/mL histone and 1 μM free Ca^{2+}. Panel B shows the binding of [^{3}H]PDBu to PKC in the presence of various phospholipid vesicles. These measurements were performed as described under Experimental Procedures. The same preparations of phospholipid vesicles were used in both measurements, and the composition of vesicles was 70% PC and 30% either PS (■), PI (●), or PG (○).

PKC activity. High levels of calcium are also known to eliminate the requirement for DAG. We therefore determined if PS was superior in supporting the binding of phorbol esters to PKC. The results (Figure 6A) indicated that, at 1 μM Ca^{2+}, phorbol dibutyrate (PDBu) stimulated the kinase activity in the presence of all three acidic phospholipid vesicles. PS was the most effective phospholipid, especially at low PDBu concentrations. The maximum binding of PDBu to PKC did not appear to be influenced by the phospholipid head group (Figure 6B), indicating that activation selectivity was not entirely due to selectivity in binding PDBu.

Phospholipid vesicles prepared by sonication are generally used in assays of PKC activity and PDBu binding. These are heterogeneous mixtures containing multilamellar and unilamellar vesicles of different sizes. Different vesicle structure and size have been reported to influence the activity of PKC (Boni & Rando, 1985). In addition, different phospholipids may have different distributions of acidic phospholipids on the inner and outer face of the vesicles. For example, the pH of the medium can influence the distribution of negatively charged phospholipids on the inner and outer surface of vesicles (Van Dijck et al., 1978; Berden et al., 1975).

In an attempt to minimize these variables, phospholipid vesicles (30% acidic phospholipid, 10% DAG, and 60% PC) were prepared by sonication and gel filtration as described under Experimental Procedures. Small unilamellar vesicles were collected and dialyzed against 40 mM HEPES (pH 7.5) to assure minimum pH differences among the preparations. The activation of PKC with these vesicles (Figure 7) indicated that vesicles containing PS, PG, or PI supported the activity of PKC to essentially the same level. This was especially true at relatively high concentrations of phospholipid (>100 μg/mL). At lower concentrations, PS was always more effective. Since the molar ratio of phospholipid to PKC required to elicit activity was always greater than 1000:1, it seemed unlikely

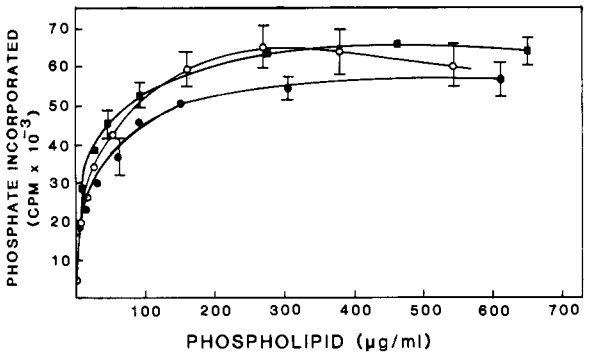

FIGURE 7: Activity of PKC in the presence of phospholipid vesicles. Phospholipid vesicles of defined size and composition were prepared as described in the text. The activity measurements were performed by using 0.2 mg/mL histone and 0.2 mM Ca^{2+}. The vesicle composition was 10% DAG, 60% PC, and 30% either PS (■), PI (●), or PG (○). The results showed the average and standard deviation of four measurements.

that the limiting factor was the availability of phospholipid for PKC binding. At low phospholipid concentrations, the order of phospholipid effectiveness shown in Figure 7 (PS > PG > PI) was the same order observed with phospholipid–Triton mixed micelles (see above and Figure 4). On the basis of these several considerations, it seemed reasonable to conclude that the condition-dependent selectivity of PKC activation for PS was the result of substrate–phospholipid interaction, and this selectivity was minimized by standardization of the physical properties of the phospholipid preparations.

Discussion

Understanding the mechanism of generating PKC activity in vitro can be important to elucidation of the function of this enzyme in vivo. Previous studies suggested a key role for substrate–enzyme interaction in determining cofactor requirements (Bazzi & Nelsestuen, 1987b). The present study furthered these findings and indicated that substrate–phospholipid interaction correlated with maximum PKC activity and appeared to account for phospholipid specificity and composition requirements. For example, when Triton–phospholipid mixed micelles were used, phosphorylation of histone or PLS always correlated with interaction of the substrates with the micelles (compare Figure 2A with Figure 1; compare also panels A and B of Figure 3). There was also direct correlation between the minimum PS composition (4 mol % in Triton) required for histone phosphorylation and that required to induce significant interaction between histone and micelles (compare Figure 2B and Figure 1B). The substrate PLS displayed selectivity for phospholipids and was phosphorylated in the presence of 2.0 mM EGTA, so that direct PKC–phospholipid binding did not occur. Therefore, the phospholipid specificity observed in micelles as well as the minimum phospholipid composition requirements reflected substrate–phospholipid interaction. Phospholipid requirements and selectivity in micelles have previously been observed but have been interpreted as enzyme–phospholipid–DAG interaction requirements (Hannun et al., 1985, 1986a; Ganong et al., 1985).

When phospholipids were provided in the form of vesicles, PKC–vesicle binding did not display significant selectivity for PS (Bazzi & Nelsestuen, 1987a). The maximum binding of PDBu to PKC did not appear to be influenced by the phospholipid head group, either (Figure 6B). Thus, it is unlikely that the phospholipid-binding characteristics of PKC were responsible for the phospholipid selectivity in this case. The results suggested that the composition of the substrate–phospholipid aggregates was a critical factor for PKC activity and that the optimum substrate to phospholipid ratio varied with the head group of the acidic phospholipid. The latter property applied to PLS or histone substrates with phospholipid–Triton mixed micelles (Figures 4 and 2) as well as to histone with phospholipid vesicles (Figure 7). Since substrate–phospholipid associations invariably involved aggregation, the actual composition and the physical states of the phospholipids in the active complex cannot be quantitatively ascertained for either vesicles or micelles.

Substrate–phospholipid interaction may prove to be a critical factor in determining apparent agonists or antagonists of PKC. For example, using the substrate histone with phospholipid vesicles containing either PG or PI, Triton X-100 would be classified as a potent inhibitor of PKC (compare Figure 1 with Figure 7). Conversely, Triton X-100 could be considered an activator of PKC if phospholipid vesicles containing PI were used with low concentrations of PLS (compare PS with PI in Figure 3B). Again, substrate–phospholipid interactions would be the dominant factor in these properties. Substrate interactions may also explain inhibition of PKC by sphingosine (Hannun et al., 1986b), lysosphingolipids (Hannun & Bell, 1987), and gangliosides (Kreutter et al., 1987). The large amounts of these materials required for inhibition approach or exceed the acidic phospholipid composition [e.g., Hannun et al., (1986b)]. While ultimate interference with PKC–membrane binding seems probable, interference with substrate–phospholipid binding may be the actual source of inhibition. Consequently, observed inhibitions may vary with the choice of substrate.

The general properties of PKC and its substrate selectivity may provide suggestions regarding physiological roles of PKC. This enzyme phosphorylates many proteins in vitro (Nishizuka, 1986b), but only a few physiological substrates have been identified. Most physiological substrates are either membrane proteins or membrane-associated proteins (Woodgett et al., 1986). An interesting example is protein kinase Pr 60^{src}, a product of the transforming gene of Rous sarcoma virus (Hunter & Cooper, 1985). Pr 60^{src} is usually a membrane-associated protein and a physiological substrate of PKC. Non-myristylated Pr 60^{src}, a mutant form of the wild-type Pr 60^{src} protein that does not associate with membrane, is not a substrate for PKC (Buss et al., 1986). While membrane–protein interaction under in vivo conditions is considerably different from phospholipid aggregation by histone, the above-cited observations are in agreement with the general conclusion that substrate–phospholipid interaction is essential for phosphorylation by PKC.

However, the criteria used in identification of physiological substrates of PKC may result in identification of a subpopulation of substrates. Sensitivity to Ca^{2+} and/or phorbol esters, the usual method of identifying PKC substrates, emphasizes only one type of in vitro substrate. It is possible that a different category of substrates is phosphorylated by PKC in the cytosol. Substrates such as PLS and myelin basic protein bind PKC and do not require calcium or DAG (Bazzi & Nelsestuen, 1987b). Physiological substrates of this type have not been sought but remain a possibility. As possible examples, Wolf and Sahyoun (1986) reported that PKC binds to two polypeptides from erythrocyte membranes and brain synaptosome preparations. These polypeptides would be potential candidates for Ca^{2+}- and DAG-independent phosphorylation by PKC.

Recently, several studies have reported the sequence specificity of PKC substrates (Ferrari et al., 1985; Turner et al., 1985; Woodgett et al., 1986; House et al., 1987). These studies

indicate that PKC requires the presence of positively charged residues near the phosphorylation site, with reported preference for either the C-terminal (Ferrari et al., 1985; Woodgett et al., 1986), the N-terminal (Turner et al., 1985), or either terminal of the peptides (House et al., 1987). An interesting observation is that the incorporation of additional positively charged residues on either side of the peptide resulted in formation of a better substrate as judged by apparent "K_m" and "V_{max}" values for the reactions (Woodgett et al., 1986; House et al., 1987). These properties are consistent with the results presented here since the interaction of peptide with anionic membrane and subsequent aggregation should be strongly correlated with its cation charge density. Preliminary results[2] have suggested that the peptide YSRRRRRRG is not dependent on DAG or Ca^{2+} for phosphorylation by PKC. The charge density of the peptide may also alter interaction with PKC, thereby altering the cofactor requirements of phosphorylation. Peptides with lower charge density should have decreased interaction with PKC and may provide substrates requiring calcium and DAG. While further studies are needed to test this possibility and other properties of the PKC reaction, the results reported here showed that the substrate had a great influence on the properties of PKC.

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[2] M. D. Bazzi and G. L. Nelsestuen, unpublished data.