Calcium and Phosphatidylserine Stimulate the Self-Association of Conventional Protein Kinase C Isoforms†

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ABSTRACT: Conflicting evidence exists as to whether "conventional" protein kinase C isoforms (cPKCs) function as monomers or oligomers. In this report, we demonstrate that purified cPKC isoforms can be rapidly cross-linked by the sulfhydryl-selective cross-linker bis(maleimido)hexane, but only in the presence of both Ca^{2+} and phosphatidylserine; cross-linking was minimal in the presence of either of these activators alone. In addition, cross-linking of these cPKCs did not require Mg^{2+} or ATP. Among the various phospholipids tested, phosphatidylserine was found to be the most effective in the promotion of cPKC self-association and for the stimulation of protein kinase activity toward the exogenous substrate histone. Phosphatidic acid and phosphatidylinositol were less effective in this regard, whereas phosphatidylcholine exhibited little ability to induce cPKC self-association or to stimulate kinase activity. An examination of the mechanism by which the cPKC isoforms self-associate in the presence of phospholipid/Ca^{2+} revealed that this process occurred independently of phospholipid aggregation. Moreover, self-association was not inhibited by saturating the enzyme active site with a peptide substrate, suggesting that self-association is distinct from an enzyme−substrate interaction. Isoform-specific antibodies revealed that all cPKC isoforms (α, *β*, and *γ*) self-associate and that, in a mixture of cPKC isoforms, PKC-α forms primarily α−α homodimers. Besides cPKC interactions detected with purified enzyme, PKC-α also appeared capable of self-association in murine B82L fibroblasts that were treated with calcium ionophore, phorbol ester, or epidermal growth factor but not in untreated cells. Collectively, these data indicate that self-association occurs in parallel with cPKC activation, that self-association is not mediated by the substrate binding site, and, at least in the case of PKC-α, that the formation of isoform homodimers predominates.

Protein kinase C (PKC)[1] was initially described as a Ca^{2+}- and phospholipid-dependent protein kinase (*1*). It is known to be critical in the control of many cellular processes, including the regulation of metabolism, receptor signal transduction and feedback, cell growth and differentiation, and hormone and neurotransmitter secretion (*2*−*4*). Because of the importance of PKC in cellular function, many aspects of its structure, regulation, and physical properties have been extensively investigated (*5, 6*).

Although PKC was originally thought to be a single protein, further analyses of purified PKC and the isolation of cDNA clones has shown PKC to be a family of at least 12 related proteins (*5*−*8*). All PKC isoforms identified so far require phospholipid for full activation. Furthermore, the various isoforms can be grouped according to their additional activator requirements: the "conventional" or "classical" PKCs (cPKCs, including α, *β*I, *β*II, and *γ* isoforms) require Ca^{2+} for full activity and are stimulated by diacylglycerol or phorbol esters; the "novel" PKCs (*δ*, *ϵ*, *ϵ*′, *θ*, *η*, and *μ* isoforms) are Ca^{2+}-independent but are stimulated by diacylglycerol or phorbol esters; and the "atypical" PKCs (*λ*/*ι* and ζ isoforms) are Ca^{2+}-independent and are not stimulated by diacylglycerol or phorbol esters. The various isoforms also have some differences in substrate specificity and their ability to be activated by certain phospholipids, fatty acids, and phorbol esters (*5, 6, 9*−*12*).

The regulation of the cPKCs by Ca^{2+} and lipids is one aspect of PKC function that has been studied in detail. Many reports show that the binding of phospholipid/Ca^{2+} to cPKCs releases an autoinhibitory pseudosubstrate site from the substrate binding region (*13*−*15*). This allows for the enhanced access of exogenous substrates and autophosphorylation sites to the catalytic domain.

Results from several investigations indicate that cPKCs may function as monomers in the presence of phospholipid−diacylglycerol−detergent micelles (*16*, *17*). However, other reports suggest that cPKCs are most active as multimers when stimulated by phospholipid vesicles and Ca^{2+} (*18*−

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[1] Abbreviations: BMH, bis(maleimido)hexane; BSA, bovine serum albumin; cPKCs, "conventional" isoforms of protein kinase C including PKC-α, -*β*, and -*γ*; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; EGF, epidermal growth factor; EGTA, ethylene glycol bis(*β*-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; SDS, sodium dodecyl sulfate.

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20). In addition, recent evidence indicates that PKC autophosphorylation and kinase activity toward heterologous substrates decrease as the PKC concentration is diluted by high lipid concentrations, which may reflect diminished enzyme−enzyme interaction on the lipid surface (*21*). This observation is consistent with our previous report that increasing the amount of cPKC relative to phospholipid/Ca^{2+} substantially enhances cPKC autophosphorylation (*22*), which would be expected if cPKCs function as oligomers. Nonetheless, these earlier studies focused on the kinase activity of cPKCs under various reaction conditions, which is a method that does not directly address whether cPKCs form oligomers. Therefore, in the current studies we utilized the sulfhydryl-selective cross-linker bis(maleimido)hexane (BMH) to directly examine cPKC self-association. The results from our studies indicate that cPKCs self-associate in an isoform-selective manner under activating conditions, suggesting that oligomer formation plays an important role in the regulation of cPKCs.

MATERIALS AND METHODS

Materials. [γ-^{32}P]ATP and enhanced chemiluminescence immunoblot detection kits were purchased from Amersham Corp. (Arlington Heights, IL). BMH was obtained from Pierce (Rockford, IL). Rabbit isoform-specific antibodies against PKC-α and -β were purchased from Life Technologies (Gaithersburg, MD), and the anti-PKC-γ antibody was obtained from Transduction Labs (Lexington, KY). The calcium ionophore A23187 was purchased from Calbiochem (San Diego, CA). Immobilon-P poly(vinylidene difluoride) membranes and ultrafiltration membranes were obtained from Millipore (Bedford, MA). Recombinant human epidermal growth factor (EGF) was received from Upstate Biotechnology Inc. (Lake Placid, NY). EGF receptor peptide (H-Lys_{652}-Arg-Thr-Leu-Arg-Arg_{657}-OH) and phorbol 12-myristate 13-acetate (PMA) were purchased from LC Laboratories (Woburn, MA). Stable transfectants of mouse B82L fibroblasts expressing the normal human EGF receptor were generated as described earlier (*23*). Dulbecco's modified Eagle's medium (DMEM) was obtained from Gibco Laboratories (Grand Island, NY), and fetal bovine serum was purchased from Hyclone Laboratories (Logan, UT). L-α-Phospholipids and all other chemicals were obtained from Sigma (St. Louis, MO).

Purification of cPKCs. cPKCs were purified from rat brain by a modification of the method of Walton et al. (*24*). The purification entails three chromatographic steps: (i) ion exchange, (ii) hydrophobic interaction, and (iii) affinity purification with protamine−agarose. The purified preparation was subjected to successive steps of ultrafiltration through membranes of 10 000 or 30 000 MW pore size (to remove contaminants and to reduce NaCl levels to a final concentration of <5 mM). The buffer containing the concentrated cPKCs was adjusted to 1 mM dithiothreitol, 2 mM ethylenediaminetetraacetic acid (EDTA), 2 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 20% glycerol, and 0.02% Triton X-100, and the preparation was stored at −70 °C. Sequencing of cPKC purified by this method indicates that this preparation contains a mixture of the α, β, and γ isoforms (*24*). However, to further confirm the profile of these cPKC preparations, we obtained isoform-specific antibodies (Pan Vera Corp., Madison, WI) that have been previously shown to react with either the α, β (I and II), γ, δ, ϵ, η, or ζ isoforms of rat PKC. Immunoblotting analysis revealed that the α, β, and γ isoforms are the predominant PKCs that can be readily detected in our preparations, although a trace amount of immunodetectable PKC-ϵ was also observed occasionally.

Cross-Linking of cPKCs. Unless otherwise stated, the reactions were performed in 100 μL of a reaction buffer consisting of 20 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.4), 10 μL of concentrated PKC (0.1−1 μg), 100 μM dithiothreitol, 200 μM EDTA, 200 μM EGTA, 0.002% Triton X-100, 2% glycerol, and the various additions as described in the figure legends. The samples were incubated at room temperature (22 °C) for 5 min, followed by the addition of BMH (final concentration 1−100 μM) in dimethyl sulfoxide. The levels of dimethyl sulfoxide in these reactions (≤4%) had no effect on cPKC activity (data not shown). The reactions were terminated by the addition of 15−25 μL of sodium dodecyl sulfate (SDS) electrophoresis sample buffer, and the mixtures were analyzed by SDS−polyacrylamide gel electrophoresis (PAGE) followed by silver staining or immunoblotting.

Phospholipid Preparation. The required amounts of phospholipid dissolved in chloroform/methanol were dried under a stream of N_2 and resuspended in 20 mM HEPES (pH 7.4). The mixture was then sonicated at 4 °C for ∼10 s with a Branson sonifer (Danbury, CT).

Assay for cPKC Enzyme Activity. Unless otherwise noted, enzyme activity was assayed in 60 μL of a reaction buffer consisting of 20 mM HEPES (pH 7.4), 100 μg/mL phosphatidylserine, 1 mM $CaCl_2$, 1 mM dithiothreitol, 1 mg/mL histone (Sigma type III-S), 10−100 ng of cPKCs, 10 mM $MgCl_2$, and 50 μM [γ-^{32}P]ATP [specific activity ∼ (0.3−3) × 10^4 cpm/pmol]. After the samples were warmed to 30 °C for 2 min, the reactions were initiated by the addition of the $MgCl_2$/ATP mixture as described previously (*24*). After 2 min, the reactions were terminated by the addition of 30 μL of a 0.5% phosphoric acid solution and cooling to 0 °C. Phosphate incorporation into histone was measured by spotting an aliquot of the reaction mixtures onto phosphocellulose filters, rinsing several times with ice-cold 0.5% phosphoric acid, drying, and measuring the radioactivity bound to the filters via scintillation counting.

Assay for PKC Autophosphorylation. The reactions were carried out under the same conditions as described for the assay for cPKC enzyme activity except that the incubations contained 1 μM [γ-^{32}P]ATP [specific activity ∼ (1−5) × 10^5 cpm/pmol], 0.1−1 μg of cPKCs, and no histone. The reactions were initiated by the addition of $MgCl_2$/ATP, incubated for 2 min at room temperature (22 °C), and terminated by the addition of 5 μL of SDS electrophoresis sample buffer. The samples were resolved by SDS−PAGE and the gels were silver-stained. The bands corresponding to cPKCs were excised and analyzed for incorporated radioactivity.

Analysis of Phospholipid Aggregation. All measurements were performed at room temperature (∼22 °C). Phospholipid aggregation was assessed by measuring the light scattering of phospholipid-containing solutions (*25*). Lipid mixtures were prepared as described above and added to a 1 mL (final volume) solution containing 20 mM HEPES (pH 7.4), 100 μM dithiothreitol, 200 μM EDTA, 200 μM EGTA, 0.002%

Triton X-100, 2% glycerol, and the various additions as indicated. The solutions were then incubated for 5 min, after which the absorbance at 350 nm was measured on a Beckman (model 2400) spectrophotometer. An increase in absorbance reflects an increase in phospholipid aggregation (*25*).

Immunoblotting. Samples separated by SDS−PAGE were electrophoretically transferred to poly(vinylidine difluoride) (PVDF) membranes. The membranes were incubated overnight in TBSTG buffer [25 mM Tris (pH 7.5), 190 mM NaCl, 0.02% Tween-20, and 0.25% gelatin]. Next, the membranes were incubated for 2 h in TBSTG buffer containing one of the following: 5 μg/mL rabbit anti-PKC-α, 5 μg/mL rabbit anti-PKC-β, or a 1:1000 dilution of the rabbit anti-PKC-γ serum. After being washed three times for 5 min with TBST buffer, the membranes were incubated for 2 h with 1:5000 horseradish peroxidase-linked goat anti-rabbit antibody in TBSTG buffer. The membranes were washed extensively in TBSTG buffer, after which cPKC isoforms were visualized by detection with enhanced chemiluminescence reagents (Amersham).

Immunoanalysis of cPKC Dimer Composition. The wells of a 96-well tissue culture plate were incubated for 2 h at 37 °C with 60 μL of 10 μg/mL anti PKC-α antibody. Control wells were incubated with 3% (w/v) bovine serum albumin (BSA). Next, all wells were incubated for 2 h with 3% BSA at 37 °C to block any unbound protein binding sites. cPKCs (1−5 μg) were cross-linked for 5 min at 22 °C in 100 μL of a solution containing 20 mM HEPES (pH 7.4), 1 mM $CaCl_2$, 100 μg/mL phosphatidylserine, and 10 μM BMH. The cross-linking reactions were terminated by the addition of ethanolamine to a final concentration of 12 μM. Next, the samples were added to the anti-PKC-α or BSA-coated microtiter wells. After a 5 h incubation at room temperature, the wells were washed with phosphate-buffered saline [10 mM Na_2HPO_4 (pH 7.2) and 145 mM NaCl]. The immunoabsorbed protein was eluted by the addition of 20 μL of SDS sample buffer, and the samples were analyzed by SDS−PAGE followed by anti-PKC-α or -β immunoblotting.

Cell Culture and Intracellular cPKC Activation. Mouse B82L cells that were transfected with the human EGF receptor and a mutant dihydrofolate reductase gene were grown in DMEM containing fetal bovine serum (10%) and 10 μM methotrexate at 37 °C in a humidified atmosphere containing 5% CO_2. After growth to approximately 80% confluence, the cells were serum-starved by incubation for 24 h in serum-free DMEM plus 0.1% BSA. The cells were then incubated for 15 min at 37 °C with DMEM containing either calcium ionophore A23187 (2 μM), phorbol ester (100 nM), or EGF (0.2−20 nM). The final concentration of dimethyl sulfoxide was ≤0.1% in all experiments, and control incubations contained 0.1% dimethyl sulfoxide. Following these treatments, the cells were washed three times with ice-cold PBS plus 1 mM EDTA and lysed in RIPA buffer [50 mM Tris buffer (pH 7.4), 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 mM phenylmethanesulfonyl fluoride]. After 30 min, the cell lysates were adjusted to 40 or 400 μM BMH and incubated for 2 h at 4 °C. The cell lysates were centrifuged at 13500*g* for 10 min at 4 °C. Following incubation with protein A−agarose for 30 min, the lysates were centrifuged at 13500*g* for 30 s to remove nonspecifically adsorbed proteins. The supernatants were incubated with 1 μg of polyclonal anti-PKC-α antibody for 4 h at 4 °C, followed by an incubation with protein A−agarose at 4 °C overnight. After extensive washing of the immunoprecipitated protein with RIPA, the samples were resuspended in 25 μL of SDS electrophoresis sample buffer, separated by SDS−PAGE, and subjected to immunoblotting with an anti-PKC-α antibody as described above.

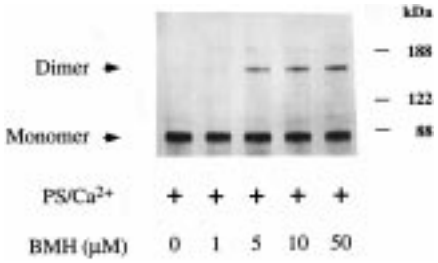

FIGURE 1: Cross-linking of cPKC by BMH. Purified cPKCs were incubated in a mixture of 100 μg/mL phosphatidylserine, 1 mM $CaCl_2$, and 20 mM HEPES (pH 7.4) for 5 min at 22 °C. The samples were then adjusted to 0−50 μM BMH, incubated at 22 °C for 2 min, and analyzed by SDS−PAGE and silver staining. The results are representative of three independent experiments.

RESULTS

Self-Association of cPKCs. To directly assess whether cPKCs self-associate, we studied the ability of the cross-linker BMH to covalently link these enzymes. At pH 7.4, BMH is highly selective for sulfhydryl groups and does not effectively cross-link to phospholipids (*26*). Furthermore, because of its short length (∼16.1 Å), BMH will only cross-link proteins that are either in direct contact or in very close proximity. In initial experiments, purified cPKCs were mixed with 1 mM $CaCl_2$ and 100 μg/mL phosphatidylserine, conditions that maximally activate cPKC kinase activity (*27*). As shown in Figure 1, under these conditions, efficient cross-linking of cPKCs was evident within 2 min and at concentrations at or above 5 μM BMH. Silver staining revealed a 160 kDa band on SDS−polyacrylamide gels, which is consistent with a dimer of the ∼80 kDa cPKC molecules. Additional experiments revealed bands on SDS−polyacrylamide gels of 240 kDa and larger, which suggests that cPKCs are not limited to forming dimers. Further analysis of cross-linked proteins by immunoblotting with isoform-specific anti-PKC antibodies (Figure 2) demonstrated that all cPKC isoforms are cross-linked in the presence of phosphatidylserine/Ca^{2+}. These immunoblots also show that the relative amounts of cPKC monomers decreased upon dimer formation, supporting the concept that the 160 kDa bands were indeed formed from cPKC monomers.

Dependence of Self-Association on Phosphatidylserine, Ca^{2+}, and Mg^{2+}. To evaluate a possible functional role of self-association in the activation of cPKC, we examined the phosphatidylserine, Ca^{2+}, and Mg^{2+} requirements for cPKC cross-linking. As shown in Figure 3, efficient cross-linking required that both phosphatidylserine and Ca^{2+} be present, whereas the combination of phosphatidylserine and Mg^{2+} did not result in cPKC cross-linking. Furthermore, cross-linking of cPKCs by Ca^{2+}/phosphatidylserine did not require Mg^{2+}, and the addition of either phosphatidylserine or Mg^{2+} alone was largely ineffective at enhancing cPKC cross-linking. In some experiments, Ca^{2+} alone resulted in a trace

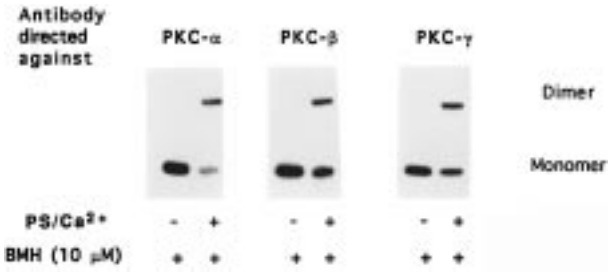

FIGURE 2: α, *β*, and *γ* isoforms of cPKC contribute to PKC dimer formation. Purified cPKCs (a mixture of α, *β*, and *γ* isoforms) were incubated for 5 min at 22 °C in 20 mM HEPES (pH 7.4) in the absence (−) or presence (+) of 100 *μ*g/mL phosphatidylserine and 1 mM $CaCl_2$. The samples were then adjusted to 10 *μ*M BMH and incubated for 2 min at 22 °C. Following SDS−PAGE, the samples were analyzed by immunoblotting with antibodies specific to PKC-α, -*β*, or -*γ*. The results are representative of three independent experiments.

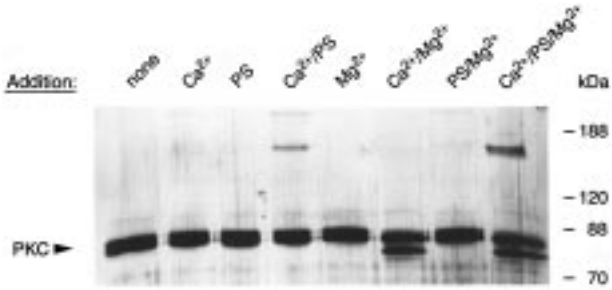

FIGURE 3: Cross-linking of cPKC is dependent on both phosphatidylserine and Ca^{2+} but not Mg^{2+}. Purified cPKCs were incubated for 5 min at 22 °C in a mixture containing 20 mM HEPES (pH 7.4) in the absence or presence of phosphatidylserine (100 *μ*g/mL), $CaCl_2$ (1 mM), and $MgCl_2$ (10 mM). The samples were then adjusted to 10 *μ*M BMH and incubated for 2 min at 22 °C. The samples were analyzed by SDS−PAGE and silver staining. The results are representative of four independent experiments.

amount of cPKC dimer formation. Interestingly, the addition of both Mg^{2+} and Ca^{2+} in the presence or absence of phosphatidylserine produced a shift in the mobility of cPKCs following SDS−PAGE. Of note, PKCs are known to be sensitive to cation-dependent protease and phosphatase activity (*5*, *7*, *12*), and it is conceivable that a trace amount of one or the other of these activities is present in the enzyme preparation. The altered mobility of the cPKCs following the addition of both Mg^{2+} and Ca^{2+} may arise from limited proteolysis and/or from the removal of phosphate from constitutively phosphorylated sites that have been shown to occur during PKC processing (*5*, *12*, *28*). Nonetheless, at this time, the nature of this shift is unclear, but it appears to be unrelated to the self-association process. Finally, under the conditions of these experiments (i.e., saturating levels of phospholipid and Ca^{2+}), the addition of $MgCl_2$/ATP or diacylglycerol did not noticeably affect the ability of cPKCs to self-associate (data not shown). Together, these results show that cPKC self-association requires the presence of both Ca^{2+} and phosphatidylserine and is largely unaffected by Mg^{2+} and ATP.

Phospholipid Dependence of cPKC Self-Association. The activation of cPKCs is principally dependent on phosphatidylserine, as opposed to other anionic phospholipids. For example, phosphatidic acid and phosphatidylinositol are less effective activators than phosphatidylserine, and phosphatidylcholine is almost inactive at stimulating cPKC activity (*16*, *27*, *29*, *30*). To determine whether this selectivity is also conserved for cPKC self-association, we concurrently measured the effects of various phospholipids on cPKC activity and BMH-mediated cross-linking. As shown in Figure 4A, in the presence of Ca^{2+}, phosphatidylserine was the most potent at inducing cPKC self-association. Phosphatidic acid and phosphatidylinositol were less effective at promoting cPKC self-association, whereas phosphatidylcholine was essentially inactive. The observed profile of phospholipid dependence for kinase activation is consistent with previous reports (*16*, *27*, *29*, *30*) and corresponds with the results from the cross-linking reactions (Figure 4B). In summary, phosphatidylserine is the most efficacious of the anionic phospholipids for promoting both kinase activation and cPKC self-association. Moreover, the results reveal that the degree of self-association parallels that of kinase activation (Figure 4B, inset).

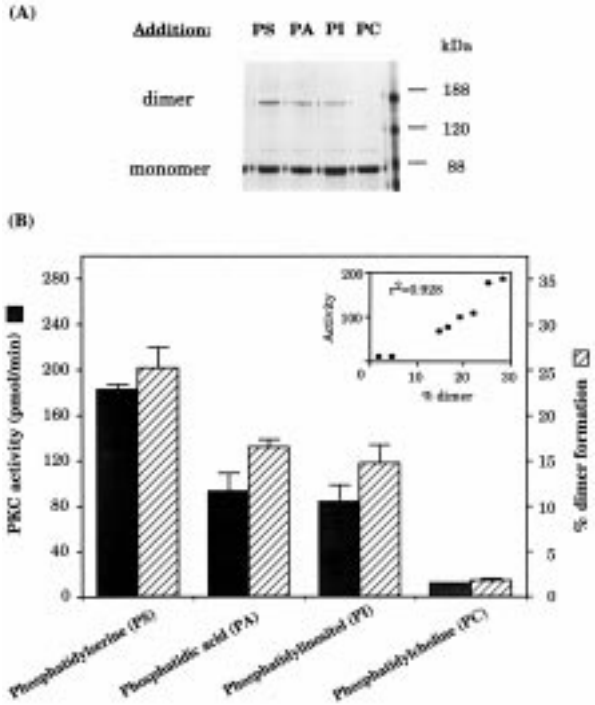

FIGURE 4: Effects of phospholipid on cPKC cross-linking and enzyme activity. Purified cPKCs were incubated for 5 min at 22 °C in a mixture containing 20 mM HEPES (pH 7.4), 1 mM $CaCl_2$, and 100 *μ*g/mL of one of the following phospholipids: phosphatidylserine (PS), phosphatidic acid (PA), phosphatidylinositol (PI), or phosphatidylcholine (PC). The samples were then divided into two portions: one for measurement of cPKC cross-linking and the other for measurement of cPKC enzyme activity. The degree of cPKC cross-linking was assessed by densitometric analysis of the silver-stained gel and is expressed as the percentage of dimer formed relative to total PKC protein. (Panel A) Silver-stained SDS−polyacrylamide gel of cross-linked cPKC as induced by treatment with different phospholipids. (Panel B) Protein kinase activity of cPKC and the percentage of total dimer formation as stimulated by the addition of different phospholipids. Inset: Protein kinase activity of cPKC as a function of percent dimer formation. In all cases, analogous results were obtained in three independent experiments.

Self-Association of cPKC Does Not Depend on Large-Scale Phospholipid Aggregation. Two previous studies have suggested that cPKCs are activated when phospholipids form large-scale aggregates (*25*, *31*). Accordingly, one explanation for the ability of cPKC to self-associate is that there is an increased interaction of cPKC monomers within large-scale

Table 1: Metal Ion Dependence of Phosphatidylserine Aggregation[a]

$CaCl_2$ (1 mM)	$MgCl_2$ (10 mM)	absorbance at 350 nm[b]
−	−	0.013 ± 0.002
+	−	0.022 ± 0.002
−	+	0.729 ± 0.001
+	+	0.761 ± 0.002

[a] The aggregation of phosphatidylserine was assessed by measuring light scattering (absorbance) at 350 nm. The solutions contained 100 μg/mL phosphatidylserine, ±1 mM $CaCl_2$, and ±10 mM $MgCl_2$. The results are representative of three independent experiments. [b] The values are presented as the mean ± range of duplicate determinations.

phospholipid aggregates. To determine the degree of phospholipid aggregation in our cross-linking reactions, we analyzed the light scattering properties of phospholipid-containing solutions at 350 nm (*25*). As shown in Table 1, large-scale phospholipid aggregation was not apparent when phosphatidylserine vesicles were prepared in a metal ion-free solution or when 1 mM $CaCl_2$ was added. However, large-scale aggregation of phosphatidylserine vesicles did appear to occur when 10 mM $MgCl_2$ was added. Indeed, the phospholipid-containing solutions became visibly cloudy when Mg^{2+} alone or both Mg^{2+} and Ca^{2+} were added, but not when Ca^{2+} alone was added. These results agree with earlier reports regarding the metal ion dependence of phospholipid aggregation (*25, 32*). Also, because efficient cross-linking of cPKCs occurs in the absence of Mg^{2+} (Figure 3), these findings indicate that self-association of cPKCs occurs independently of large-scale phospholipid aggregation.

Self-Association of cPKC Is Not Due to Enzyme−Substrate Recognition. Another possible mechanism for cPKC self-association is that one monomer binds in the active site of another monomer. Indeed, phosphatidylserine/Ca^{2+} causes a release of the pseudosubstrate domain (*33, 34*) leaving the substrate binding site accessible for potential intermolecular interaction. To test if this occurs, a peptide substrate [EGF receptor peptide, H-Lys_{652}-Arg-Thr-Leu-Arg-Arg_{657}-OH (*35*)] was added to the reaction mixtures to block the active site. The reaction mixtures were split into two portions, one for cross-linking with BMH and a second for measurement of cPKC autophosphorylation. As shown in Figure 5A, increasing concentrations of the EGF receptor peptide did lead to a progressive inhibition of cPKC autophosphorylation, indicating that this peptide does block the catalytic site. In contrast, the self-association of cPKCs was unaffected by concentrations of the EGF receptor peptide as high as 2 mM (Figure 5B), which is a saturating concentration for the enzyme active site [K_m = 0.13 mM (*35*)]. Therefore, self-association of cPKCs is likely mediated by elements outside the enzyme active site and can occur even when this site is occupied.

PKC-α Preferentially Forms Homodimers. In initial studies, we demonstrated that self-association is shared by all cPKC isoforms, but it was unclear whether these isoforms self-associate as homo- or heterodimers. Because our preparations of cPKC contain a substantial amount of the α and β isoforms (see Figure 2), we examined whether these two isoforms interact. In these experiments, the cPKC mixture was cross-linked in the presence of phosphatidylserine and Ca^{2+}. The cPKC dimers were then added to microtiter wells that had been coated with an anti-PKC-α-specific antibody. Unbound PKC was removed and the immunoabsorbed protein was analyzed by immunoblotting with antibodies recognizing either PKC-α or PKC-β.

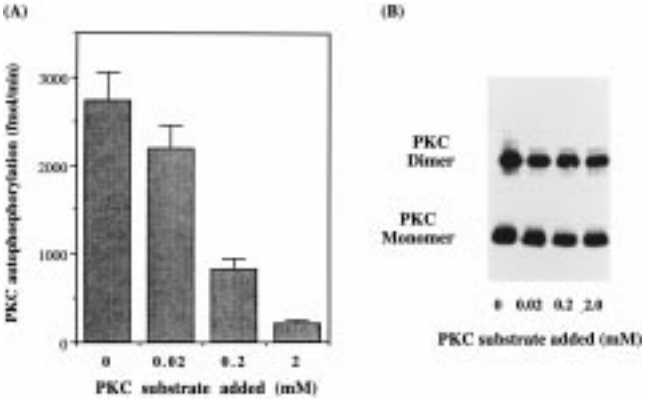

FIGURE 5: Effect of a peptide substrate on cPKC cross-linking and autophosphorylation. Purified cPKCs were incubated for 5 min at 22 °C in a mixture containing 20 mM HEPES (pH 7.4), 1 mM $CaCl_2$, 100 μg/mL phosphatidylserine, and various concentrations (0−2 mM) of the EGF receptor peptide. The samples were then divided into two portions: one for measurement of cPKC cross-linking and the other for measurement of cPKC autophosphorylation. (Panel A) Level of cPKC autophosphorylation in the presence of various concentrations of the EGF receptor peptide. (Panel B) Silver-stained gel of cross-linked cPKC at various concentrations of EGF receptor peptide. The results are representative of three independent experiments.

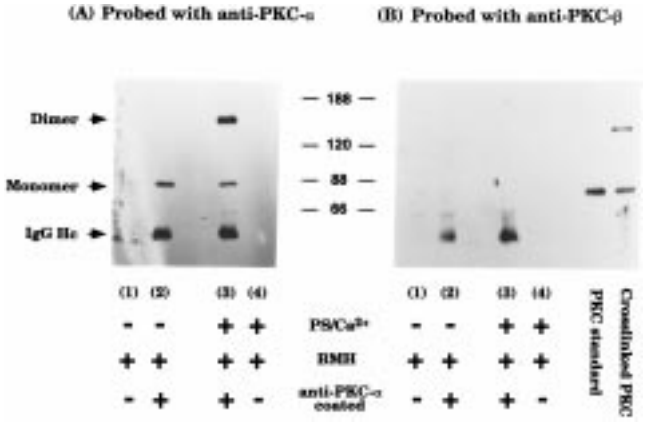

FIGURE 6: PKC-α preferentially forms homodimers. Purified cPKCs were incubated for 5 min at 22 °C in 20 mM HEPES (pH 7.4) in the absence (−) or presence (+) of 100 μg/mL phosphatidylserine and 1 mM $CaCl_2$. The samples were then adjusted to 10 μM BMH and incubated for 2 min at 22 °C. The samples were subsequently added to tissue culture wells coated with (+) or without (−) anti-PKC-α antibody. The immunoabsorbed cPKC was eluted and analyzed by SDS−PAGE, followed by immunoblotting with antibodies against PKC-α (panel A) or PKC-β (panel B). The two lanes on the far right of panel B illustrate the level of PKC-β immunoreactivity in the original and cross-linked cPKC preparation before immunoabsorption with anti-PKC-α. Similar data were obtained in three independent experiments.

In these experiments, neither PKC-α nor PKC-β bound to BSA-coated wells (Figure 6A,B, lanes 1 and 4). Also, PKC-β monomer did not bind to anti-PKC-α-coated wells (Figure 6B, lane 2). We found that PKC-α monomer and cross-linked dimer bound to the anti-PKC-α-coated microtiter wells (Figure 6A, lanes 2 and 3, respectively). In contrast, PKC-β was not detected following immunoabsorption of cross-linked cPKC dimers with anti-PKC-α (Figure 6B, lane 3), indicating that PKC-β does not readily form dimers with PKC-α. Together, these results demonstrate that, under our

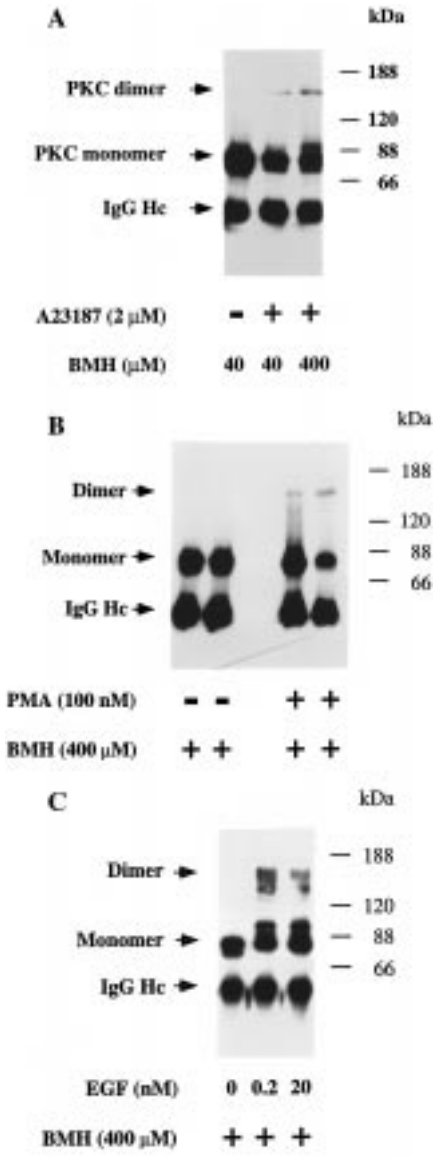

FIGURE 7: Effect of the calcium ionophore A23187, phorbol ester, or EGF on cPKC self-association in intact mouse B82L fibroblasts. Mouse B82L fibroblasts transfected with the human EGF receptor were treated for 15 min at 37 °C with 0.1% dimethyl sulfoxide in the absence or presence of 2 μM calcium ionophore A23187 (panel A), 100 nM phorbol ester (PMA) (panel B), or 0.2−20 nM EGF (panel C). BMH was added after lysis of the cells with RIPA buffer as described under Materials and Methods. Cell lysates were subjected to immunoprecipitation with anti-PKC-α antibody, followed by SDS−PAGE and immunoblotting with anti-PKC-α antibody. The results are representative of two independent experiments.

experimental conditions, activation of PKC-α is primarily associated with the formation of homodimers.

Effect of Pharmacological Agents and EGF on cPKC Self-Association in Mouse B82L Fibroblasts. Although the above experiments demonstrate that cPKCs self-associate in vitro in the presence of phosphatidylserine and Ca^{2+}, it is important to determine whether this process can occur in intact cells. Accordingly, we examined whether treatment with EGF or pharmacological agents that stimulate cPKCs in vivo can also induce cPKC self-association in mouse B82L fibroblasts. After incubation of these cells with the various agents indicated in Figure 7, the cells were lysed and mixed with BMH. Following termination of the cross-linking reaction, PKC-α was immunoprecipitated and analyzed via anti-PKC-α immunoblotting. In these studies, we focused on PKC-α because it is ubiquitously expressed (*7*) and because it is a major isoform in fibroblasts (*12*).

The experiments shown in Figure 7 reveal the presence of 80 and 160 kDa immunoreactive PKC-α bands in lysates from cells that were first treated with the calcium ionophore A23187 (2 μM; panel A), PMA (100 nM; panel B), or EGF (0.2−20 nM; panel C). Only the 80 kDa (monomer) band was apparent in lysates of untreated cells. The presence of the 160 kDa band in the stimulated cells is consistent with the presence of a cPKC dimer, although it is also possible that the 160 kDa band is composed of a stable complex of PKC-α and a different 80 kDa protein. In preliminary experiments, we have assessed whether PKC activation by EGF, ionophore A23187, or PMA treatment of B82L cells can also promote PKC cross-linking to other potential interacting proteins, such as Src and Raf-1. However, no immunodetectable Src or Raf-1 was found in the BMH-cross-linked PKC-α complexes following treatment with these agents (data not shown). In sum, these observations support the concept that PKC-α can stably and selectively self-associate in intact cells following stimulation with growth factor, calcium ionophore, or phorbol ester.

DISCUSSION

Activator-dependent self-association is a common mechanism for the regulation of protein kinases and various metabolic enzymes (*36*−*38*). In our studies, we explored the role of cPKC self-association by examining the ability of these enzymes to be rapidly cross-linked under various conditions. We found that cPKC self-association occurs in vitro preferentially in the presence of phosphatidylserine and Ca^{2+}. In addition, apparent cPKC self-association was observed in the extracts of cells stimulated by EGF and pharmacological agents that are known to activate the cPKCs. These findings indicate that cPKC self-association occurs in parallel with, and may participate in, the activation of these enzymes in vitro and in vivo.

Our results indicate that cPKC self-association is an event that is closely related to the activation of these protein kinases and independent of secondary events such as large-scale phospholipid aggregation or substrate recognition. First, both phosphatidylserine and Ca^{2+} together were required for cPKC cross-linking, which are conditions that also activate kinase activity. Phosphatidylserine alone or together with another divalent metal ion, Mg^{2+}, stimulated neither PKC self-association nor its autophosphorylation. Second, cPKC cross-linking was observed in the absence of large-scale phospholipid aggregation. Third, phosphatidylserine was the most effective anionic phospholipid at stimulating both kinase activity and self-association. The other anionic phospholipids tested were less potent in their ability to promote self-association or kinase activation, and in general, kinase activation closely paralleled self-association. These results further define the specificity of cPKC self-association for phosphatidylserine/Ca^{2+} and suggest that cPKC cross-linking is not due to nonspecific interactions with phospholipid vesicles. Finally, cPKC cross-linking was unaffected by the presence of saturating levels of an exogenous peptide

substrate, demonstrating that self-association is not simply an enzyme−substrate interaction and that it is not limited to reactions containing only purified cPKC. Collectively, these results indicate that cPKC self-association occurs in parallel with, and may be directly linked to, kinase activation.

The concept of a connection between cPKC self-association and kinase activation is in agreement with several earlier observations. First, Sando and co-workers (*19, 20*) reported that diluting cPKCs with short chain phospholipid micelles decreases kinase activity. Because cPKCs most likely exist as monomers in these micelles, they interpreted this as evidence for a role of PKC self-association in kinase activation. Second, Le Peuch et al. (*39*) found that aggregation of phospholipids increases cPKC activity. Although our results indicate that phospholipid aggregation alone does not induce cPKC self-association, it is possible that phospholipid aggregation in the presence of Ca^{2+} could drive cPKC self-association and activation. Third, Bazzi and Nelsestuen (*40*) showed that cPKCs form large aggregates with exogenous substrates. In this case, activation of cPKCs could correspond to enhanced self-association within large cPKC−substrate aggregates.

In previous studies, we examined the effect of increasing the ratio of PKC to phospholipid/Ca^{2+} on kinase autophosphorylation (*22*). Under these conditions, increasing the concentration of cPKC monomers on the vesicle surfaces should lead to a greater likelihood of intermolecular interactions. We found that increasing the ratio of cPKC to phospholipid/Ca^{2+} substantially enhanced the rate of cPKC autophosphorylation. Recently, Sando et al. (*21*) provided evidence that there is an optimal mole percent of phosphatidylserine in lipid mixtures for PKC activation. They found that PKC activity is decreased as the mole percent of phosphatidylserine is increased beyond maximally effective concentrations and that this may occur because of a diluting out of enzyme−enzyme aggregates on the lipid surface. Together, these data are consistent with cPKCs functioning as oligomers in the presence of Ca^{2+} and phospholipid vesicles and suggest that cPKC self-association can participate in the conformational changes that promote kinase activation (*13*−*15*).

The observation that cPKC cross-linking occurs in the presence of a saturating amount of a peptide substrate reveals that self-association is not solely mediated by the active site. Furthermore, in previous experiments, we have shown that efficient substrate binding by cPKCs requires MgATP (*41*). Therefore, the finding that cPKC cross-linking occurs in the absence of MgATP supports the hypothesis that self-association involves regions other than the enzyme active site. Collectively, these results demonstrate that cPKC self-association is not restricted to the binding of one cPKC monomer to the active site of another monomer.

The ability of BMH, a sulfhydryl-selective agent, to rapidly and efficiently cross-link cPKCs suggests that the association of monomers is mediated by regions in proximity to cysteines that are highly reactive (i.e., reduced, accessible, and in close apposition). Many cysteines are located within the phorbol ester/diacylglycerol binding site in the regulatory domain of the cPKCs, and several others are found in the catalytic domain. Kikkawa et al. (*42*) reported that cPKC activity is inhibited by the sulfhydryl alkylating agent *N*-ethylmaleimide (NEM) but that phorbol ester binding is largely unaffected by this compound. This suggests that free cysteines in the carboxy-terminal domain rather than the amino-terminal phorbol ester binding domain are essential for cPKC activity. Interestingly, image analyses of electron micrographs of PKC β_1 microcrystals suggests that cPKC molecules may form contacts between their carboxy-terminal domains (*43*). Given these findings, it is likely that cysteines in the carboxy-terminal domain are involved in, or proximal to, the sites required for both cPKC activation and self-association. Two of the four cysteines in the carboxy-terminal domain (Cys-488 and Cys-502 of PKC-β) are particularly good candidates because they are conserved among all the PKCs and they lie within the "activation loop", a region that is necessary for cPKC activation (*12, 15, 44, 45*).

Our results show that all cPKC isoforms (α, β, and γ) are cross-linked by BMH in the presence of phosphatidylserine and Ca^{2+}. Moreover, our data indicate that PKC-α preferentially forms homodimers, and immunochemical analyses suggest that PKC-α self-associates upon activation in vivo. In these latter studies, a cross-linked 160 kDa band was observed in anti-PKC-α immunoblots of lysates from B82L fibroblasts stimulated by either a calcium ionophore, a phorbol ester, or EGF. These three agents stimulate cPKCs by distinct mechanisms, suggesting that self-association generally corresponds to cPKC activation in vivo.

In summary, the present studies reveal that cPKCs self-associate under activating conditions in vitro and that this process is not mediated by the active site but occurs in parallel with kinase activation. Our results also support the possibility that cPKCs selectively form homodimers and that PKC-α self-associates in vivo. Of note, this isoform-specific self-association could relate to the differential localization of certain cPKC isoforms within the cell (*46*−*48*).

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