Association of Protein Kinase C with Phospholipid Vesicles†

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ABSTRACT: The Ca^{2+}- and phospholipid-dependent protein kinase, protein kinase C (PKC), was purified from bovine brain by a modified procedure that provided sufficient quantities of stable protein for analysis of physical properties of protein–membrane binding. The binding of PKC to phospholipid vesicles of various compositions was investigated by light-scattering and fluorescence energy transfer measurements. The binding properties for membranes of low phosphatidylserine (PS) content were consistent with a peripheral membrane association; PKC showed Ca^{2+}-dependent binding to phospholipid vesicles containing phosphatidylserine, phosphatidylinositol, or phosphatidylglycerol. Membranes containing 0–20% PS (the remainder of the phospholipid was phosphatidylcholine) bound less protein than membranes containing greater than 20% PS; the factor limiting protein binding to membranes containing low PS appeared to be the availability of acidic phospholipids. Increasing the PS content above 20% did not increase the amount of membrane-bound protein at saturation, and the limiting factor was probably steric packing of protein on the membrane surface. The membranes bound about 1 g of protein/g of phospholipid at steric saturation. Binding was of relatively high affinity (K_d < 5 nM), and the association rate was rapid on the time scale of the experiments. Addition of ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid to phospholipid-bound PKC caused dissociation of the complex, and the properties of this dissociation indicated an equilibrium binding of protein to membrane. However, only partial dissociation of PKC was achieved when the PS content of the vesicles exceeded 20%. A number of comparisons revealed that binding of protein to the membrane, even in the presence of phorbol esters, was insufficient for development of enzyme activity. For example, PS was much more effective in promoting activity than the other acidic phospholipids but was not greatly superior in protein binding. Diolein (DAG) and phorbol esters, potent activators of PKC activity, had little effect on the binding of the enzyme to phospholipid vesicles or on the Ca^{2+} requirement of binding. Binding required only equal weight concentrations of phospholipid and protein while activity always required a large excess of phospholipid. These studies suggest that factors other than formation of a PKC–PS–Ca^{2+}–DAG complex are critical to development of enzyme activity.

Phospholipid- and Ca^{2+}-dependent protein kinase C (PKC)[1] is involved in phosphorylation of many proteins in the cell (Kuo et al., 1984), and possibly in regulation of many cell functions (Nishizuka, 1984a; Takai et al., 1984). This enzyme, described by Takai et al. (1977), is widely distributed in various tissues (Ashendel et al., 1983; Minakuchi et al., 1981; Kuo et al., 1980). Diacylglycerol, generated in vivo by the turnover of inositol phospholipids (Berridge, 1984; Nishizuka, 1984b), is known to reduce the Ca^{2+}-requirement of the enzyme to physiological concentrations (Takai et al., 1979f Kishimoto et al., 1980). Phorbol esters have a similar effect on PKC (Ashendel, 1985). Extensive efforts have been directed toward characterization of the enzyme, including the phospholipid and Ca^{2+}-requirement, and the substrate specificity of the enzyme (Kuo et al., 1984; Takai et al., 1984).

The mechanism of enzyme binding to membranes and its activation by phospholipids and Ca^{2+} are not well understood. Many previous studies have concentrated on activity measurements. The development of activity when titrated with Ca^{2+} or phospholipid has often been interpreted as a simple equilibrium constant (K_a). This assumes that the activation of PKC is a simple equilibrium process and that binding correlates with activity. However, full understanding of the PKC mechanism requires knowledge of the physical entity, or species, which generates kinase activity. To describe this species, it is necessary to characterize the various physical interactions related to this enzyme and its cofactors. These include enzyme–membrane binding, substrate–membrane binding, diacylglycerol–enzyme interaction, and calcium binding. Properties of the physical associations can then be related to the appearance of enzyme activity to determine the total requirements of activity. This protein also provides an example of a membrane-associating protein, and characterization of its membrane binding properties will contribute to understanding of protein–membrane interactions in general.

Biophysical techniques, such as light-scattering intensity and fluorescence energy transfer, have been shown to be excellent tools for studying certain peripheral protein–phospholipid interactions [e.g., see Silversmith & Nelsestuen (1986), Griep et al. (1985), Pusey et al. (1982), and Nelsestuen & Lim (1977)]. Using these methods, we studied the binding of PKC to phospholipid vesicles, and the Ca^{2+} and DAG requirements of binding. PKC bound to membranes in a manner typical of several other peripheral phospholipid binding proteins and

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[1] Abbreviations: PKC, protein kinase C; PS, phosphatidylserine (bovine brain); PI, phosphatidylinositol; PG, phosphatidylglycerol (egg yolk); dansyl-PE, dipalmitoyl-*N*-dansyl-L-α-phosphatidylethanolamine; PC, phosphatidylcholine (egg yolk); DAG, diolein; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); PDBu, phorbol-12,13-dibutyrate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin.

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displayed relatively high affinity. The phospholipid, Ca^{2+}, and DAG requirements of PKC activity were strongly interrelated. In contrast, binding of PKC to the membranes did not appear to be influenced by DAG and occurred under conditions where kinase activity was not displayed. Development of activity appeared to be dependent on factors other than simple formation of a protein–membrane complex.

Experimental Procedures

Materials. Unless indicated, all the chemicals and the reagents were from Sigma Chemical Co. and were of the highest grade available. Sepharose 4-B and Sephacryl S-300 are trade names of Pharmacia Fine Chemicals and were obtained through Sigma Chemical Co. DE52 (DEAE-cellulose) and glass fiber filters were from Whatman. [γ-^{32}P]ATP (3 Ci/mmol) was from Amersham Corp.

Preparation of Phospholipids. Phospholipid vesicles were prepared by a procedure similar to that of Huang (1969) [also see Nelsestuen & Lim (1977)]. The phospholipids (>98% pure, manufacturer's assay) were mixed at the desired composition and dried from the organic solvent under a stream of argon. They were then suspended in 20 mM Tris buffer (pH 7.5, containing 100 mM NaCl) and subjected to brief direct probe sonication (30–60 s). The suspension was applied to a Sepharose 4-B column (30 × 1.5 cm) equilibrated with the same buffer. Small single-bilayer phospholipid vesicles obtained from the column elution profile were used for light-scattering intensity and fluorescence energy transfer measurements. For PKC activity measurements, the phospholipids were suspended in a buffer containing 20 mM Tris (pH 7.5) and were used without gel filtration. The membrane compositions are expressed as the percentage of PS, PI, PG, or DAG in the membrane. The remainder of the lipid in every case was PC.

PKC Assay. PKC was routinely assayed by measuring the incorporation of ^{32}P into histone. The assay conditions were similar to those reported by Kikkawa et al. (1982).

PKC Binding. The binding of PKC to the phospholipid vesicles was measured by light-scattering intensity measurement and by fluorescence energy transfer.

Light-scattering intensity measurements were performed as described by Nelsestuen and Lim (1977). This method applied to systems such as this where dilute solutions of nonaggregating particles that are small compared with the wavelength of the light are used. Briefly, the phospholipid vesicles and PKC were mixed in 1.5 mL of a buffer containing 20 mM Tris, pH 7.5, and 100 mM NaCl. The increase in the light-scattering intensity as a function of Ca^{2+} addition was attributable to PKC–phospholipid binding. Similarly, the decrease of the light-scattering intensity as a function of EGTA addition was the result of PKC–phospholipid dissociation. The data are reported as the relative molecular weight, M_2/M_1, where M_2 is the molecular weight of the protein–lipid complex and M_1 is the molecular weight of the lipid only. M_2/M_1 is related to I_2/I_1 by the general relationship:

$$I_2/I_1 = (M_2/M_1)^2[(\partial n/\partial c_1)/(\partial n/\partial c_2)]^2$$

where I_2 is the light-scattering intensity of the protein–lipid complex, I_1 is the light-scattering intensity of the phospholipid, and $\partial n/\partial c$ is the refractive index increment of each species (Nelsestuen & Lim, 1977). In computing M_2/M_1, I_2/I_1 was corrected for dilution and light scattering from free protein. The latter was usually insignificant. Light-scattering intensity of the phospholipid alone was not altered by the addition of Ca^{2+} or EGTA at the concentrations employed. The measurements were performed on a Perkin-Elmer spectrofluorometer (Model MPF 44 A) with excitation and emission wavelengths set at 320 nm. The temperature was maintained at 25 °C.

Fluorescence energy transfer was also used to measure protein–membrane binding. The phospholipid vesicles contained 10% dansyl-PE. The excitation and the emission wavelengths were 284 and 500 nm, respectively. A 340-nm cutoff filter was placed in front of the emission monochromator. Fluorescence energy transfer is expressed as a percentage change in emission intensity, $100(I - I_0)/I_0$, or as a percentage of the maximum change observed ($100\Delta I/\Delta I_{max}$). In these equations, I is the fluorescence intensity of the protein–lipid complex, and I_0 is the intensity of the phospholipid alone. Excitation at 284 nm caused minor direct excitation of the dansyl moiety (I_0). This intrinsic intensity provided an internal standard that allowed comparison of energy transfer in different experiments, and the energy transfer could be expressed relative to this internal standard.

PKC Purification. The following purification procedure has been applied to preparations starting with two, three, or four bovine brains. Thus, when applicable, the reagents used are reported per bovine brain used (about 500 g of tissue). All manipulations were performed at 4 °C.

Fresh brains were obtained from a local abattoir and chilled immediately by placing on ice. The brains were first washed with tap water and then homogenized in the extraction buffer (20 mM Tris, pH 7.9, containing 0.3 M sucrose, 10 mM EGTA, 5 mM EDTA, and about 2 L of the buffer/bovine brain) using a Waring blender. About 30 s of homogenization was adequate. The homogenate was centrifuged at 13000g for 2 h, and the supernatant was saved.

The supernatant was mixed with DEAE-cellulose (DE52) using about 1 L of packed bead resin per bovine brain. The DEAE-cellulose beads were previously equilibrated with the standard buffer (20 mM Tris, pH 7.9, containing 1 mM EGTA, 1 mM EDTA, and 30 mM β-mercaptoethanol). The mixture was allowed to stand for 2 h with occasional stirring. The resin was collected in a large Büchner funnel and washed with the same buffer (about 4 L/L of packed resin). The resin was then suspended in the same buffer and packed into columns (40 × 6 cm). Each column was washed with an additional 4 L of the standard buffer. The enzyme was eluted by a 4-L gradient of 0–0.3 M NaCl in the standard buffer. The fractions containing phospholipid-dependent kinase activity (Figure 1A) were pooled (about 600–800 mL/column) and dialyzed against 10 L of the standard buffer (two changes).

The following step was performed essentially as described by Parker et al. (1984). The dialyzed active fractions from the previous step were made 10% (v/v) with glycerol and loaded onto a DEAE-cellulose column (40 × 3 cm) previously equilibrated with a buffer containing 20 mM HEPES, 30 mM β-mercaptoethanol, 1 mM EGTA, 1 mM EDTA, and 10% glycerol, pH 7.5. The column was extensively washed with the same buffer and eluted isocratically with a buffer containing 20 mM PIPES, 30 mM β-mercaptoethanol, 1 mM EGTA, 1 mM EDTA, and 10% glycerol, pH 6.5. The active fractions (Figure 1B) were pooled and concentrated to about 20 mL by pressure dialysis in an ultrafiltration cell (Amicon). The concentrated sample was centrifuged at 48000g for 20 min, and the precipitate was discarded.

Phospholipid vesicles composed of 20% PS, 10% diolein, and 70% PC were prepared by sonication and filtration on Sepharose 4-B as described above. Only the vesicles that eluted

in the void volume of the column were used for purification. The amount of phospholipid required for quantitative binding of PKC depended upon the protein concentration and the nature of the contaminating proteins. However, 0.4 mg of phospholipid/mg of total protein was sufficient to bind PKC in most cases. The phospholipid vesicles were mixed with the concentrated sample of PKC, leupeptin was added to a final concentration of 10 μg/mL, and Ca^{2+} was added to a final concentration of 1 mM. The mixture was applied to a Sepharose 4-B column (80 × 4.5 cm), previously equilibrated with 20 mM Tris buffer containing 30 mM β-mercaptoethanol, 100 mM NaCl, and 1 mM $CaCl_2$, pH 7.9. The enzyme eluted with the phospholipid vesicles in the void volume of the column (Figure 1C). The active fractions were pooled, made 10 mM with respect to EGTA, and concentrated to about 10 mL by pressure dialysis.

The concentrated sample was applied to a Sephacryl S300 column (120 × 2.5 cm) equilibrated with the standard buffer and eluted with the same buffer. Some (15–40%) of the kinase activity usually remained bound to the phospholipid vesicles, while the free PKC eluted near the position expected for a protein of 80000 molecular weight (Figure 1D). The fractions containing the free PKC were collected, concentrated by pressure dialysis, and made 50% with respect to glycerol. The enzyme was pure by the criterion of gel electrophoresis in SDS (Figure 1D, inset) and could be stored at −20 °C for at least 1 month with no detectable loss of activity. The specific activities of various PKC preparations were 2800–3500 nmol of phosphate transferred min^{-1} (mg of protein)$^{-1}$ under the standard assay conditions.

Protein concentrations were determined by the method of Bradford (1976) using BSA as the standard. SDS gel electrophoresis was performed as described by Laemmli (1970). Protein bands were visualized by the silver staining method (Merril et al., 1981). Phospholipid concentrations were determined from the total organic phosphate by the method of Chen et al. (1956) using a phosphorus to phospholipid weight ratio of 1:25. Dansyl-PE was prepared as described by Pusey et al. (1982).

The apparent free Ca^{2+} concentration in solutions containing Ca^{2+} chelators was calculated as described by Storer and Cornish-Bowden (1976). The K_a values for various complexes involving Ca^{2+}, Mg^{2+}, EGTA, EDTA, and ATP were taken from Fabiato and Fabiato (1979).

Results

Purification of PKC. A modified procedure for PKC purification was developed in order to provide sufficient quantities of PKC to perform biophysical analysis of protein–membrane binding. The purification procedure described here has consistently produced an apparently homogeneous preparation of PKC. A critical step, in terms of enzyme yield, was the DEAE-cellulose (pH 6.5) step (Figure 1B), which gave yields ranging from 20% to 50% of the kinase activity applied to the column. This step was necessary to separate PKC from other contaminant proteins and was similar to a purification step described by Parker et al. (1984). After this step, the preparation contained contaminants, presumed to be lipid in nature, which were removed by high-speed centrifugation. The final yield of PKC ranged from 50 to 200 μg of PKC per bovine brain (seven preparations to date).

Occasionally, it was essential to remove final traces of phospholipid from the protein preparation by inserting an additional gel filtration step before the Sephacryl S-300 chromatography (Figure 1D). This column consisted of a 2.5 × 50 cm Sepharose 4-B column eluted with 20 mM Tris, 1 mM EGTA, 1 mM EDTA, 50 mM NaCl, and 30 mM β-mercaptoethanol, pH 7.9. The vesicles eluted near the exclusion volume of this column, and the free PKC eluted near the inclusion volume. The fractions containing PKC were concentrated by pressure dialysis and applied to the Sephacryl

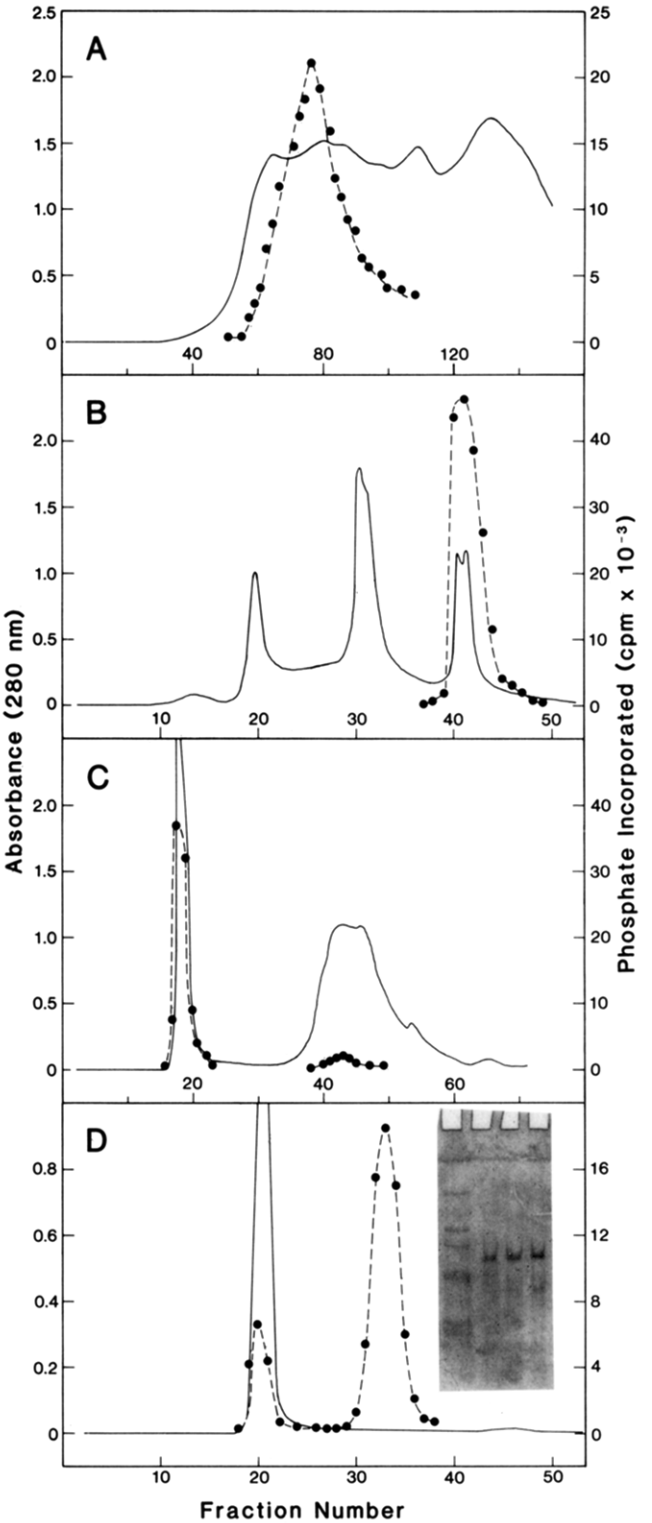

FIGURE 1: PKC purification steps. In each panel, the absorbance (—) and the kinase activity (---) are shown. Each column fraction was 25 mL except in panel D where each fraction was 8.5 mL. (A) DEAE-cellulose chromatography with gradient elution; (B) DEAE-cellulose (pH 6.5) chromatography; (C) Sepharose 4-B gel filtration chromatography; (D) Sephacryl S-300 gel filtration chromatography. The inset shows SDS gel electrophoresis of three different preparations of purified PKC. The molecular weights of the standards shown are 200000, 116000, 92500, 66000, and 45000.

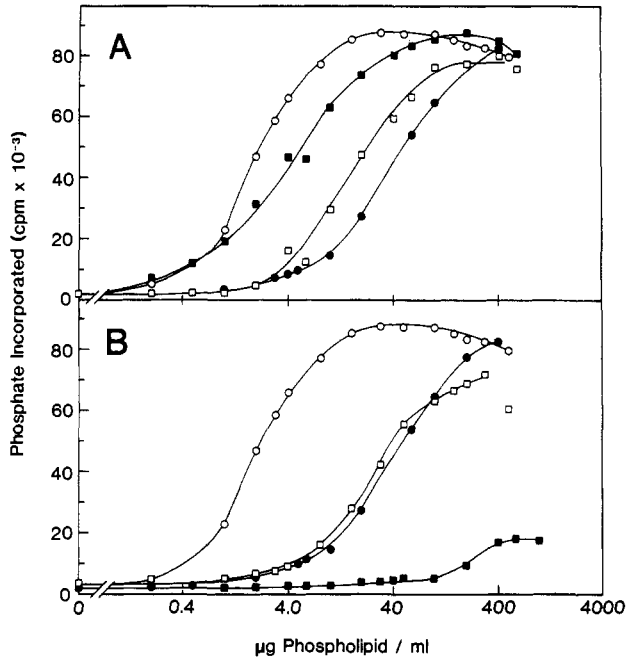

FIGURE 2: Interrelationship of Ca^{2+} and phospholipid requirements for PKC activation. Panel A shows the effect of Ca^{2+} on the phospholipid requirement. Phospholipid vesicles composed of PS–DAG–PC (30:10:60) were used. The Ca^{2+} concentration was (●) 5 μM, (□) 20 μM, (■) 100 μM, and (○) 300 μM. Panel B shows the effect of DAG. Phospholipid vesicles (PS–PC, 30:70) were used in the presence of 5 (■) or 600 μM Ca^{2+} (□). The activity was measured with phospholipids containing DAG in the presence of 5 (●) and 300 μM Ca^{2+} (○), respectively. The latter data are reploted from Figure 2A for reference.

S-300 column shown in Figure 1D.

Activity Requirements. This protein was similar to other reported preparations of PKC in that phospholipid, calcium and DAG greatly enhanced activity (Figure 2). In addition, there was a strong synergism between the three components evidenced by lower requirement for phospholipid if calcium was present at higher levels. For example, half-maximal activity was obtained at 1.8–58 μg of phospholipid/mL when the Ca^{2+} concentration was varied from 300 to 5 μM (Figure 2A). Plotting these data according to the Hill equation gave Hill coefficients of about 1 (range from 0.95 to 1.02). However, this treatment of the data may not be valid since development of activity did not appear to be a simple equilibrium binding process (see below) and these plots are not shown.

DAG was an important activator of this enzyme as shown by the results in Figure 2B. The presence of DAG reduced the phospholipid requirement by about 20-fold at a constant Ca^{2+} concentration (600 μM). At the lower Ca^{2+} concentration used in Figure 2B, DAG was essential for expression of maximum activity. However, at higher Ca^{2+} concentrations, the same maximum activity was obtained in the presence or absence of DAG. DAG was therefore not absolutely essential for activity. Experiments at 600 μM Ca^{2+} and in the absence of DAG showed the same phospholipid requirements as experiments at 5 μM calcium but in the presence of DAG. Clearly, the three components, phospholipid, Ca^{2+}, and DAG, showed strong interactive characteristics where an increase of one component greatly decreased the requirement for the other components. Similar effects of DAG on the Ca^{2+} and phospholipid requirements of PKC have been reported by others (Kaibuchi et al., 1982; Wise et al., 1982; Kishimoto et al., 1980; Takai et al., 1979). The PKC produced by this purification procedure therefore appeared similar to several other preparations (Parker et al., 1984; Uchida & Filburn, 1984; Kikkawa et al., 1983a). The molecular weight of the protein was 80 000 (Figure 1D) which also agreed with that of other PKC preparations.

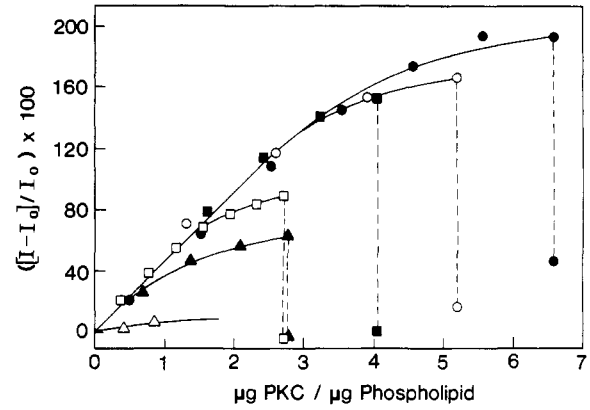

FIGURE 3: PKC binding to phospholipid vesicles as a function of PS content. All the phospholipid vesicles contained 10% DAG and 10% dansyl-PE. The PS content was (△) 0%, (▲) 5%, (□) 10%, (■) 15%, (○) 20%, and (●) 30%. The remaining lipid was PC. The dashed lines indicate the addition of EGTA.

Membrane Binding Characteristics of PKC. Many studies have investigated the requirements for PKC activity. However, direct observation of protein–lipid binding has not been reported for purified systems. In the cell, PKC is distributed between soluble and membrane-bound fractions. The relationship between binding and appearance of activity is not known.

Figure 3 shows results of fluorescence energy transfer from tryptophan residues in the protein to dansyl-PE in the membrane. Energy transfer is dependent on close proximity of the donor and acceptor molecules and will only occur when the protein is bound to the membrane. Light of 280-nm wavelength will directly excite the dansyl moiety to a small extent, and an emission intensity was observed in the absence of protein. This intrinsic intensity of the dansyl moiety (I_0 in Figure 3) served as a useful internal reference when comparing results from different experiments. As shown in Figure 3, dansyl-PE dispersed in PC did not show significant energy transfer from protein added to the solution. Acidic phospholipids were essential for PKC–membrane binding. Membranes containing PS showed fluorescence due to energy transfer from tryptophan residues, and the maximum fluorescence energy transfer was approximately proportional to the PS content for 5%, 10%, and 15% PS. It appeared likely that the factor limiting maximum binding of protein to these membranes was the availability of acidic phospholipids. This general conclusion was also supported by the fact that a greater amount of protein (abscissa in Figure 3) was needed to saturate the signal for lipids containing more PS. Above 20% PS, the maximum intensity due to energy transfer was approximately constant, and it appeared that the factor limiting protein binding was steric packing of proteins on the membrane surface.

Addition of EGTA caused dissociation of the PKC–membrane complex, but total reversibility was only observed for membranes containing less than 20% PS. For example, upon EGTA addition, the emission intensity from membranes of 30% PS did not return to that of the phospholipid alone. Similar lack of total reversibility was observed with the light-scattering intensity measurements (see below).

Binding occurred with a variety of acidic phospholipids as shown by the results in Figure 4. PG and PI were nearly as efficient in binding PKC as was PS; protein–membrane

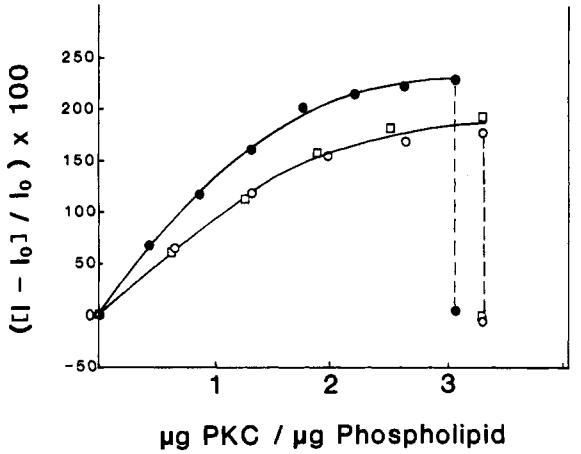

FIGURE 4: PKC binding to vesicles of various acidic phospholipid compositions. The binding was measured by fluorescence energy transfer in 1.6 mL of a medium containing 1.0 mM Ca^{2+}. All the phospholipid vesicles contained DAG–dansyl-PE–PC–X (10:10:60:20), where X is PS (●, 3.7 μg), PG (□, 2.6 μg), or PI (○, 3.1 μg). The dashed line shows the effect of 1.0 mM EGTA.

binding appeared to have little selectivity for other structures in the acidic phospholipids. This result was interesting since PI and PG were not very effective in promoting the activity of PKC (Kaibuchi et al., 1981; Wise et al., 1982; Schatzman et al., 1983). In our studies, at similar concentrations, phospholipid vesicles containing 30% PG or PI produced only 5% or 28%, respectively, of the PKC activity obtained with membranes of 30% PS. There appeared to be no simple and direct correlation between protein–membrane binding and the appearance of activity.

Effect of DAG on PKC–Membrane Binding. Figure 5A shows the binding of PKC to membranes monitored by either light scattering or fluorescence energy transfer. Light scattering is dependent on the mass of protein bound to the membrane while energy transfer is dependent on a number of parameters such as the molar concentration of the donor–acceptor pair, their distance, spectral overlaps, quantum yields, and orientation. Both methods of monitoring membrane binding showed similar titrations with half-maximal binding at 10–40 μM Ca^{2+}. A very interesting observation was that the binding event seemed to be influenced to a small or negligible degree by the presence of DAG. This contrasted dramatically with the appearance of activity which, for these membranes, was totally dependent on the presence of DAG (Figure 5A). Once again, the binding event was clearly distinct from the appearance of activity. Activity required much higher Ca^{2+} concentrations than did binding.

Decreasing the Ca^{2+} concentration in the medium by successive additions of EGTA caused dissociation of PKC from the vesicles (Figure 5B). The Ca^{2+} concentration at half-dissociation (Figure 5B) was about the same as that at half-association (Figure 5A). This property suggested that membrane binding was an equilibrium process. As observed with the association measurement, the presence of DAG appeared to have little influence on the Ca^{2+} requirement during dissociation.

From knowledge of the concentrations of all the added materials, the absolute free calcium concentration can be calculated by the methods of Storer and Cornish-Bowden (1976). Binding and activity calculated by this approach are shown in Figure 5C. It is clear that the midpoint for the appearance of activity occurred at higher Ca^{2+} than the midpoint for binding. The difference is actually greater than suggested in Figure 5 since the activity measurements were

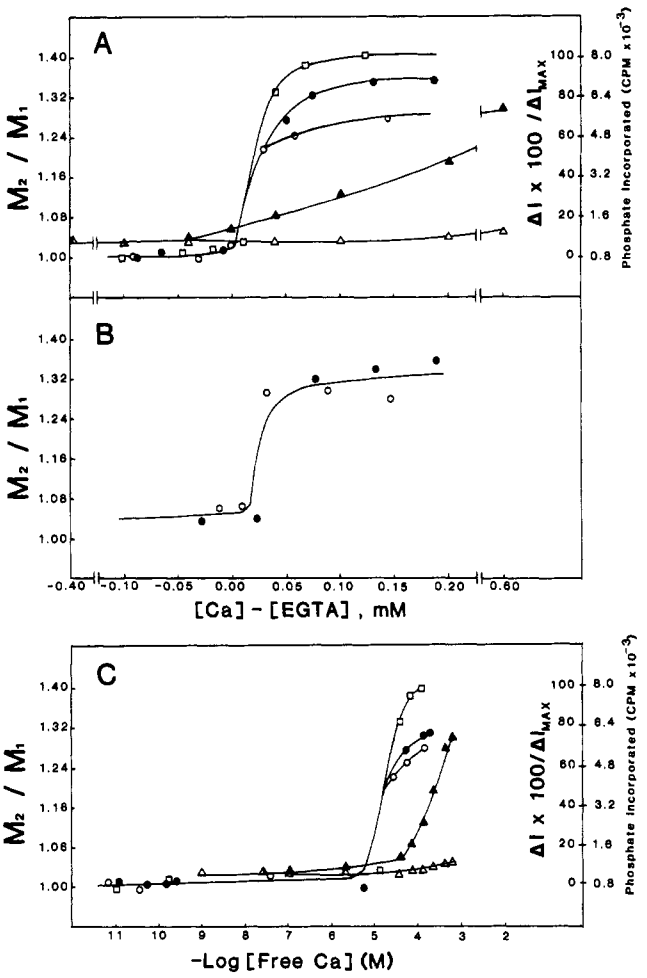

FIGURE 5: Ca^{2+} requirement of PKC binding. Phospholipid vesicles containing PS–DAG–PC–dansyl-PE (20:10:70:0, 20:10:60:10, or 20:0:80:0) were prepared by sonication and gel filtration. The reagents were added to a total volume of 1.6 mL. The abscissa gives the difference between total Ca^{2+} and total EGTA concentrations. Panel A shows the Ca^{2+} requirement of binding (association direction) and activation. PKC (4 μg) was mixed with 4 μg of vesicles with (●) or without (○) DAG, and the binding was measured by light-scattering intensity measurements. Binding of PKC (5.7 μg, □) to 6.6 μg of vesicles containing DAG and dansyl-PE, as measured by fluorescence energy transfer, is also shown. The activity of PKC (25 ng in a total volume of 0.25 mL) in the presence of 42 μg/mL phospholipid with (▲) or without DAG (△) is shown. Panel B shows dissociation of the PKC–vesicle complex by EGTA (titration proceeded from right to left) as measured by light-scattering intensity. Two experiments with phospholipid vesicles containing DAG (●) and phospholipid vesicles without DAG (○) are shown. Panel C shows the Ca^{2+} requirement of PKC binding and activation plotted as a function of the free Ca^{2+} concentration in the medium. The symbols used are the same as in panel A.

performed at higher phospholipid concentrations. Due to the interaction between Ca^{2+} and phospholipid titration curves (Figure 2A), the increase in phospholipid concentration would reduce the Ca^{2+} requirement for appearance of activity.

Figure 6 shows experiments conducted with phospholipid vesicles containing 30% PS. Once again, the Ca^{2+} requirement of binding appeared to be independent of DAG (Figure 6A). Decreasing the concentration of Ca^{2+} by EGTA addition reduced the extent of binding (Figure 6B). Again, the binding measured by light-scattering intensity was not totally reversible; about 25–40% of the bound protein was not dissociated by EGTA (Figure 6B). Neither the phospholipid alone nor the protein alone showed a detectable change in light-scattering intensity or fluorescence emission signal upon the addition of up to 2.0 mM Ca^{2+} or 10 mM EGTA. The irreversible

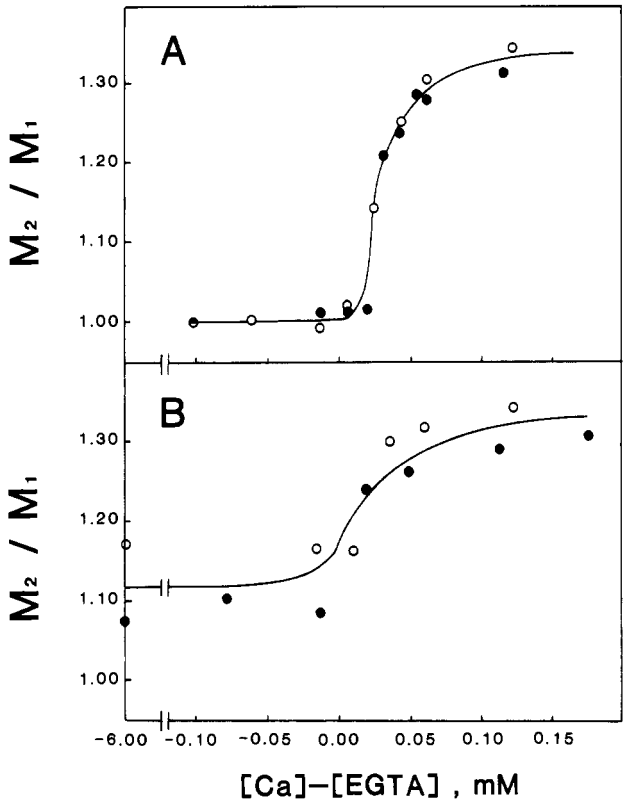

FIGURE 6: Ca^{2+} requirement of PKC binding to 30% PS vesicles as determined by light-scattering intensity. The phospholipid vesicles contained PS–DAG–PC (30:10:60 or 30:0:70). Panel A shows the Ca^{2+} requirement of binding (association direction): 2.6 μg of PKC was mixed with 3.4 μg of vesicles with (●) or without (○) DAG. Panel B shows dissociation (titration proceeded from right to left) by successive additions of EGTA. Phospholipid vesicles containing DAG (●) and without DAG (○) are shown.

changes therefore arose from the protein–membrane complex and not from the individual components.

Experiments, parallel to those in Figure 6, showed, once again, a strong synergetic effect of Ca^{2+}, phospholipid, and DAG on production of PKC activity. At 160 μg/mL of phospholipid vesicles which did not contain DAG, PKC was half-activated by 150 μM Ca^{2+}. In contrast, only 0.1 μM Ca^{2+} was required for half-maximal activation of PKC by the vesicles containing DAG. Conversely, in the presence of DAG, 1 mM Ca^{2+} produced activity with only 0.2 μg of phospholipid/mL. These results contrasted with the binding properties which appeared insensitive to DAG. In fact, no appreciable kinase activity could be developed at all under the minimum conditions required for binding (Figure 6). It is also interesting to note that these requirements contrasted greatly from those needed for activity by membranes of 20% PS (Figure 5A). Activity was quite sensitive to PS content in this range while binding did not appear highly sensitive to this range of PS contents (see above).

Binding and Activity Development with Phorbol Esters. DAG may undergo isomerization (Sardarevich, 1967) to less active forms (Ganong et al., 1986; Boni & Rando, 1985). In addition, DAG is imbedded in the membrane bilayer and may be relatively inaccessible to protein. Therefore, the effective concentration of DAG in these studies is unknown and may be the limiting factor for development of activity.

Phorbol esters activate PKC in a manner that is presumed to be similar to that of DAG. They bind to PKC in a 1:1 stoichiometry with high affinity (König et al., 1985; Parker et al., 1984). These properties allow measurement of PKC–PDBu binding in aqueous solutions and in the presence of

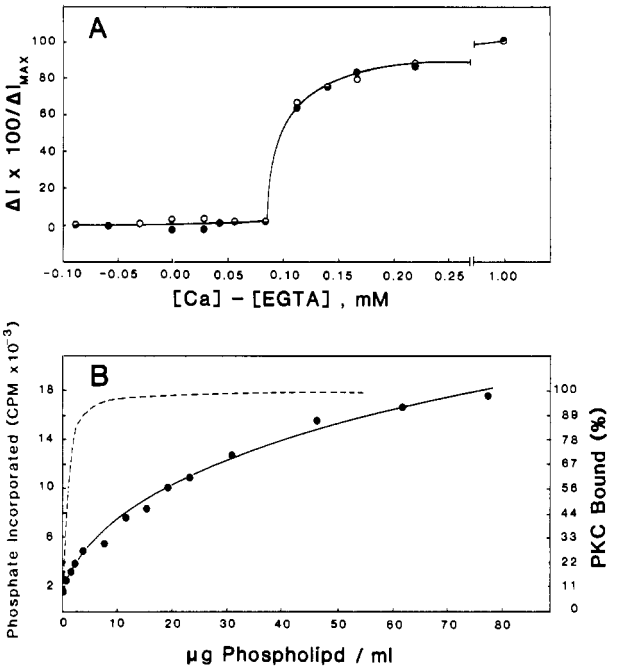

FIGURE 7: Ca^{2+} requirement of PKC binding in the presence of PDBu. Phospholipid vesicles containing PS–PC–dansyl-PE (20:70:10) were used. Panel A shows the binding of PKC to phospholipid vesicles as measured by fluorescence energy transfer in the presence (○) or absence (●) of 73 μM PDBu. Twelve micrograms of PKC was mixed with 6 μg of vesicles in a total volume of 1.7 mL, and the increase in emission intensity was plotted as a percentage of that observed at 1.0 mM Ca^{2+}. Panel B shows the activity and fraction of PKC bound as a function of phospholipid concentration. The activity (●) was measured in the presence of 0.2 mM Ca^{2+} and 73 μM PDBu. The percentage of PKC bound (- - -) was calculated by using a K_d value of 5 nM, the upper limit for the estimated K_d (see Discussion).

membranes (Tanaka et al., 1986; Parker et al., 1984; Kikkawa et al., 1983b; Ashendel et al., 1983). Figure 7A shows the Ca^{2+} titration of PKC–membrane binding in the presence and the absence of 73 μM PDBu. The binding constant for phorbol esters at 0.2 mM Ca^{2+} is reported to be about 8 nM (Tanaka et al., 1986; Kikkawa et al., 1983) which is similar to results obtained in this laboratory (unpublished data). Consequently, the use of 73 μM PDBu assured that all PKC molecules were saturated with the phorbol ester. PDBu appeared to have little effect on the calcium requirement of PKC–membrane binding.

Figure 7B shows the phospholipid requirement of activity in the presence of PDBu. Again, a large excess of phospholipid was required for development of activity. On the basis of binding properties, the dashed line shows the approximate amount of phospholipid required for binding of PKC to the membrane. At phospholipid concentrations to the right of the dashed curve in Figure 7B, all PKC should be bound to the membrane with calcium and phorbol ester associated with it. The results indicated that a complex of PKC–Ca^{2+}–phorbol ester–membrane was not adequate to generate maximum kinase activity in this assay system. Other parameters must be satisfied for activity.

DISCUSSION

This study provides preliminary characterization of the membrane binding properties of PKC with comparison to development of activity. Binding was dependent on the presence of acidic phospholipids although there was neither an absolute requirement nor an apparent strong preference for one acidic phospholipid over another. Protein reached steric saturation of the membrane surface at membrane compositions

above about 20% PS. Light-scattering and fluorescence energy transfer results showed that about 1.0 g of protein could bind to 1.0 g of phospholipid. The latter relationship was suggested from the observation that a protein to lipid ratio giving M_2/M_1 of about 1.35 (indicating 0.35 g of protein bound to 1 g of lipid, Figures 5 and 6) gave about one-third of maximum fluorescence energy transfer. This approximate capacity of protein–membrane binding was similar for a number of peripheral membrane binding proteins including prothrombin (Nelsestuen & Broderius, 1977) and blood clotting factors X (Nelsestuen & Broderius, 1977), V (Pusey et al., 1982), and XII (Griep et al., 1985). An acidic phospholipid content of 20% also appears to correlate with the content of cytosolic membranes of at least some animals cells. Consequently, maximum binding capacity was achieved at physiological membrane compositions.

While Ca^{2+} was essential for membrane binding, the other major effectors of PKC activity, DAG and PDBu, had little influence on membrane binding. This was observed by both fluorescence energy transfer and light-scattering intensity measurements (Figures 5–7). It is possible that these components have a more subtle effect on membrane binding that was not detected by the studies reported here. However, a lack of DAG effect on the amount of protein bound or on the Ca^{2+} requirement of binding seemed consistent with several observations showing that membrane binding was not correlated with the appearance of kinase activity. This conclusion contradicted those of Wolf et al. (1985a,b), who reported that PKC–membrane binding is regulated by Ca^{2+}, Mg^{2+}, ATP, and phorbol esters. However, Wolf et al. (1985a,b) assumed a correlation between phorbol ester binding and protein–membrane binding; the latter was not monitored directly.

The reversibility of PKC binding to phospholipid vesicles was dependent on the density of acidic phospholipid in the vesicles. With membranes containing 5%, 10%, and 15% PS, the binding was total reversible (Figure 3). With 30% PS, however, the binding was not totally reversible (Figures 3 and 6B). Use of both light-scattering and fluorescence techniques to measure dissocation was important since they are sensitive to very different properties of the complex. Possible artifacts that might influence reversibility measured by one technique should not influence the other. One possible explanation for lack of reversibility could be penetration of some PKC into the hydrocarbon region of the membrane to produce an integral membrane protein. Such a penetration might account for the observation that nearly one-third of PKC activity in the brain is membrane bound and can be extracted only in the presence of detergent (Kikkawa et al., 1983a).

In the presence of saturating Ca^{2+}, actual equilibrium binding constants for PKC–membrane associations were not measured in this study. The techniques used did not appear capable of estimating the low levels of free protein that would be in equilibrium with bound protein. That is, when membrane binding sites exceeded protein concentration, the results suggested that all of the protein was membrane bound. For example, similar intensity changes were obtained when similar amounts of protein were added to different phospholipid compositions (Figure 3). Even membranes containing the lowest acidic phospholipid compositions appeared to bind PKC tightly. In addition, higher concentrations of components did not increase the relative yield of fluorescence energy transfer as protein was added to phospholipid. That is, a protein: phospholipid ratio of 1.2 gave the same relative energy transfer whether the phospholipid was present at 0.5 or 10 μg/mL (data not shown). If significant amounts of protein were free in solution due to an equilibrium, increasing the phospholipid would increase the percentage of protein bound and give relatively more fluorescence energy transfer at the same protein:lipid ratio. The lack of significant free protein at the lowest phospholipid concentration suggested that the equilibrium dissociation constant was less than 5 nM.

The results indicated that, while binding of PKC to membranes was required for activity, binding did not always correlate with the appearance of activity. Wolf et al. (1985b) also observed that the phorbol ester binding to PKC required lower Ca^{2+} concentrations than were needed for activity. They proposed that calcium had multiple effects. However, a number of factors may be needed for expression of enzyme activity. A possible requirement may be substrate binding to the membrane. Preliminary studies on substrate binding show a further complicating factor; histones cause extensive aggregation of the phospholipid vesicles under conditions of PKC activity measurements (unpublished data). Consequently, systems that use histones contain extensively aggregated substrate–enzyme–membrane complexes. This aggregation also applies to the detergent-solubilized PS system described by Hannun et al. (1985). Another well-known substrate for PKC, myelin basic protein, also causes extensive aggregation of vesicles (Lampe et al., 1983). Consequently, it is possible that activity measurements are made in complex aggregates. Substrate and cofactor requirements of activity may be related to properties of these aggregates.

After completion of the manuscript, Hannun et al. (1986) reported analysis of the interrelationships of calcium, acidic phospholipids, and diacylglycerol in the detergent-solubilized phospholipid assay system. In agreement with our studies using vesicles, the micellular system showed strong interdependence of these components for appearance of activity. In addition, PG and PI were less effective in promoting PKC activity than was PS, and activity required a large excess of micellular phospholipid over protein. Since direct binding of enzyme to the micelles was not measured and other aspects of the system such as aggregation of the micelles in the presence of substrate were not considered, it is not possible to interpret the mechanistic implications of the activity requirements of the micelle system for the reasons outlined above for vesicle-based activity.

The factors influencing activity of PKC appear quite complex. Better understanding of the physical state of the total complex is needed to elucidate the physical entity that actually generates PKC activity. The studies presented here show properties of PKC–membrane events. Further studies on substrate–membrane binding as well as its influence on PKC–membrane binding are in progress.

Registry No. PKC, 9026-43-1; DAG, 25637-84-7; Ca, 7440-70-2.

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