Properties of Membrane-Inserted Protein Kinase C†

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ABSTRACT: Protein kinase C (PKC) interacted with phospholipid vesicles in a calcium-dependent manner and produced two forms of membrane-associated PKC: a reversibly bound form and a membrane-inserted form. The two forms of PKC were isolated and compared with respect to enzyme stability, cofactor requirements, and phorbol ester binding ability. Membrane-inserted PKC was stable for several weeks in the presence of calcium chelators and could be rechromatographed on gel filtration columns in the presence of EGTA without dissociation of the enzyme from the membrane. The activity of membrane-inserted PKC was not significantly influenced by Ca^{2+}, phospholipids, and/or PDBu. Partial dissociation of this PKC from phospholipid was achieved with Triton X-100, followed by dialysis to remove the detergent. The resulting free PKC appeared indistinguishable from original free PKC with respect to its cofactor requirements for activation (Ca^{2+}, phospholipid, and phorbol esters), molecular weight, and phorbol 12,13-dibutyrate (PDBu) binding. The binding of PDBu to free and membrane-inserted PKC was measured under equilibrium conditions using gel filtration techniques. At 2.0 nM PDBu, free PKC bound PDBu with nearly 1:1 stoichiometry in the presence of Ca^{2+} and phospholipid. No PDBu binding to the free enzyme was observed in the absence of Ca^{2+}. In contrast, membrane-inserted PKC bound PDBu in the presence or the absence of Ca^{2+}; calcium did enhance the affinity of this interaction. These results indicated that neither Ca^{2+} nor phorbol esters were needed to maintain the configuration of an active PKC complex once the enzyme was inserted into a membrane. Insertion into membranes could be the mechanism by which phorbol esters or diacylglycerols activate or induce long-term activation of PKC.

The Ca^{2+}- and phospholipid-dependent protein kinase C (PKC)[1] is a key regulatory enzyme believed to be involved in many cellular events [for reviews, see Nishizuka (1986) and Kikkawa and Nishizuka (1986)]. Activation of this enzyme under in vitro conditions requires the presence of cofactors that vary depending on the nature of the substrate (Bazzi & Nelsestuen, 1987a). For many purposes, category C substrates such as histone are the most interesting since their phosphorylation by PKC requires the presence of Ca^{2+}, phospholipid, and phorbol esters. Also, there is a strong synergism among these cofactors (Wolf et al., 1985a; Hannun et al., 1986; Bazzi & Nelsestuen, 1987b). However, the process of PKC activation and the mechanisms by which various activators function have been unclear.

Understanding the mechanism of PKC activation should demonstrate the biochemical basis for the effect of diacylglycerol (DAG) and/or phorbol esters on PKC. Previous models for PKC activation have suggested that Ca^{2+} and phorbol ester (or DAG) are needed to form a quaternary complex, PKC–phospholipid–Ca–DAG, that is essential for enzyme activity (Castagna et al., 1982; Kikkawa et al., 1983; Ashendel, 1985; Ganong et al., 1985; Parker et al., 1986). Phorbol esters have been proposed to activate PKC by increasing Ca^{2+} affinity. These ideas suggest that a sustained signal (continuous generation of DAG, for example) would be needed for long-term activation of PKC in the cell. However, DAG is only transiently found in biological membranes (Kikkawa et al., 1985; Nishizuka, 1984) so that long-term effects of PKC may require mechanisms other than continuous generation of DAG.

Recent studies with phospholipid monolayers (Bazzi & Nelsestuen, 1988a) or vesicles (Bazzi & Nelsestuen, 1988b) suggested that the binding of PKC to phospholipids consisted of two steps: simple and reversible binding that required a low concentration of calcium, followed by insertion of the enzyme into the membrane to form a structure that was not dissociable by calcium chelation. Once the enzyme was inserted into the membrane, the kinase activity was independent of Ca^{2+}, phospholipids, and/or phorbol esters (Bazzi & Nelsestuen, 1988b). While activation by insertion into membranes may be a general property of PKC, many questions remain about this form of the enzyme.

In this paper, we examined the activity, phorbol ester binding, and other properties of membrane-inserted and free PKC. The results showed that membrane-inserted PKC bound phorbol 12,13-dibutyrate (PDBu) in the presence or the absence of Ca^{2+}, but this binding event did not influence the activity of the enzyme. In contrast, the free form of PKC bound PDBu only in the presence of Ca^{2+} and phospholipid; this binding stimulated the kinase activity and appeared to be accompanied by membrane insertion. Free enzyme could be regenerated from membrane-inserted PKC by solubilization with detergent. These studies extend our knowledge of PKC activation in vitro and may suggest roles of second messengers (or lack thereof) in sustaining PKC activity in vivo.

Experimental Procedures

Materials. Bovine brain phosphatidylserine (PS), egg yolk phosphatidylcholine (PC), histone III-S, and protamine sulfate were purchased from the Sigma Chemical Co. [γ-^{32}P]ATP (3 Ci/mmol) was purchased from Amersham Corp. Nitro-

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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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cellulose filters (pore size 0.45 μm) were purchased from Millipore Corp. Phorbol 12,13-dibutyrate (PDBu) was purchased from LC Services Corp. [20-^{3}H(N)]PDBu (20 Ci/mmol) was purchased from New England Nuclear. Sepharose 4B and Sephacryl S300 are trade names of Pharmacia Fine Chemicals and were obtained through Sigma Chemical Co. Polycarbonate filters (0.1-μm diameter) were puchased from Nucleopore Corp.

Purification of Membrane-Inserted and Free PKC. Both membrane-inserted and free PKC were isolated from bovine brain according to a procedure outlined previously (Bazzi & Nelsestuen, 1987b). Briefly, bovine brains were extracted with buffer containing 10 mM EGTA and centrifuged at 13000*g*. The supernatant was chromatographed on a DEAE-cellulose column eluted with a NaCl gradient (0–0.3 M). The active fractions were dialyzed and loaded on another DEAE-cellulose column that was eluted by shifting the pH from 7.8 to 6.5 (Parker et al., 1984). PKC was bound to phospholipid vesicles (25% PS and 75% PC) in the presence of 0.5 mM Ca^{2+} and then applied on a Sepharose 4B column. Fractions containing PKC, which eluted with the phospholipid vesicles in the void volume of the column, were combined and made 10 mM in EGTA. The sample was rechromatographed on a Sepharose 4B column equilibrated with buffer containing 20 mM Tris (pH 7.8) plus 1.0 mM EGTA, 1.0 mM EDTA, 30 mM β-mercaptoethanol, and 150 mM NaCl. The elution profile from this column showed two peaks of protein kinase activity (Figure 1). The activity that eluted at the exclusion volume of the column was termed membrane-inserted PKC, whereas free PKC eluted near the inclusion volume of the column (largest peak in Figure 1). The fractions containing free PKC were collected, concentrated, and gel filtered on Sephacryl S300 (Bazzi & Nelsestuen, 1987b).

Isolation of Free PKC from Membrane-Inserted PKC. Triton X-100 was added to a portion (20 mL) of the membrane-inserted PKC preparation (Figure 1) to solubilize the phospholipid component [final concentration of about 1% Triton (v/v) was usually sufficient]. The sample was dialyzed against 4 L (two changes) of buffer containing 20 mM Tris (pH 7.5), 30 mM β-mercaptoethanol, 1.0 mM EGTA, and 1.0 mM EDTA. Final traces of Triton X-100 were removed by 10 cycles of sample dilution/concentration (20–200 mL) with an ultrafiltration cell equipped with a PM-10 membrane. The final sample was applied on a Sephacryl S300 column (2.5 × 120 cm) and eluted with same buffer used in dialysis.

PKC–PDBu Binding. The binding of PDBu to PKC was measured at equilibrium by gel filtration chromatography as described by Hummel and Dreyer (1962). Sephacryl S300 columns (1.5 × 40 cm) were equilibrated and eluted (5 mL/h) with buffer containing 20 mM Tris (pH 7.5), 2.0 nM [^{3}H]-PDBu, 10% glycerol, 0.5 mg/mL bovine serum albumin, 30 mM β-mercaptoethanol, and either 0.5 mM Ca^{2+} or 1.0 mM EGTA. A known amount of free PKC was made 2.0 nM in [^{3}H]PDBu (1-mL volume) and loaded on the Sephacryl S300 column in the presence of phospholipid vesicles (0.2 mg/mL) and either 0.5 mM Ca^{2+} or 1.0 mM EGTA. Binding of PDBu to membrane-inserted PKC was determined under the same conditions except that no additional phospholipid was added. The PKC–PDBu binding was estimated by measuring the radioactivity and the kinase activity associated with each fraction. A peak and a trough of radioactivity are generated, and both of these were used to determine the amount of bound PDBu. The kinase activity in each fraction was measured with protamine sulfate as a substrate since its phosphorylation by PKC is not influenced by Ca^{2+} (Takia et al., 1977).

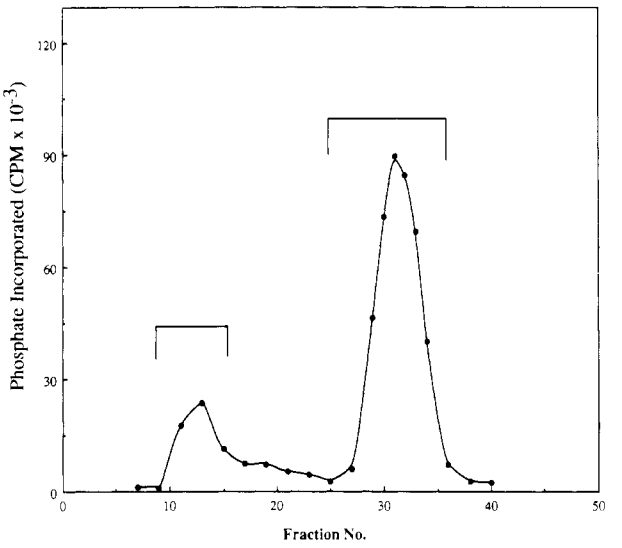

FIGURE 1: Isolation of free and membrane-inserted PKC. Membrane-bound PKC, isolated by gel filtration in the presence of Ca^{2+}, was made 10 mM with EGTA and applied on a Sepharose 4B column (4.5 × 80 cm) equilibrated with a buffer containing 20 mM Tris, pH 7.8, 150 mM NaCl, 1.0 mM EGTA, 1.0 mM EDTA, and 30 mM β-mercaptoethanol. The column was eluted with the same buffer, and fractions (22 mL) were collected. Membrane-inserted PKC eluted at the void volume (first peak), while free PKC eluted near the inclusion volume of the column (second peak).

Preparation of Phospholipid Vesicles. Large unilamellar vesicles composed of PS:PC (25:75) were prepared by extrusion (Hope et al., 1985). The phospholipids were mixed, dried from organic solvent under a stream of nitrogen, and then suspended in 20 mM Tris buffer (pH 7.5) by vigorous agitation. The suspension was freeze-thawed five times and extruded through a polycarbonate filter (0.1 μm) ten times.

Other Methods. 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). Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as the standard. Phospholipid concentrations were determined from organic phosphate by the method of Chen et al. (1956) using a phosphorus to phospholipid weight ratio of 1:25.

RESULTS

Characterization of Membrane-Inserted PKC. Addition of EGTA to membrane-associated PKC showed the presence of two populations. The majority of PKC activity dissociated from the phospholipid vesicles and eluted near the inclusion volume of the column while a smaller portion of the protein kinase activity remained associated with the phospholipid vesicles and eluted at the exclusion volume of the column (Figure 1). The latter form of PKC was not the result of a partial adsorption that would slowly dissociate. The kinase activity in these fractions was stable to storage in the presence of calcium chelators for up to 4 weeks at 4 °C and could be rechromatographed on gel filtration columns in the presence of EGTA without dissociation from the phospholipids (see below). Qualitatively, these results are in agreement with a previous study (Bazzi & Nelsestuen, 1987b) which indicated that the binding of PKC to phospholipid vesicles was not totally reversible once the PS content of the vesicles exceeded 20%. Studies with phospholipid monolayers suggested that irreversible PKC–phospholipid binding was the result of insertion of PKC into membranes (Bazzi & Nelsestuen, 1988a).

The activity of membrane-inserted PKC was measured and compared to that of free PKC. The results (Figure 2) showed

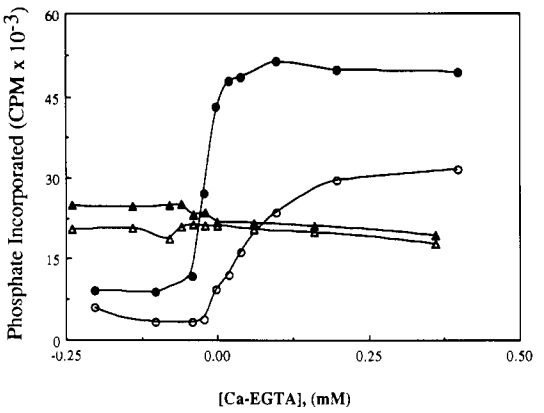

FIGURE 2: Cofactor requirements of free and membrane-inserted PKC. The activities of free PKC (●, ○) and membrane-inserted PKC (▲, △) were measured in the presence (solid symbols) or the absence (open symbols) of 30 nM PDBu. Activity was measured in 20 mM Tris, pH 7.5, 10 mM $MgCl_2$, 0.2 mg/mL histone III-S, 0.10 mg/mL phospholipid vesicles, and either free PKC (40 ng) or membrane-inserted PKC (10 μL of the fractions pooled in Figure 1).

that the activity of free PKC displayed the usual properties, was dependent on phospholipid and Ca^{2+}, and was stimulated by PDBu especially at low calcium concentrations (Figure 2). In contrast, the activity of membrane-inserted PKC was not influenced by the presence of phorbol esters and/or Ca^{2+}. Consequently, insertion of PKC into membrane produced protein kinase with constitutive activity toward the substrate histone. These properties corroborate previous findings (Bazzi & Nelsestuen, 1988b).

One possible explanation for the differential effect of PDBu on the PKC preparations would be that free enzyme binds PDBu in the presence of phospholipids and Ca^{2+}, while membrane-inserted PKC does not. The binding of phorbol esters to PKC has previously been reported (Tanaka et al., 1986; Kikkawa et al., 1983; Niedel et al., 1983; Sando & Young, 1983). While the innovative methods developed for this binding assay have been valuable for many important qualitative observations, the measurements were not performed at equilibrium since they included precipitation, ionic or filter trapping, and washing or gel filtration of the complex to remove free or loosely bound PDBu. Recent observations show that substantial artifacts can arise from use of polycations such as DEAE-cellulose in the assay[2] (Thompson et al., 1988). To avoid these problems, the binding of [^{3}H]PDBu to both populations of PKC was measured under equilibrium conditions with a gel filtration procedure (Hummel & Dreyer, 1962).

Free PKC bound to phospholipid vesicles in the presence of Ca^{2+} and eluted at the exclusion volume of the column (Figure 3A). The PKC–phospholipid complex bound PDBu as indicated by increased PDBu in fractions containing the enzyme and the subsequent trough in the PDBu elution pattern. In the absence of Ca^{2+} (Figure 3B), free PKC did not bind phospholipid vesicles and eluted at its expected position. At this concentration of PDBu there was no detectable PKC–PDBu binding (Figure 3B). In the presence of Ca^{2+}, membrane-inserted PKC bound PDBu and eluted at the exclusion volume of the column (Figure 3C). In the absence of Ca^{2+} (Figure 3D) membrane-inserted PKC remained associated with phospholipid and still bound some PDBu. The experiments in Figure 3C,D were conducted with membrane-inserted PKC that had been stored in the presence of EGTA for 4 weeks (4 °C). This illustrated the stability of the complex.

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

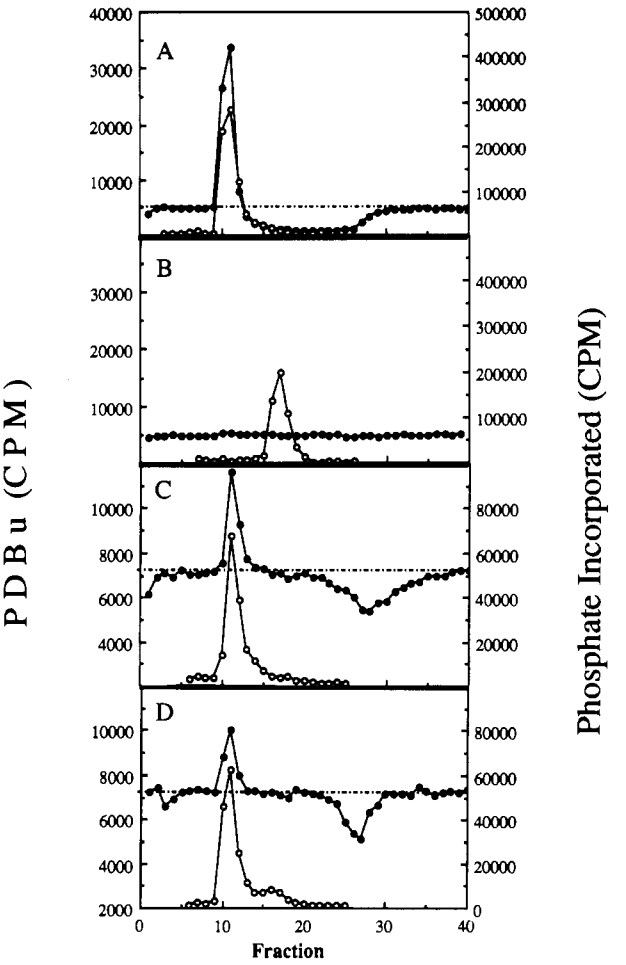

FIGURE 3: Binding of PDBu to free and membrane-inserted PKC. Columns were equilibrated and eluted (2-mL fractions) with a buffer containing 20 mM Tris, pH 7.8, 2.0 nM [^{3}H]PDBu, 10% glycerol, 0.5 mg/mL BSA, 30 mM β-mercaptoethanol, and either 0.5 mM Ca^{2+} (panels A and C) or 1.0 mM EGTA (panels B and D). The measurements were performed with either free PKC (panels A and B) or membrane-inserted PKC (panels C and D). The PDBu (solid symbols) represents radioactivity in 250 μL. Kinase activity (214-μL samples) toward protamine sulfate (open symbols) is shown.

Table I: Quantitative Estimation of PKC–PDBu Binding[a]

PKC sample + condition	PDBu bound (peak) (pmol)	PDBu bound (trough) (pmol)	av[b]	PDBu bound/ PKC (pmol/ pmol)
free PKC + PL + Ca^{2+}	44.2	46.8	45.5	0.90[c]
free PKC + PL + EGTA	0.90	1.2	1.1	0.02[c]
mem-ins PKC + Ca^{2+}	3.10	4.54	3.8	0.92[d]
mem-ins PKC + EGTA	1.45	2.36	1.9	0.46[d]

[a] Quantitative estimation of PKC–PDBu binding was obtained by the equilibrium measurements presented in Figure 3. Nonspecific binding of PDBu to phospholipids was estimated by similar measurements in experiments where PKC was omitted. This low contribution [0.35 pmol for free PKC and 0.63 pmol for mem-ins (membrane-inserted) PKC] has been subtracted as a background. [b] Average of peak and trough estimates. [c] Measurements with free PKC were performed with 4 μg (50.6 pmol) of PKC and 250 μg of phospholipids (25% PS and 75% PC; the presence of phospholipid is designated by the abbreviation +PL). [d] The amount of membrane-inserted PKC (4.1 pmol) was estimated from activity measurements. Free PKC and membrane-inserted PKC were assumed to have identical specific activity toward protamine sulfate.

Quantitative estimation of PDBu binding could be obtained from the PDBu peak associated with the phospholipid–PKC complex or from the trough of PDBu in the elution profile

(Table I). At a PDBu concentration of 2.0 nM and in the presence of Ca^{2+} and phospholipid, approximately one molecule of PDBu was bound per PKC molecule. Similar columns run at 4–16 nM PDBu gave similar stoichiometry.[2] Since this stoichiometry was based on the amount of PKC loaded on the column rather than on actual protein mass present in the same fractions, it is tentative (i.e., the enzyme recovery was assumed to be 100%). While a similar stoichiometry was obtained in the previous determinations (Tanaka et al., 1986; Kikkawa et al., 1983), the equilibrium measurements in Figure 3 showed tighter binding with one molecule of PDBu bound per PKC molecule at 2.0 nM PDBu. Previous studies have reported K_d values of 4–11 nM (Tanaka et al., 1986; Kikkawa et al., 1983; Niedel et al., 1983; Sando & Young, 1983).

Quantitative estimation of PDBu binding to membrane-inserted PKC was more difficult, and the concentration of membrane-inserted PKC had to be estimated from activity measurements. This assumed that both forms of PKC (free and membrane inserted) had constant specific activity toward protamine sulfate. Within the limits of this assumption, membrane-inserted PKC bound PDBu with approximately unit stoichiometry (Table I) in the presence of Ca^{2+}. In the absence of Ca^{2+} about half as much PDBu was bound. It is possible that membrane-inserted PKC had higher affinity for PDBu in the presence of Ca^{2+}. Higher affinity could arise directly, from interaction between the calcium and phorbol ester binding sites, or indirectly, from the effects of 0.5 mM calcium on the phospholipid. In any event, it is clear that membrane-inserted PKC did bind PDBu in the presence and the absence of calcium. Consequently, the failure of PDBu to influence the activity of membrane-inserted PKC (Figure 2) was not due to a failure of PDBu to interact with PKC.

Isolation of Free PKC from the Membrane-Inserted Preparation. Previous studies showed that irreversible binding of PKC to membrane was influenced by factors such as the concentration of Ca^{2+}, the duration of PKC-membrane binding, and/or the presence of phorbol esters (Bazzi & Nelsestuen, 1988b). However, it was not known whether insertion of PKC into the membrane produced an irreversible change in PKC. Membrane-inserted PKC obtained from material shown in Figure 1 was solubilized with Triton X-100, and the detergent was removed by dialysis (see Experimental Procedures). Gel filtration (Figure 4) showed partial release of PKC. The newly generated free enzyme displayed the expected properties of free PKC; it phosphorylated histone III-S only in the presence of Ca^{2+} and phospholipid (Figure 4), the activity was greatly stimulated by PDBu at low Ca^{2+} concentrations (data not shown), and the protein eluted from gel filtration at the correct position. In contrast, the remaining membrane-inserted enzyme phosphorylated histone in a Ca^{2+}- and phospholipid-independent manner (Figure 4). Thus, the membrane-inserted protein was highly stable to storage at 4 °C in the presence of EGTA but returned to its free state when released by detergent.

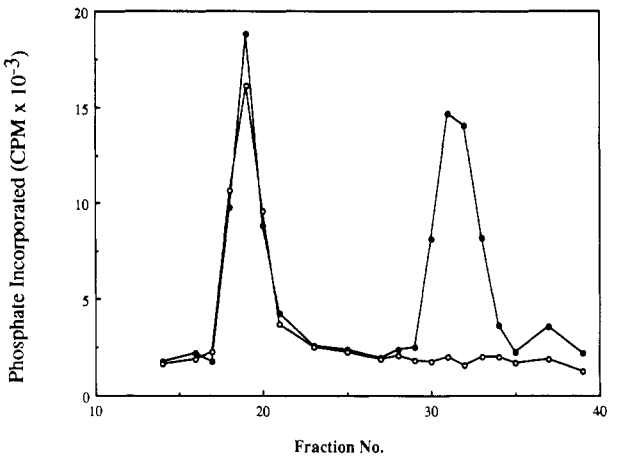

FIGURE 4: Solubilization of membrane-inserted PKC. Membrane-inserted PKC (20 mL from Figure 1) was solubilized with Triton X-100, and the detergent was removed as described under Experimental Procedures. The resulting sample was chromatographed on a Sephacryl S300 column (2.5 × 120 cm) equilibrated with 20 mM Tris, pH 7.8, 1.0 mM EGTA, 1.0 mM EDTA, and 30 mM β-mercaptoethanol. The kinase activity toward histone was measured in the presence of either 0.2 mg/mL phospholipid vesicles plus 0.5 mM Ca^{2+} (●) or 1.0 mM EGTA (○).

Discussion

This study investigated in vitro properties of PKC with the objectives of understanding the properties of the purified enzyme, especially those related to in vivo behavior of PKC. In agreement with observations made with phospholipid monolayers (Bazzi & Nelsestuen, 1988b), PKC formed a chelator-resistant, irreversible complex with purified phospholipid vesicles that could be dissolved with detergents. This form of PKC is referred to as "membrane-inserted PKC", which implies interaction of some portion of the protein with the hydrocarbon region of the membrane. The characteristics of this protein appeared analogous to a form observed in whole cells or cell membranes, which is described as chelator-stable and detergent-extractable membrane-associated PKC (Gopalakrishna et al., 1986). The population of irreversibly membrane-bound PKC (membrane-inserted PKC) described here had unusual activity and phorbol ester binding properties. The possibility that these properties might be applicable in the cell has important implications for PKC regulation by its second messengers.

Binding of phorbol esters and activation of PKC were found to be separable. While phorbol esters bound to membrane-inserted PKC, neither they nor Ca^{2+} had significant effect on the activity of this form of the enzyme. Previous studies showed that irreversible PKC–membrane binding was enhanced by phorbol esters, high Ca^{2+} concentrations, and membranes of high PS content (Bazzi & Nelsestuen, 1988b). It is possible that many in vitro activators of PKC, including phorbol esters, may function via the single mechanism of causing PKC–membrane insertion (Bazzi & Nelsestuen, 1988b). Phorbol esters and calcium would then serve transient and irreversible roles in PKC activation. Membrane insertion appeared to be a relatively rare process since only about 15% of the PKC that was membrane bound by calcium became irreversibly bound (Figure 1). Activation of PKC via an irreversible step also indicated that in vitro titrations of PKC activity cannot be analyzed by the usual steady-state kinetic expressions.

Recently, changes in PKC have been implicated in sustained cellular responses (Alkon & Rasmussen, 1988, and references cited therein). Sustained activation of PKC under in vivo conditions would appear to require regulatory mechanisms other than continuous generation of DAG. The results presented here and previously (Bazzi & Nelsestuen, 1988b) suggest that a protein conformation change followed by insertion into the membrane is a viable mechanism for generating the tightly bound PKC–membrane complex. A rate-limiting process consisting of membrane insertion, as observed in vitro, provides a mechanism for gradual cell regulation; multiple cell stimulations may be needed to significantly alter the amount of PKC that is inserted into the membrane. While it is possible that Ca or DAG still influences the activity of membrane-associated PKC toward its specific in vivo substrates, the in

vitro properties shown here suggest that this form of PKC may no longer be regulated by DAG and calcium but may be regulated by mechanism(s) that is (are) not yet identified. Overall, the in vitro properties of PKC illustrate a possible mechanism by which Ca^{2+} and DAG could produce accumulating effects that function in long-term cell potentiation or memory (Alkon & Rasmussen, 1988; Bazzi & Nelsestuen, 1988b).

It is well established that there are multiple PKC isozymes, and several forms have been isolated from the brain (Huang et al., 1986; Ono et al., 1987; Jaken & Kiley, 1987). The preparations of enzyme used here were mixtures, but virtually all activity could become irreversibly associated with the membrane.[2] If different isozymes of PKC insert into membranes with different levels of ease, response to the second messengers might differ between cell types. Response to messengers could also be influenced by cell membrane or organelle phospholipid distribution since composition had a strong effect on irreversible binding (Bazzi & Nelsestuen, 1987b). While future studies may find such quantitative differences, Shearman et al. (1987) recently reported that PKC isolated from membranes had about the same isozyme distribution as the cytosolic protein.

Activity of PKC toward the commonly used in vitro substrates is dependent on binding of the substrate to the membrane and subsequent aggregation (Bazzi & Nelsestuen, 1987a). A recent study with phospholipid monolayers (Bazzi & Nelsestuen, 1988a) suggested that assembly of PKC and histone III-S on planer phospholipid monolayers in the presence of Ca^{2+} and phorbol esters was not sufficient for avid phosphorylation of substrate. Aggregation may also be critical to activation by short-chain PC (Walker & Sando, 1988). A counterpart to aggregation may or may not be required under in vivo conditions; PKC may have much greater substrate specificity or unidentified mechanism(s) for delivering substrate to the active site of PKC may exist.

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