Properties of the Protein Kinase C–Phorbol Ester Interaction†

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ABSTRACT: The properties of the protein kinase C (PKC)–phorbol ester interaction were highly dependent on assay methods and conditions. Binding to cation-exchange materials or adsorption to gel matrices resulted in PKC that was capable of binding phorbol 12,13-dibutyrate (PDBu). The extraneous interactions were eliminated by measuring phorbol ester binding with a gel filtration chromatography assay in the presence of bovine serum albumin (BSA). In the absence of calcium, free PKC did not bind PDBu or phospholipids. Calcium caused structural changes in PKC which enhanced its interaction with surfaces such as the gel chromatography matrix. While BSA prevented this interaction, it did not interfere with PKC association with acidic phospholipids. Interaction of PKC with phospholipid resulted in two forms of membrane-associated PKC. The initial calcium-dependent and reversible form of membrane-associated PKC was capable of binding PDBu. Both PKC and PDBu were released from this complex by calcium chelation. Sustained interaction with phospholipid vesicles resulted in a PKC–membrane complex that could not be dissociated by calcium chelation and appeared to result from insertion of PKC into the hydrocarbon portion of the phospholipid bilayer. Membrane insertion was observed at calcium concentrations of 2–500 μM and with membrane compositions of 10–50% acidic phospholipid. However, the extent of insertion was dependent on the binding conditions and was promoted by high phospholipid to PKC ratios, high calcium, the presence of phorbol esters, high membrane charge, and long incubations. Once PKC was inserted into a phospholipid bilayer, it bound PDBu in the presence (K_d = <0.5 nM, k_{diss} = 10^{-4} s^{-1} at 4 °C) and in the absence of Ca^{2+}. Calcium enhanced the affinity of PKC–PDBu interaction and decreased the dissociation rate. These results showed that dramatic changes occurred in the in vitro properties of PKC upon the formation of the irreversible PKC–membrane complex. These properties may be related to cellular events that induce formation of the chelator-resistant form of membrane-bound PKC.

The calcium- and phospholipid-dependent protein kinase (PKC)[1] is considered to be a major regulatory enzyme which is sensitive to the second messengers calcium and diacylglycerol (Nishizuka, 1986; Kikkawa & Nishizuka, 1986). PKC appears to be the primary receptor for tumor-promoting phorbol esters, and these compounds are thought to function in a role analogous to that of diacylglycerol. Many cellular effects of phorbol esters are attributed to either activation or down-regulation of PKC (Blumberg et al., 1984; Ashendel, 1985). In vitro, phorbol esters bind to PKC and reduce the calcium needed for generating maximum kinase activity (Castanga et al., 1982). Phorbol esters also alter the cellular distribution of PKC; treatment of cells with phorbol esters causes a decrease in the cytosolic PKC and an increase in a chelator-resistant form of membrane-associated PKC (Kraft et al., 1982; Kraft & Anderson, 1983; Gopalakrishna et al., 1986).

Several previous studies have examined binding of phorbol esters to PKC by innovative techniques that are rapid and convenient. These techniques have been invaluable for demonstrating the fact that PKC is a phorbol ester receptor and for detecting the presence of this receptor (Kikkawa et al., 1983; Sando & Young, 1983; Niedel et al., 1983; Leach et al., 1983). However, many phorbol ester binding measurements involve conditions inconsistent with equilibrium; the PKC–phospholipid–PDBu complex is typically washed or gel filtered to remove unbound PDBu. This treatment would allow loosely bound material to dissociate. Some procedures trap the PKC–phospholipid–PDBu complex by ionic binding to cationic materials such as glass-fiber filters treated with poly(ethylenimine) (Tanaka et al., 1986) or DEAE-cellulose (Parker et al., 1984) or by precipitation of the complex with poly(ethylene glycol) [Sharkey et al., 1984; see also Jaken (1987)]. Because of minimum assay requirements, it is often difficult to examine secondary effects which these components or procedures might exert on the binding process.

The cofactor requirements for binding of phorbol esters to PKC have not been entirely consistent. While most studies suggest that phorbol ester binding requires acidic phospholipids (Kikkawa et al., 1983; Sando & Young, 1983; Parker et al., 1984; Blumberg et al., 1984; Konig et al., 1985), the Ca^{2+} requirement appears to be influenced by assay conditions. For example, measurement of PKC–PDBu binding by filtration on glass-fiber filters showed an absolute requirement for Ca^{2+} (Sando & Young, 1983; Huang & Huang, 1986; Dougherty & Niedel, 1986), while measurements of binding by gel filtration (Kikkawa et al., 1983) or by filtration on glass-fiber filters treated with poly(ethylenimine) (Tanaka et al., 1986) indicated that Ca^{2+} only enhanced binding. The calcium requirement also appeared to depend on the phospholipid composition of the membranes (Konig et al., 1985) or on whether the enzyme was proteolytically cleaved (Huang & Huang, 1986). Phospholipid-dependent but calcium-independent binding of phorbol esters appears difficult to reconcile with the observation that PKC–phospholipid interaction has been reported to have an absolute requirement for Ca^{2+} (Bazzi & Nelsestuen, 1987a; Wolf et al., 1985).

Recent in vitro studies showed a condition under which calcium-independent binding of PDBu occurred. Protein

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[1] Abbreviations: BSA, bovine serum albumin; DAG, diacylglycerol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; PC, phosphatidylcholine; PDBu, phorbol 12,13-dibutyrate; PKC, Ca^{2+}- and phospholipid-dependent protein kinase; PS, phosphatidylserine.

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kinase C was shown to exist in two membrane-bound forms, a form that was reversibly bound in a calcium-dependent manner and a form that was irreversibly associated with the membrane and which could only be removed by detergents (Bazzi & Nelsestuen, 1988a; Gopalakrishna et al., 1986). The latter form bound phorbol esters in the presence or the absence of calcium and displayed cofactor-independent kinase activity (Bazzi & Nelsestuen, 1988a). Insertion of PKC into the membrane was proposed to be a mechanism of PKC activation and could be the basis for phorbol ester function.

The present study was initiated to improve our understanding of PKC–phorbol ester interaction. It was found that, while free PKC did not bind phorbol esters, a variety of surfaces supported this interaction. Calcium was essential for reversible PKC–membrane binding, and this complex bound phorbol esters. Both PKC and phorbol esters were released from this complex upon calcium chelation. While bound in this reversible manner, a number of factors including calcium, phorbol esters, and membrane composition contributed to formation of membrane-inserted PKC which then bound PDBu in the presence or the absence of calcium.

Experimental Procedures

Materials. Bovine serum albumin (BSA), bovine brain phosphatidylserine (PS), egg yolk phosphatidylcholine (PC), histone III-S, and protamine sulfate were purchased from Sigma Chemical Co. [γ-^{32}P]ATP (3 Ci/mmol) was purchased from Amersham Corp. Phorbol 12,13-dibutyrate and 4α-phorbol 12,13-dibutyrate (4α-PDBu) were purchased from LC Services Corp. [^{3}H]PDBu (15–20 Ci/mmol) was purchased from New England Nuclear. Polycarbonate filters (0.1-μm diameter) were purchased from Nucleopore Corp. Other chemicals and reagents were from the Sigma Chemical Co. and were of the highest grade available.

PKC was purified to apparent homogeneity from bovine brains according to a published procedure (Bazzi & Nelsestuen, 1987a). The enzyme activity was assayed by measuring ^{32}P incorporation into histone by a procedure similar to that reported by Kikkawa et al. (1982). Protein concentrations were determined by the method of Bradford (1976) using BSA as the standard.

PKC–PDBu Binding. Two methods were used. The first was similar to that reported by Parker et al. (1984). Binding was performed in a total of 250 μL of Tris buffer (20 mM, pH 7.5) containing phospholipid (0.2 mg/mL), PKC (4 μg), β-mercaptoethanol (30 mM), $MgCl_2$ (10 mM), glycerol (50% v/v), [^{3}H]PDBu (0–50 nM), and either Ca^{2+} (0.5 mM) or EGTA (1.0 mM). After a 15-min incubation at room temperature, 50 μL of a 50% suspension of DEAE-cellulose was added. The mixture was vortexed and incubated for an additional 15 min. The enzyme–DEAE-cellulose complex was collected by rapid filtration on glass-fiber filters (GF/F, Whatman). The filters were washed three times (2 mL each) with ice-cold Tris buffer (20 mM, pH 7.5) containing glycerol (50%) and either 0.5 mM Ca^{2+} or 1.0 mM EGTA to match the binding assay conditions. PDBu binding was estimated from radioactivity associated with the filters. Nonspecific PDBu binding was determined under the same conditions except that PKC was omitted.

The second method utilized gel filtration chromatography (Hummel & Dreyer, 1962) at 4 °C on Sephacryl S-300 columns (1.0 × 40 or 1.5 × 40 cm) equilibrated and eluted with buffer consisting of Tris (20 mM, pH 7.5), glycerol (10% v/v), and β-mercaptoethanol (30 mM). Other buffer components included in various experiments were Ca^{2+} (0.5 mM or 2 μM), EGTA (1.0 mM), [^{3}H]PDBu (0–4 nM), BSA (0.5 mg/mL), and/or 4α-PDBu (100 nM). If PDBu–PKC interaction occurred, the elution pattern of [^{3}H]PDBu showed a peak (corresponding to the elution position of the protein) and a trough of radioactivity at the inclusion volume with a constant level of radioactivity before, between, and after these features. The areas of both the peak and the trough were used to determine the amount of bound PDBu (Hummel & Dreyer, 1962). The concentration of PDBu was estimated from the radioactivity present in each fraction (250-μL aliquots). The kinase activity in each fraction (50- or 100-μL aliquot) was measured with protamine sulfate as the substrate since its phosphorylation by PKC is not influenced by Ca^{2+} (Takai et al., 1977). Concentrations of PKC were determined by comparison of this activity with that of pure PKC, assuming equal activity of all species. Total yield of enzyme activity from the columns was usually greater than 80% and was independent of the amount of enzyme applied to the column.

Fractions from the column elution profiles which contained membrane-bound PKC (from above) were used to measure the degree of dissociation of PKC–PDBu from the membrane and to observe phorbol ester binding to membrane-inserted PKC. One-milliliter samples of PKC–PDBu–membrane complex were made 2.0 mM in EGTA, incubated for 1 h at 4 °C, and applied on Sephacryl S-300 columns eluted with buffer containing EGTA. The amounts of membrane-inserted and free PKC as well as bound and released PDBu were determined from the elution profiles. Total yield of PKC, as determined by both kinase activity and PDBu recovery, was greater that 85%, and yield was independent of amount of PKC applied to the column or incubation time. Low levels of nonspecific binding of PDBu to phospholipid vesicles alone (0.03–0.54 pmol) was subtracted as a background.

Approximation of PKC–PDBu Dissociation Rate. PKC–PDBu–membrane complexes were formed by gel chromatography of PKC in buffer containing BSA, Ca^{2+} (0.5 mM), and [^{3}H]PDBu (2 nM) as outlined above. One-milliliter samples of this complex (containing a total of 5 pmol of [^{3}H]PDBu, 3 pmol of which were bound to PKC) or of buffer (contained only 2 pmol of free [^{3}H]PDBu) were applied to Sephacryl S-300 columns (1.0 × 40 cm) equilibrated with the same buffer except that the PDBu was unlabeled. Fractions (1 mL) were collected at a flow rate of 4.8 mL/h, and the radioactivity (250 μL) was measured. The amount of [^{3}H]PDBu dissociated from PKC in each fraction was estimated as the radioactivity present in the sample column *minus* the radioactivity present in the control column (see Figure 7). The time of dissociation at each point was estimated from the position of the fraction. For example, PDBu appearing at the inclusion volume had dissociated at zero time while PDBu separated from the inclusion volume by 4.8 mL had dissociated at time = 1 h. The total amount of [^{3}H]PDBu dissociated from the PKC–phospholipid complex at any given point in the chromatogram was estimated from the running sum of radioactivity in the elution profile. That is, at any point in the elution profile, the total amount of PDBu that had dissociated was equal to the sum of radioactivity from that fraction to the inclusion volume.

Phospholipid Preparations. Large unilamellar vesicles composed of PS–PC (10:90, 20:80, or 50:50) were prepared by the extrusion method of Hope et al. (1985) using a 0.1-μm polycarbonate filter. Phospholipid concentrations were determined from organic phosphate (Chen et al., 1956) with a phosphorus to phospholipid weight ratio of 1:25.

Results

Influence of Methodology on PKC–PDBu Binding. The

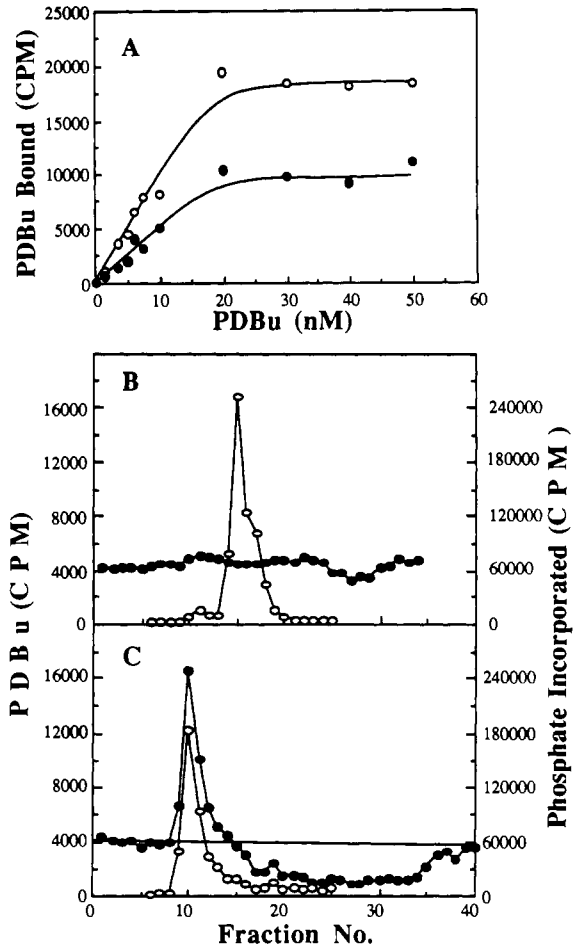


FIGURE 1: Cofactor requirements of PDBu binding to PKC. Panel A shows the PDBu binding as determined by the DEAE-cellulose technique. This method, described under Experimental Procedures, used PKC (4 μg), phospholipid vesicles (200 μg/mL), and buffer containing either 0.5 mM Ca^{2+} (○) or 1.0 mM EGTA (●). Panels B and C show binding as determined by gel filtration in the presence of 2.0 nM PDBu. In this method, Sephacryl S-300 columns (1.5 × 40 cm) were equilibrated and eluted (2-mL fractions) with buffer containing 2 nM [^{3}H]PDBu and either 1.0 mM EGTA (panel B) or 0.5 mM Ca^{2+} (panel C). PKC (4 μg) was mixed with 0.6 mL of the equilibration buffer in the presence of 200 μg of phospholipid vesicles and applied to the column. The [^{3}H]PDBu (●) and kinase activity (○) were measured as described under Experimental Procedures. The phospholipid vesicles for all experiments were composed of PS–PC (50:50).

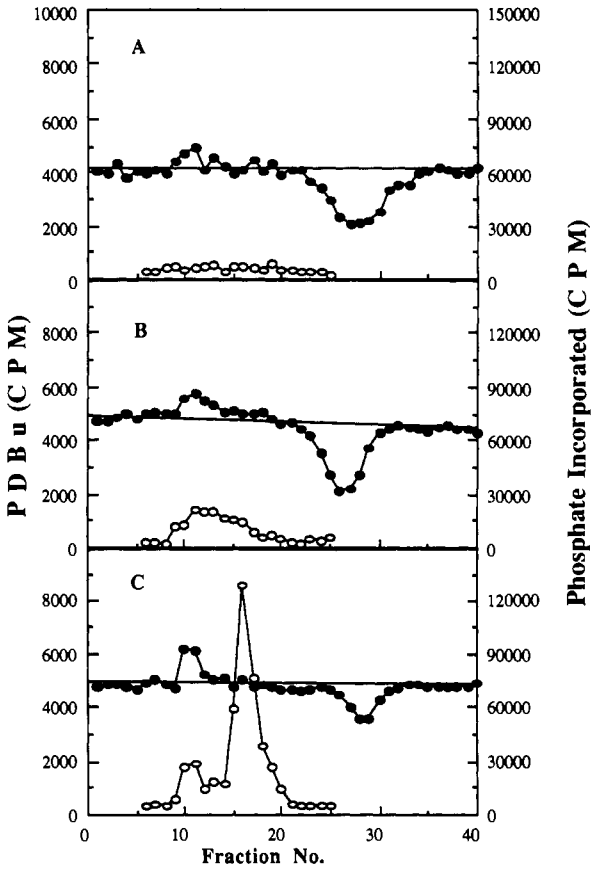


FIGURE 2: Influence of calcium on PKC–PDBu interactions. Panel A shows the elution profile of PKC (4 μg) applied to Sephacryl S-300 columns (1.5 × 40 cm) and eluted (2-mL fractions) with buffer containing 0.5 mM Ca^{2+} and 2.0 nM [^{3}H]PDBu. Panels B and C show similar experiments except that the buffer also contained 100 nM 4α-PDBu (panel B) or 0.5 mg/mL BSA (panel C). Kinase activity (○) and [^{3}H]PDBu (●) were determined as in Figure 1.

binding of PDBu to PKC was first measured with the DEAE-cellulose binding assay described by Parker et al. (1984). Binding was saturable, and half-maximum occurred at about 10 nM PDBu (Figure 1A). This was similar to that in previous reports (Kikkawa et al., 1983; Leach et al., 1983; Sando & Young, 1983; Blumberg et al., 1984; Parker et al., 1984). Also in agreement with a number of studies, calcium had only a minor influence on binding and increased association by about 2-fold (Kikkawa et al., 1983; Tanaka et al., 1986). This assay procedure also showed partial binding of PDBu to PKC in experiments where phospholipid was omitted (data not shown). The latter binding was quantitatively variable but often attained the level of calcium-independent binding seen in Figure 1A. Quantitative interpretation of these binding results by Scatchard or other equilibrium analyses appeared unwarranted (see below).

The cofactor requirements of PDBu binding were dependent on methodology. Results in Figure 1B,C show PDBu binding measured by the gel filtration procedure of Hummel and Dreyer (1962). In the absence of Ca^{2+}, there was no detectable PDBu binding to PKC in the presence of phospholipid (Figure 1B). The results in Figure 1B also showed that nonspecific adsorption of PDBu to phospholipid vesicles was negligible. In the presence of Ca^{2+}, PKC bound to the phospholipid vesicles and eluted at the exclusion volume of the column (Figure 1C). Phospholipid-bound PKC also bound PDBu, as indicated by increased radioactivity associated with the enzyme. The amount of PKC loaded on the column in Figure 1C was adequate to nearly deplete the PDBu from the entire column. This emphasized the lack of PDBu binding to free enzyme (Figure 1B).

These studies showed that the calcium-independent binding of PDBu observed in Figure 1A was probably due to artificial aspects of the method, perhaps to the DEAE-cellulose component. A similar effect of cationic polymers has recently been reported in an independent study (Thompson et al., 1988).

Gel filtration of PKC on Sephacryl S-300 columns revealed some unexpected in vitro properties of PKC. Figure 2 shows profiles from columns eluted with buffer containing calcium and [^{3}H]PDBu. In the absence of phospholipid, PKC activity did not appear in the elution profile (Figure 2A), suggesting adsorption to the column matrix. These columns had not been exposed to phospholipids so prior contamination was not possible. Furthermore, a significant trough appeared in the PDBu elution profile. Since no corresponding peak of [^{3}H]-PDBu appeared, the trough must have arisen from binding of [^{3}H]PDBu to column-associated PKC. While the extent of this binding was less than that observed in the presence of phospholipids, the results in Figure 2A suggested that PKC

had adsorbed to the column and bound some PDBu in that state.

Upon binding calcium, PKC has been reported to undergo conformational changes which may result in exposure of a hydrophobic site on the protein (Walsh et al., 1984; Anderson & Salomon, 1985). It was possible that column-adsorbed PKC bound PDBu via nonspecific interactions. To test this possibility, 4α-PDBu was added to the equilibration buffer. Similar levels of PDBu binding were observed in experiments conducted in the absence (Figure 2A) or in the presence of a 50-fold excess of unlabeled 4α-PDBu (0.1 μM, Figure 2B). This suggested that the troughs in Figure 2A,B arose from a specific binding event rather than from nonspecific hydrophobic interactions.

Inclusion of BSA in the buffer prevented calcium-dependent adsorption of PKC to the column matrix (Figure 2C). Since the majority of PKC activity eluted at the position of the monomeric protein, BSA appeared to function by covering adsorption sites on the column matrix rather than by interacting with PKC. Columns eluted with a single large addition of BSA also showed no peak or trough of [^{3}H]PDBu (data not shown), indicating the lack of significant interaction between BSA and PDBu. In addition, BSA did not interfere with the binding of PKC to phospholipid vesicles or with the binding of PDBu to membrane-bound PKC (see below). In the presence of calcium, a small amount of enzyme eluted at the exclusion volume (Figure 2C) and also bound [^{3}H]PDBu. This material was not investigated as a part of the current study although it may suggest some enzyme aggregation under the conditions of the experiment.

A further benefit of BSA in the elution buffer appeared to be stabilization of the [^{3}H]PDBu concentration. This was apparent from the elution profiles in Figures 1 and 2; when the columns were eluted with buffers that did not contain BSA, 2 nM [^{3}H]PDBu gave steady-state levels of sampled radioactivity of about 4000 cpm (Figure 1B,C and 2A); when an excess of another surface-active component was present (BSA in Figure 2C or 4α-PDBu in Figure 2B), the radioactivity in a similar volume (250 μL containing 0.5 pmol of PDBu) was 5000 cpm. The difference could arise from interaction of BSA or 4α-PDBu with the air–water interface, thereby excluding [^{3}H]PDBu which would remain in bulk solution. Previous studies showed that BSA prevented interaction of PKC with the air–water interface and depletion of the enzyme from bulk solution (Bazzi & Nelsestuen, 1988b). The stabilizing effect of BSA or γ-globulin on low concentrations of PDBu has been previously recognized (Delclos et al., 1980; Leach et al., 1983; Sharkey et al., 1984; Tanaka et al., 1986). It is possible that added proteins function by a common mechanism consisting of covering hydrophobic sites (on the column matrix or at the air–water interface), thereby stabilizing the amount of PKC and/or [^{3}H]PDBu in bulk solution.

The results in Figure 3 indicated that gel filtration in the presence of BSA provided a reliable method for quantitation of PKC–PDBu interaction. The quantity of bound [^{3}H]PDBu estimated from the peak and trough gave indistinguishable results. There was a linear relationship between the amount of PKC loaded onto the column and the amount of bound [^{3}H]PDBu (Figure 3). Due to several factors, the stoichiometry of 0.93 PDBu/PKC (slope of the line, Figure 3) remained tentative (see Discussion).

Factors Influencing the Forms of PKC and Their PDBu Binding Properties. Previous studies demonstrated two types of membrane-bound PKC; one was released by calcium chelation, and another could only be removed by detergents (Bazzi & Nelsestuen, 1988a; Gopalakrishna et al., 1986). The latter form was constitutively active toward in vitro substrates and bound PDBu in the absence of calcium (Bazzi & Nelsestuen, 1988a). Several factors which influenced the distribution of PKC between these two forms were examined.

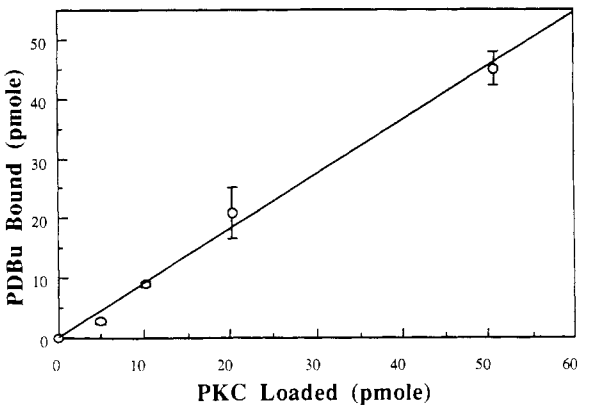


FIGURE 3: Ratio of PDBu to PKC. The PKC was mixed with 0.6 mL of the equilibration buffer and phospholipid vesicles [200 μg; PS–PC (50:50)]. After 20 min, the sample was applied to the column and eluted (2-mL fractions) with a buffer containing 20 mM Tris, pH 7.5, 10% glycerol, 30 mM β-mercaptoethanol, 0.5 mg/mL BSA, 0.5 mM Ca^{2+}, and 2.0 nM [^{3}H]PDBu. The amount of bound PDBu was calculated from the areas of the peak and trough in the PDBu elution profile. The results show the average and standard deviation of three experiments. The slope of the line is 0.93 PDBu/PKC (mol/mol).

Phospholipid vesicles composed of PS–PC (50:50) and a high phospholipid to PKC ratio were used to generate a high level of the nondissociable PKC–membrane complex. Gel filtration (Figure 4A) showed PKC–vesicle association in the presence of Ca^{2+} and [^{3}H]PDBu binding with approximately unit stoichiometry (0.93 PDBu/PKC, mol/mol). Figure 4B shows chromatography of PKC in the presence of EGTA. The enzyme used in this experiment was bound to phospholipid vesicles for 20 min before EGTA was added. The elution profile showed that the majority of the PKC activity dissociated from the vesicles and only about 11% had become inserted into the membrane. The membrane-inserted population bound [^{3}H]PDBu in the presence of EGTA (Figure 4B). Prolonged PKC–phospholipid interaction in the presence of Ca^{2+} and PDBu enhanced the extent of PKC insertion into the membranes. A sample of PKC that had been bound to phospholipid for approximately 36 h (fractions 10–12, Figure 4A) showed that the majority of PKC activity (open circles, Figure 4C) remained associated with the phospholipid in the presence of EGTA and bound a high level of PDBu (PDBu:PKC = 0.8; closed circles, Figure 4C). The elution of PDBu in Figure 4C was not expected to show a trough because the loaded sample had already been equilibrated with 2.0 nM PDBu.

Important features of the elution profile in Figure 4C were the large peak of [^{3}H]PDBu associated with membrane-inserted PKC, a slightly higher level of free [^{3}H]PDBu eluting between this peak and the inclusion volume (fractions 15–27), and the lack of a peak of free [^{3}H]PDBu coincident with the inclusion volume of the column. The latter indicated that removal of calcium did not cause extensive release of PDBu from the membrane-inserted PKC at 2 nM free [^{3}H]PDBu.

There were two possible explanations for the slight but real increase in free PDBu in fractions 15–27. Continual dissociation (of PDBu or PKC from the PDBu–PKC–vesicle complex) during column elution could result from an extremely slow dissociation rate so that equilibrium was never reached

Table I: Effect of Conditions on Formation of Membrane-Inserted PKC

binding conditions[a]			binding[b]			dissociation[c]		
PS (%)	Ca (μM)	PDBu	PKC applied (pmol)	PKC bound (pmol)[d]	PDBu/PKC[e]	PKC applied (pmol)	PKC inserted (pmol)[d]	PDBu/PKC[e]
50	500	+	20.25	18.5 ± 1	0.80 ± 0.13	5.3	3.4 ± 0.8	0.82 ± 0.02
20	500	+	20.25	15.1 ± 3	0.77 ± 0.16	5.0	2.0 ± 1.2	0.66 ± 0.28
10	500	+	20.25	12.7 ± 4	0.48 ± 0.03	3.9	1.0 ± 0.1	0.63 ± 0.1
50	2	+	15.2	10.9	0.81	4.2	1.8	0.88
20	2	+	15.2	7.8	0.61	2.3	0.5	0.82
50	2	-	15.2	10.1		2.9	0.8	
20	2	-	15.2	8.7		2.5	0.6	

[a] The gel filtration columns were equilibrated with buffer containing 20 mM Tris, pH 7.5, BSA (0.5 mg/mL), 10% glycerol, 30 mM β-mercaptoethanol, and Ca^{2+} (as indicated), with (+) or without (-) 2.0 nM [^{3}H]PDBu. The phospholipid vesicles (50 μg) were composed of PS (the percentage indicated) and PC (to 100%). [b] Binding of PKC to vesicles was determined as illutrated in Figure 6A. Free PKC (amount indicated) was mixed with phospholipid vesicles in 0.6 mL of the equilibration buffer containing BSA. After 30 min, the sample was applied on the column. The amount of phospholipid-bound PKC was calculated from the kinase activity eluting at the exclusion volume of the column. [c] Dissociation was determined as illustrated in Figure 6B. Sixteen hours after initial sample preparation, fractions containing membrane-associated PKC (corresponding to fractions 10–12 in Figure 6A) were combined, made 2.0 mM in EGTA, incubated for 1 h, and applied onto columns equilibrated with buffer containing BSA, 2 nM [^{3}H]PDBu (as appropriate), and 1.0 mM EGTA. In each case, the applied sample (1.0 mL) contained the amount of PKC indicated. The amount of membrane-inserted PKC was calculated from the amount of kinase activity that remained associated with the vesicles (as in Figure 6B). [d] Where presented, error estimates represent the average and standard deviation of three experiments. [e] This is the estimated molar ratio of bound PDBu–PKC in the membrane-containing fractions.

during the chromatography time. Alternatively, PKC may have a lower affinity for PDBu in the absence of Ca^{2+}; 2 nM free PDBu may not be sufficient to maintain total saturation so that some PDBu would be released due to continual maintenance of equilibrium. To distinguish between these two possibilities, another portion of the PKC–phospholipid–[^{3}H]PDBu complex from Figure 4A was chromatographed on a column equilibrated with buffer containing EGTA but without PDBu. The result (triangles, Figure 4C) showed that all the [^{3}H]PDBu was rapidly released (<1-h estimate) and eluted at the inclusion volume. Therefore, PDBu that was bound to PKC in the presence of EGTA and 2 nM PDBu (circles, Figure 4C) was in equilibrium and exchanged many times during chromatography. Continual release during chromatography (Figure 4C) appeared to be due to lower binding affinity in the absence of calcium. Other experiments indicated that lower affinity arose from the dissociation rate constant for the PKC–vesicle complex (see below).

A further question concerned whether the reversibly membrane-associated PKC could bind PDBu. Figure 5A shows an elution pattern for PKC in the presence of calcium, [^{3}H]PDBu, and phospholipid. After 16 h, 32% of the PKC dissociated from the vesicles (Figure 5B). An important new property observed in Figure 5B was a peak of free [^{3}H]PDBu near the inclusion volume that was approximately equal to the amount of PKC that had been released from the membrane by EGTA. This indicated that PKC that was reversibly bound to the membrane did bind PDBu and that, upon dissociation from the membrane, this PDBu was released. This property was more obvious in subsequent experiments that are summarized in Table I. Consequently, there were at least two forms of PKC that were capable of binding PDBu.

The results in Figures 4 and 5 show the time dependence of insertion of PKC into membranes. The extent of PKC–membrane insertion was 11%, 68% and >75% after 20-min (Figure 4B), 16-h (Figure 5B), and 36-h (Figure 4C) incubations at 4 °C, respectively. Gopalakrishna et al. (1986) reported a strong temperature dependence for formation of the chelator-resistant form of membrane-bound PKC in cell membranes. The conditions used in this study were selected to allow investigation of PKC–membrane insertion on the time scale of gel chromatography. Insertion of PKC into membranes was much more rapid and efficient at higher temperatures or when certain crude phospholipid mixtures were used (Bazzi & Nelsestuen, 1988c). With purified components, the rate of this insertion could be enhanced by increasing the concentration of total phospholipid and decreased by lowering

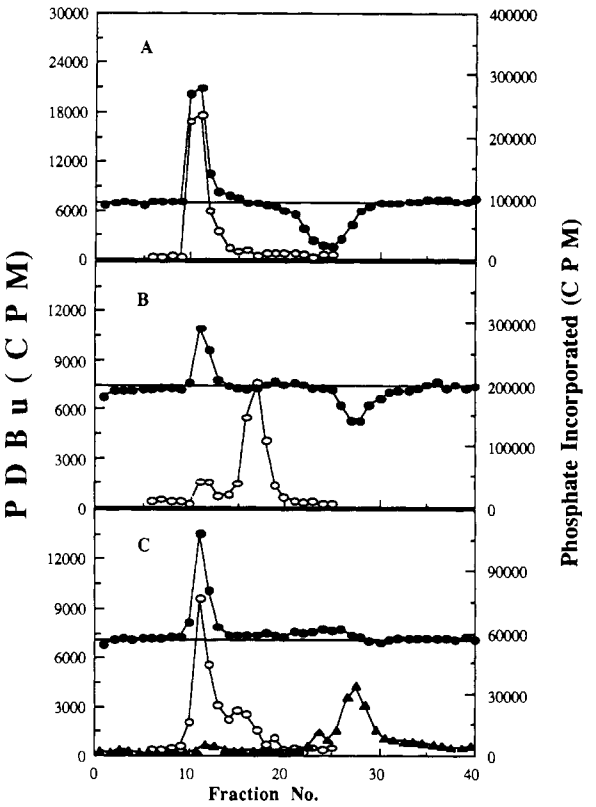


FIGURE 4: Binding of PDBu to membrane-inserted PKC. Two samples of PKC (1.6 μg) were each mixed with 200 μg of phospholipid vesicles [PS–PC (50:50)] in 0.6 mL of buffer containing BSA, 2 nM [^{3}H]-PDBu, and 0.5 mM Ca^{2+}. After 20 min, one sample was applied to a column (1.5 × 40 cm) equilibrated and eluted (2-mL fractions) with buffer containing BSA, 2 nM [^{3}H]PDBu, and 0.5 mM Ca^{2+} (panel A). EGTA (2.0 mM total concentration) was added to the second sample which was applied on a column equilibrated with buffer containing BSA, 2 nM [^{3}H]PDBu, and 1.0 mM EGTA (panel B). PDBu (●) and the kinase activity (○) of the fractions are shown. At 36 h after mixing of the original sample, the fractions containing maximum kinase activity (fractions 10–12 of panel A) were combined and made 2.0 mM in EGTA. Two aliquots (1.0 mL each) were immediately chromatographed on columns (panel C) equilibrated with buffer containing BSA and 1.0 mM EGTA plus (○, ●) or minus (▲) [^{3}H]PDBu. The elution of [^{3}H]PDBu (●, ▲) and kinase activity (○) are shown in panel C.

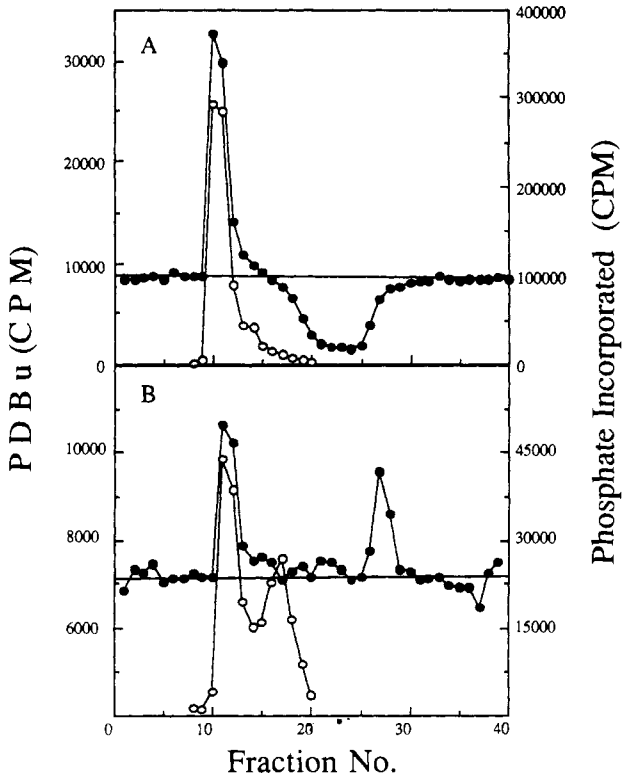


FIGURE 5: Reversible binding of PDBu to membrane-associated PKC. PKC (1.6 μg) was mixed with phospholipid vesicles (50 μg) in 0.6 mL of buffer containing BSA, 2 nM [^{3}H]PDBu, and Ca^{2+}. This sample was applied to a column (1.0 × 40 cm) and eluted (1-mL fractions) with the same buffer (panel A) Sixteen hours after mixing of the samples, the fractions containing maximum kinase activity (fractions 10–12, panel A) were combined, made 2.0 mM in EGTA, and incubated for an additional hour. One milliliter of that sample was applied to a column equilibrated with buffer containing BSA, 2 nM [^{3}H]PDBu, and 1.0 mM EGTA (panel B). In both panels, [^{3}H]PDBu (●) and kinase activity (○) were measured.

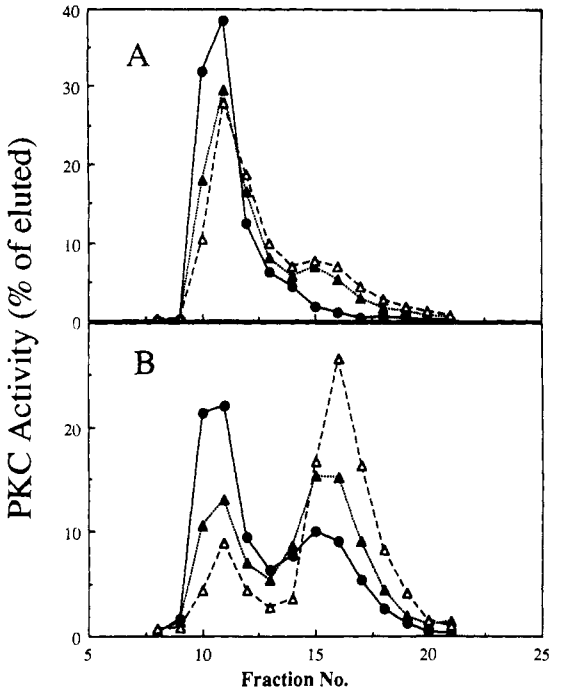


FIGURE 6: Factors influencing insertion of PKC into membranes. Phospholipid vesicles [50 μg of PS–PC (50:50)] and PKC (1.6 μg) were mixed in 0.6 mL of buffer containing BSA plus PDBu and/or Ca^{2+}, as appropriate. The sample was chromatographed on columns equilibrated with the same buffers: 0.5 mM Ca^{2+} and 2.0 nM PDBu (●, solid line), 2 μM Ca^{2+} and 2.0 nM PDBu (▲, dotted line), or 2.0 μM Ca^{2+} without PDBu (△, dashed line). Panel B shows dissociation of the PKC–membrane complexes generated in panel A. Fractions 10–12 from panel A containing maximum kinase activity were combined (16 h after the initial sample was prepared), made 2.0 mM in EGTA, and incubated for an additional hour. The samples were chromatographed on a column equilibrated with buffer containing BSA, PDBu (as appropriate), and 1.0 mM EGTA. The symbols correlate with the samples in panel A. The kinase activity associated with each fraction is plotted as a percentage of that eluted (i.e., the sum of kinase activity in all fractions is 100%).

the PS content of the membrane (see below). Under the experimental conditions used, PKC appeared stable and there was no evidence of proteolytic degradation. Proteolytic cleavage of PKC would release a catalytically active fragment (M_r = 55 000) which is easly distinguishable from the intact (M_r = 80 000) or membrane-associated kinase ($M_r > 2 \times 10^6$) by gel filtration.

Membrane composition, Ca^{2+} concentration, and PDBu each influenced the association of PKC with vesicles. PKC was first bound to phospholipid vesicles composed of PS–PC (50:50) in the presence of either 0.5 mM or 2.0 μM Ca^{2+} and in the presence or the absence of PDBu. At 2 μM Ca^{2+}, the small peak of free PKC indicated that binding of PKC to the membrane was not quantitative (triangles, Figure 6A). Similar experiments conducted with vesicles of 50%, 20%, and 10% PS showed 91%, 75% and 63% membrane-bound kinase activity, respectively (Table I). The peak of free PKC (2 μM Ca^{2+}) was approximately the same in the presence or absence of 2 nM PDBu (Figure 6A, Table I). Consequently, PDBu did not appear to influence calcium-dependent PKC–membrane binding to an extent detectable by this experiment. This agreed with previous observations which showed that phorbol esters did not change the Ca^{2+} requirements of PKC–membrane association (Bazzi & Nelsestuen, 1987a). Since PDBu binding was absolutely dependent on PKC–membrane association (see above), the lack of a large reciprocal effect was unexpected.

The extent of PKC insertion into these vesicles was assessed by dissociation after 16 h. An aliquot of fractions containing phospholipid-bound PKC (fractions 10–12, Figure 6A) was mixed with EGTA and rechromatographed (Figure 6B). The results, summarized in Table I, showed two patterns. First, less insertion of PKC into membranes occurred with membranes containing lower PS content. Second, with phospholipid vesicles of the same composition (20% or 50% PS), insertion of PKC into membrane was most pronounced at high Ca^{2+} and in the presence of PDBu. In all the cases involving PDBu, the PKC released from the phospholipid by EGTA also released a corresponding amount of PDBu; the membrane-inserted PKC continued to bind PDBu in the presence of EGTA at essentially the same molar ratio as was observed in the presence of Ca^{2+}.

Estimation of Binding Constants. A constant amount of PKC was applied to columns equilibrated with varying concentrations of PDBu (Figure 7A). At ≥2.0 nM PDBu, the equilibrium condition was satisfied; the peak of bound [^{3}H]-PDBu was followed by a region of constant [^{3}H]PDBu (e.g., fractions 15–25, Figure 7A) and then a trough. At 0.5 and 1.0 nM PDBu, the PKC depleted much of the [^{3}H]PDBu from the column so the amount of bound [^{3}H]PDBu may not represent an equilibrium measurement. Binding was clearly saturable (Figure 7B). A Scatchard plot of these data (Figure 7B, inset) showed a linear relationship with a dissociation constant of 0.5 nM. Since not all points were clearly at equilibrium, this represented the upper limit for the dissociation constant.

More accurate estimation of the equilibrium binding con-

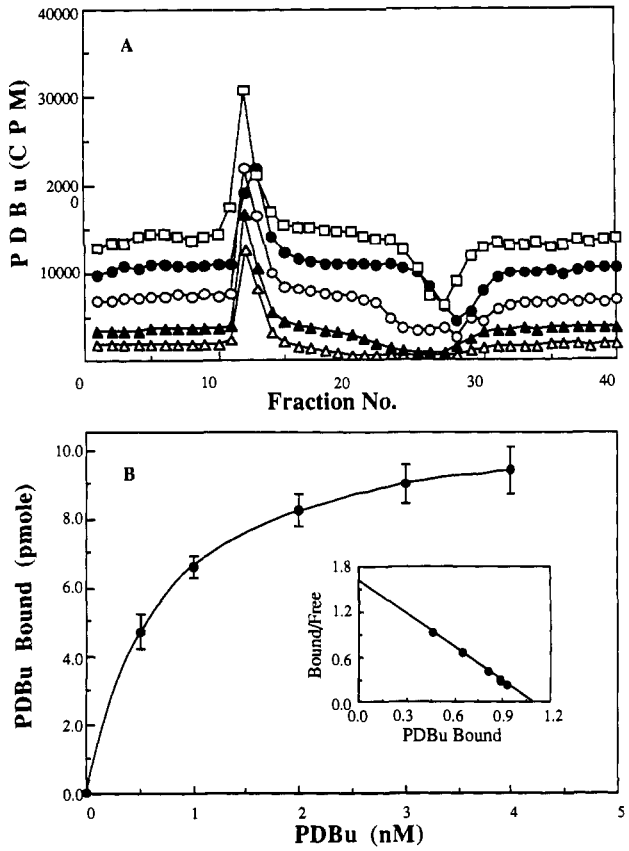


FIGURE 7: Estimation of PDBu binding parameters. PKC (0.8 μg) was mixed with phospholipid vesicles [200 μg of PS–PC (50:50)] in 0.6 mL of buffer. After 30 min, the sample was applied to columns of Sephacryl S-300 (1.0 × 40 cm) equilibrated and eluted (1.0-mL fractions) with buffer containing BSA, 0.5 mM Ca^{2+}, and PDBu [0.5 (△), 1.0 (▲), 2.0 (○), 3.0 (●), or 4 nM (□)]. The amount of PDBu bound was estimated from the peak and trough areas in the PDBu elution profile. Panel B shows bound PDBu as a function of free PDBu. The results show the average and standard deviation of two measurements. The inset shows a Scatchard plot of the same data.

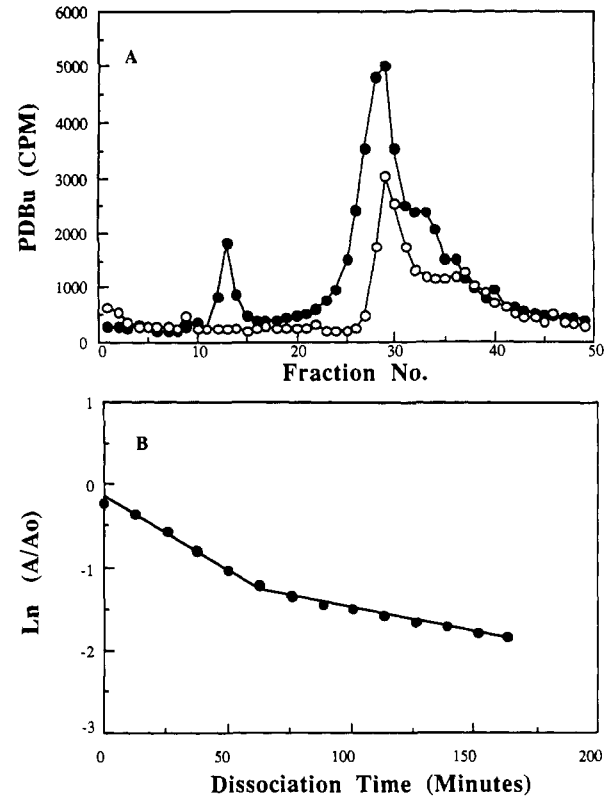


FIGURE 8: Estimation of the rate of PDBu dissociation from the PKC–phospholipid complex. A sample containing membrane-bound PKC plus [^{3}H]PDBu was obtained by the procedure outlined in Figure 5A. One milliliter of that sample (●) or 1.0 mL of the buffer used for column elution [containing free [^{3}H]PDBu (○)] was applied on a column equilibrated with buffer containing BSA, 0.5 mM Ca^{2+}, and 2.0 nM unlabeled PDBu (elution at 4.8 mL/h). Radioactivity that had dissociated from PKC was determined from the difference between the two elution profiles (see Experimental Procedures). Panel B shows a semilogarithmic plot of undissociated PDBu (A/A_0, where A is the amount of [^{3}H]PDBu bound at the time shown and A_0 is the amount bound at zero time). The slopes of the lines drawn correspond to dissociation rate constants of 1×10^{-4} and 2.7×10^{-4} s^{-1}.

stant will require use of much lower amounts of PKC and lower concentrations of PDBu. Unfortunately, these conditions pose substantial technical problems arising from measurement of PDBu, PKC, and quantitation of recovery. Many samples may contain mixtures of the two forms of membrane-associated PKC (inserted and reversible) which may have different binding constants. Furthermore, the low concentration of PDBu needed ($\sim 10^{-10}$ M) may give rise to artifacts that are difficult to document. More extensive investigations are needed before absolute binding constants for a single form of PKC can be determined.

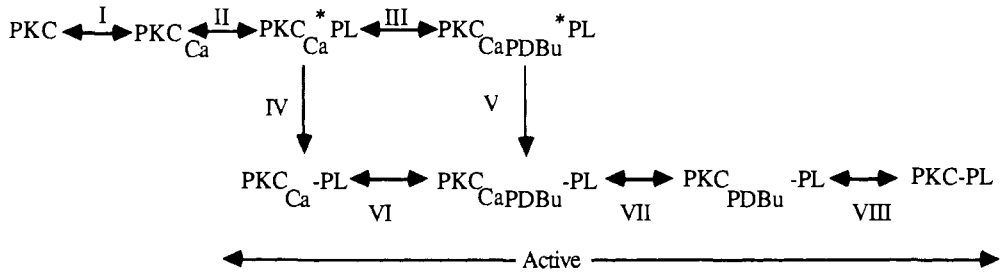
However, other properties of the interaction can give information about binding parameters that are valuable to experimental design. Slow dissociation rates are diagnostic of tight binding interactions. Figure 8A shows elution profiles of a column loaded with PKC–phospholipid–[^{3}H]PDBu complex (similar to the peak in Figure 4A) and eluted with buffer containing unlabeled PDBu. The process of PDBu exchange took place as the column was eluted and the released [^{3}H]-PDBu trailed behind the phospholipid–PKC complex. The peak of radioactivity coincident with the exclusion volume showed that dissociation was not complete within the 8 h of this chromatography. Dissociation was therefore much slower in the presence of Ca^{2+} (>8 h, Figure 8A) than in the absence of calcium (<1 h, Figure 4C). This relationship corroborated the observations that membrane-bound PKC bound PDBu with lower binding affinity in the absence of Ca^{2+} (Bazzi & Nelsestuen, 1988a).

The column elution profiles shown in Figure 8A were used to estimate the dissociation rate constant. The PKC–phospholipid–PDBu sample applied to the column contained both free and bound [^{3}H]PDBu. Contributions from free PDBu were determined by a control column (Figure 8A) and were subtracted as background. The first-order dissociation rate plot (Figure 8B) obtained from the data was biphasic with slopes corresponding to dissociation rate constants of (1.0–2.7) $\times 10^{-4}$ s^{-1}. The faster dissociation rate constant appeared to have greater error since it was obtained from fractions where the background from free PDBu was most prominent (fractions 30–25, Figure 8A). While the slower dissociation rate was probably more reliable, the difference was not great, and this approach was intended to provide only an approximation of the exchange rate. These observations showed that exchange was slow on the time scale of most in vitro experiments.

Discussion

PKC activity is usually considered to be phospholipid, diacylglycerol (or phorbol ester), and calcium dependent. However, observation of these requirements requires careful control of in vitro experimental conditions and the selection of appropriate substrates. Kinase activity and phorbol ester binding may also be generated, under a wide range of conditions, without some or all of the cofactors. Many in vitro situations may artificially give rise to kinase activity or phorbol

Scheme I



ester binding, and only a few conditions may be important physiologically. A full description of PKC, however, requires that even artificial activations (or phorbol ester binding) be consistent with enzyme properties. For example, previous studies showed that cofactor-independent activity toward protamine sulfate and calcium-independent activity toward other substrates arose from an aggregation process that appeared essential to phosphorylation of most common in vitro substrates (Bazzi & Nelsestuen, 1987b).

The properties of PKC–PDBu binding were dependent on the interactions of PKC with other components. The results presented in this and previous studies indicated that PKC–cofactor interactions produced several structures, perhaps in the sequence shown in Scheme I. In Scheme I, PL designates a phospholipid membrane. The reversible PKC–membrane complexes (upper line of equilibria) are designated by an asterisk (*) while irreversible PKC–membrane complexes (lower line) are designated by a dash (–).

Little is known about the interaction of Ca^{2+} with PKC (step I). In fact, calcium binding is largely inferred from the fact that calcium is needed to cause reversible PKC–membrane binding (step II). PKC would appear to provide a novel calcium binding site(s) since the sequence shows no homology to known sites such as EF-hand structures (Parker et al., 1986; Ohno et al., 1987). Calcium binding appeared to enhance the interaction of PKC with certain surfaces to form a complex that could bind PDBu with high affinity. Under physiological conditions, it is probable that only membranes provide such a surface (step III). In vitro, however, a suitable surface could be provided by phospholipid (Figure 1C), by a column matrix (Figure 2B), or possibly by an aggregated form of the enzyme itself (Figure 2C). In the absence of such a surface, calcium binding to the free enzyme did not allow detectable PDBu binding.

The existence of the reversible complex, $PKC_{Ca\cdot PDBu}$*PL (step III), was clearly documented by the results in Figures 5 and 6 and in Table I. In this complex, maintenance of the phorbol ester binding site appeared to require simultaneous interaction of PKC with a surface component, which in turn required the presence of Ca^{2+}. Dissociation of PKC from membrane by calcium chelation resulted in release of the PDBu (see Figure 5 for an example). A surprising observation was that PDBu enhanced the insertion of PKC into membranes but did not greatly alter the PKC–membrane binding equilibrium (2 μM calcium, Figure 6 and Table I). Earlier investigations (Bazzi & Nelsestuen, 1987a) also suggested that the reversible binding event (step II) was not altered to a detectable degree by PDBu. The phorbol esters therefore appeared to associate largely with the enzyme with minimal simultaneous interaction with the phospholipid component; the latter should have considerable effect on PKC–membrane interaction. It is possible that the more potent phorbol esters with long hydrocarbon chains (phorbol 12-myristate 13-acetate for example) may allow simultaneous interactions and may have a marked influence on reversible PKC–membrane interaction. Further studies are needed to clarify this point.

Studies with phosphatidylserine monolayers suggested that PKC–phospholipid interaction consisted of two steps: a simple Ca^{2+}-dependent binding step followed by insertion of PKC into the hydrocarbon region of the bilayer to form a structure with properties of an integral membrane protein (Bazzi & Nelsestuen, 1988b). The latter form of PKC displayed constitutive kinase activity toward the in vitro substrate, histone, which was not influenced by Ca^{2+} or phorbol esters (Bazzi & Nelsestuen, 1988a,c). Insertion of PKC into phospholipid vesicles could occur in the absence (step IV) or presence of PDBu (step V; data in Figure 6 and Table I). The resulting membrane-inserted PKC could bind Ca^{2+} and phorbol esters independently (step VII). While not essential for activity or PDBu binding, calcium enhanced the affinity of membrane-inserted PKC for PDBu via decreasing the rate of dissociation (Figures 4C and 8). That calcium completely dissociated from membrane-inserted PKC (step VII) has been shown by formation of the irreversible complex in the presence of ^{45}Ca followed by gel filtration in the presence of EGTA. Less that 0.1 equiv of ^{45}Ca per PKC remained associated after this treatment.[2]

Insertion of PKC into membranes may also be an important event in vivo. PKC is distributed between two states in the cell, a free or reversible membrane-bound form that can be extracted by calcium chelation and a tightly membrane-bound form that can only be released by detergents (Kikkawa et al., 1982; Shearman et al., 1987). Formation of the irreversible membrane-bound PKC population is frequently observed in whole cells [e.g., see Gopalakrishina et al. (1986)] and is often referred to as "translocation" of PKC to the membrane (Kraft & Anderson, 1983; Kraft et al., 1982). Translocation occurs upon treatment of cells with phorbol esters and appears analogous to step V of Scheme I. If in vivo activation is similar to that observed in vitro, the chelator-resistant PKC–membrane complex may no longer be regulated by diacylglycerol or calcium. These second messengers might serve a transient role in PKC activation, and their appearance would cause an accumulating signal (membrane-inserted PKC). This type of signal could provide mechanisms for long-term cell potentiation or cell memory (Alkon & Rasmusson, 1988; Bazzi & Nelsestuen, 1988c). Of course, in vitro properties cannot eliminate the possibility that phosphorylation of specific in vivo substrates is still influenced by phorbol esters and/or calcium. In fact, it is possible that the different forms of PKC shown in Scheme I may all have different in vivo substrates.

Despite several attempts (data not shown), we could not clearly determine whether the reversible complex, $PKC_{Ca,PDBu}$*PL (step III), was enzymatically active toward histone. The difficulty arose from multiple membrane-associated events in the assay. Activity is dependent on substrate–membrane aggregation (Bazzi & Nelsestuen, 1987b),

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

and the kinase assay employs high levels of magnesium. These factors could contribute to irreversible binding during the time of the assay. Nevertheless, a chelator-resistant membrane-bound form of PKC is clearly formed in vivo, and this may represent one major pathway for in vivo PKC activation.

Due to high-affinity interactions, accurate estimation of the PDBu–PKC binding parameters was very difficult and subject to considerable uncertainty. The PKC preparations used in this study probably contained a mixture of isozymes (Huang et al., 1986; Jaken & Kiley, 1987; Ono et al., 1987). While the different isozymes of PKC may bind PDBu with different affinities, this problem appeared to be relatively minor compared with other factors. For example, the level of free PDBu available for binding to PKC may be altered by significant migration of PDBu to the air–water interface. The existence of at least two forms of PKC capable of binding PDBu (reversible and irreversible membrane-bound forms) may produce two binding affinities for each isozyme. While species variability may contribute to observed differences, methodology and possible nonequilibrium conditions may be the major factors contributing to the difference between K_d obtained in this study ($\leq$0.5 nM) and those previously reported (generally 4–15 nM).

Another binding parameter that was difficult to measure with certainty was stoichiometry. The results strongly suggested a saturation stoichiometry of 1 mol of PDBu/mol of PKC (Figure 7B, inset). A major difficulty is related to determination of protein concentration (BSA may not be an appropriate standard for the protein assay). In addition, enzyme activity could under- or overestimate the amount of PDBu binding protein eluting from the column. Despite apparent agreement with previously reported values (Kikkawa et al., 1983; Tanaka et al., 1986), the 1:1 stoichiometry remained tentative. Overall, the K_d obtained was viewed as an upper limit, while the 1:1 stoichiometry was considered a minimum limit. More thorough knowledge of the structures and processes of PKC is needed before accurate binding parameters for a single PKC species can be obtained.

Registry No. PKC, 9026-43-1; PDBu, 37558-16-0; Ca, 7440-70-2.

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