Synergistic Activation of Protein Kinase Cα, -βI, and -γ Isoforms Induced by Diacylglycerol and Phorbol Ester: Roles of Membrane Association and Activating Conformational Changes†

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ABSTRACT: Protein kinase Cα (PKCα) has been shown to contain two discrete activator sites with differing binding affinities for phorbol esters and diacylglycerols. The interaction of diacylglycerol with a low-affinity phorbol ester binding site leads to enhanced high-affinity phorbol ester binding and to a potentiated level of activity [Slater, S. J., Ho, C., Kelly, M. B., Larkin, J. D., Taddeo, F. J., Yeager, M. D., and Stubbs, C. D. (1996) *J. Biol. Chem. 271*, 4627−4631]. In this study, the mechanism of this enhancement of activity was examined with respect to the Ca^{2+} dependences of membrane association and accompanying conformational changes that lead to activation. The association of PKCα with membranes containing 12-*O*-tetradecanoylphorbol 13-acetate (TPA) or 1,2-dioleoylglycerol (DAG), determined from tryptophan to dansyl-PE resonance energy transfer (RET) measurements, was found to occur at relatively low Ca^{2+} levels (≤1 μM). However, PKCα was found to be inactive even though membrane association was complete at these Ca^{2+} levels and further titration of Ca^{2+} to a concentration of ∼100 μM was required for activation. This increase in Ca^{2+} concentration also led to a further increase in RET, which was due to a Ca^{2+}-induced activating conformational change, as verified by an accompanying increase in the PKCα tryptophan fluorescence anisotropy. Coaddition of DAG and TPA resulted in a reduction in the Ca^{2+} levels required for both the conformational change and enzyme activation. Also, it was found that incubation of the enzyme with TPA alone resulted in a time-dependent increase in the Ca^{2+}-independent PKCα activity, the rate and extent of which was further enhanced upon coaddition with DAG. The results suggest that the enhanced level of activity induced by coaddition of DAG and TPA involves both Ca^{2+}-dependent and Ca^{2+}-independent activating conformational changes which result in active conformers of PKCα distinct from those formed by interaction with either activator separately.

Protein kinase C (PKC)[1] consists of an extended family of 11 isoforms which are centrally involved in diverse signal transduction processes (*1*). It has been shown in a wide variety of cell types that the "conventional" PKCα, -βI, -βII, and -γ (cPKC) isoforms translocate from the cytosol to the membrane as a response to an increase in the intracellular levels of Ca^{2+} and diacylglycerol, an event that is often assumed to straightforwardly lead to the expression of enzymatic activity. This rather oversimplistic view ignores increasing evidence which shows that different activators such as diacylglycerol and phorbol esters interact quite differently with PKC (*2*−*4*). Indeed, recent studies indicate that these two activators interact with two discrete binding sites on PKC, alluding to fundamental features of the mechanism of PKC activation that remain to be resolved (*2*, *3*, *5*).

The phorbol ester binding activity of PKC resides within the C1 domain, which is comprised of C1A and C1B cysteine-rich zinc finger-like repeats (*6*, *7*). Recent studies from this laboratory indicating that phorbol esters bind PKC with distinct high and low affinities would be consistent with these two sites being the C1A and C1B domains (*2*, *3*, *5*, *8*). This observation was recently extended to the isolated C1 domain of PKCα (*5*), in line with the observation that both C1A and C1B are functionally active phorbol ester binding domains (*9*−*22*). Studies in which the phorbol ester binding ability of either the C1A or C1B was removed in turn reveal a complex interplay between the two subdomains. Mutation of a single consensus proline in the binding loop of either the C1A or the C1B of PKCδ, which has been shown to

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[1] Abbreviations: BPS, bovine brain phosphatidylserine; DAG, 1,2-dioleoylglycerol; dansyl-PE, *N*-[5-(dimethylamino)naphthalenyl-1-sulfonyl]-1,2-dihexadecanoyl-*sn*-glycero-3-phosphocholine; Me_2SO, dimethyl sulfoxide; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; N-Rho-PE, *N*-(lissamine rhodamine-B-sulfonyl)dipalmitoyl-L-α-phosphatidylethanolamine; LUV, large unilamellar vesicles; MBP_{4-14}, myelin basic protein peptide substrate; PC, phosphatidylcholine; PKCα, -βI, -βII, and -γ, "conventional" protein kinase C isoforms; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; PS, phosphatidylserine; RET, resonance energy transfer; SAPD, sapintoxin-D; SDS, sodium dodecyl sulfate; SLV, sucrose-loaded vesicles; TPA, 4β-12-*O*-tetradecanoylphorbol 13-acetate; TOE, tryptophan octyl ester.

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markedly reduce the level of phorbol ester binding to the isolated domains (*10*), was found to result in a relatively small effect on the phorbol ester dose−response curve for PKC translocation in Swiss 3T3 cells (*22*). However, mutation of both proline residues in both zinc finger domains was found to cause a relatively large increase in the phorbol ester concentration requirement for translocation. While it was concluded from this that the C1A and C1B domains are nonequivalent, these findings also point to an interplay between these domains, an observation consistent with the findings of earlier studies (*13*).

The nonequivalence of the two zinc finger domains and the interplay between them are in keeping with studies from this laboratory which show that low-affinity phorbol ester binding can be displaced by DAG, resulting in an increase in the level of high-affinity phorbol ester binding and an elevation of enzyme activity beyond that which can be achieved by either activator alone (*2*, *3*). The importance of this observation is that this type of effect is likely to occur quite commonly in both experimental and physiological paradigms. For example, it may occur in cells in which DAG production is instigated and where phorbol esters are present. The various compounds that interact with the low- and high-affinity phorbol ester binding sites may do so with asymmetric or opposing affinities. For example, the potent antitumor agent, bryostatin-1, competes primarily for binding to the high-affinity site, while the "inactive" phorbol ester, 4α-12-*O*-tetradecanoylphorbol 13-acetate (4α-TPA), binds to the low-affinity site (*3*). Further, the low-affinity phorbol ester binding site was shown to constitute a hydrophobic site for *n*-alkanols and anesthetics, which, like diacylglycerol, were also found to interact with and enhance high-affinity phorbol ester binding and the level of PKC activity (*8*, *23*). On the basis of these results, a model for PKC activation was proposed in which interaction of a ligand with the low-affinity phorbol ester binding site leads to an enhanced level of binding of either the same or a second ligand to the high-affinity phorbol ester binding site, and consequently to an elevated level of PKC activity (*3*, *8*).

The parallel but independent interactions of diacylglycerol or the tumor-promoting phorbol esters with the C1 domain and that of Ca^{2+} with the second conserved (C2) domain result in an apparent "cooperative" enhancement of membrane binding affinity and activation of cPKC isoforms (*24*−*26*). However, the Ca^{2+} concentrations required to induce the association of these isoforms with membranes containing diacylglycerol or phorbol ester have been shown to be lower than those required to induce activity, suggesting the possibility that membrane association and activation may be functionally distinct processes (*27*−*31*). Evidence supporting this contention has been provided recently by the finding that diacylglycerol selectively induces a high-affinity interaction of PKCβII with phosphatidylserine/detergent mixed micelles at Ca^{2+} concentrations lower than that required to induce activity (*31*). It was suggested that the diacylglycerol-induced association of PKCβII with membranes and the ensuing activation may be accompanied by distinct conformational changes, the former resulting in the exposure of the V3-hinge region to proteolysis and the latter in the removal of the pseudosubstrate from the substrate binding site. Consistent with this, another study showed that inducing the activating conformational change in PKCβII in the absence of lipids by interaction with the arginine-rich substrate, protamine sulfate, resulted in relative insensitivity to proteolytic cleavage at the hinge region compared to that associated with lipids (*32*). Furthermore, a recent mutagenesis study showed that the C2 domain of PKCα may contain two discrete Ca^{2+}-binding sites, one of which may mediate the association of this isoform with membranes and the other of which may induce a conformational change that results in activation (*33*).

While the membrane association and activation of cPKC isoforms induced by diacylglycerols have been shown to display an absolute requirement for Ca^{2+}, the effects of phorbol esters on these processes appear to differ in being relatively insensitive to inhibition by Ca^{2+} chelation (*34*, *35*). This difference between the activators has been suggested to result from the relatively high affinity of PKC for phorbol esters compared to that of diacylglycerols (*24*, *36*). However, the Ca^{2+} independence of phorbol ester-induced membrane association and activation may also result from the relatively slow formation of an irreversibly membrane-bound, constitutively active form of PKC (*4*, *34*, *35*, *37*). Diacylglycerols were found to be relatively ineffective at generating this Ca^{2+}-independent form of the enzyme compared to phorbol esters (*4*), which is in keeping with the reported existence of two distinct activator binding sites on PKC (*2*−*5*).

The aim of this study was to determine the role of Ca^{2+}-induced and Ca^{2+}-independent membrane binding events and activating conformational changes in the mechanism by which interaction of diacylglycerol with the low-affinity phorbol ester binding site, combined with occupancy of the high-affinity site by phorbol ester, results in a synergistic enhancement of cPKC activity (*2*, *3*, *5*). It was found for PKCα, -βI, and -γ that the enhanced level of activity resulting from coaddition of phorbol ester and diacylglycerol may proceed by simultaneous Ca^{2+}-dependent and Ca^{2+}-independent mechanisms. Thus, coaddition of the two activators resulted in both an increased level of Ca^{2+}-independent membrane association and activation and a decreased Ca^{2+} concentration requirement for these processes. Evidence is provided supporting the contention that the active cPKC conformer arising from simultaneous interaction of phorbol ester and diacylglycerol with the low- and high-affinity phorbol ester binding sites may be distinct from those formed by the interaction of either phorbol ester or diacylglycerol individually.

MATERIALS AND METHODS

Materials. A peptide substrate corresponding to the PKC phosphorylation site of myelin basic protein (QKRPSQRSKYL, MBP_{4-14}) was custom synthesized by the Jefferson Cancer Institute peptide synthesis facility. Lipids were from Avanti Polar Lipids, Inc. (Alabaster, AL), except *N*-[5-(dimethylamino)naphthalenyl-1-sulfonyl]-1,2-dihexadecanoyl-*sn*-glycero-3-phosphocholine (dansyl-PE) which was from Molecular Probes (Eugene, OR). Sapintoxin-D (SAPD) was from Calbiochem (La Jolla, CA) or Alexis Biochemicals (San Diego, CA). TPA, tryptophan octyl ester (TOE), and trypsin were from Sigma (St. Louis, MO). Adenosine 5′-triphosphate (ATP) was from Boehringer Mannheim (Indianapolis, IN), and [γ-^{32}P]ATP was from New England Nuclear (Boston, MA). All other chemicals were of analytical grade and were obtained from Fisher Scientific (Pittsburgh, PA).

Assay of Ca^{2+}-Dependent cPKC Activity. Recombinant cPKC isoforms PKCα, -βI, and -γ (rat brain) were prepared using the baculovirus-insect cell expression system (*38*) and purified as previously described (*2*). The activities of cPKC isoforms were determined using MBP_{4-14} as a substrate, as previously described (*39*). Briefly, large unilamellar vesicles (LUV) 100 nm in diameter, prepared as described elsewhere (*40*), consisted of 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) and bovine brain phosphatidylserine (BPS) at a 4:1 molar ratio. The assay system (75 μL) consisted of 50 mM Tris-HCl (pH 7.40) containing 0.1 mM EGTA, 50 μM MBP_{4-14}, and LUV (150 μM) containing TPA (0.3 mol %) or DAG (4 mol %) or both activators, as indicated. Finally, Ca^{2+} was added at a level calculated to yield the required concentration when buffered by EGTA (*41*). After thermal equilibration to 30 °C, assays were initiated by the simultaneous addition of PKCα, PKCβI, or PKCγ (0.3 nM) along with a solution containing 5 mM Mg^{2+}, 15 μM ATP, and 0.3 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) and terminated after 30 min with 100 μL of 175 mM phosphoric acid. Following this, a 100 μL aliquot was transferred to P81 filter papers, which were then washed three times in 75 mM phosphoric acid. The amount of phosphorylated peptide was determined by scintillation counting.

Assay of Ca^{2+}-Independent cPKC Activity. Time-dependent effects of TPA or DAG, either separately or together, on Ca^{2+}-independent PKCα activity were investigated using a procedure identical to that used for measurements of Ca^{2+}-dependent activity, except that the enzyme was preincubated at 30 °C in the assay mixture containing either 0.1 mM EGTA or 0.1 mM Ca^{2+} for the indicated time periods. The assay was then initiated with the addition of 15 μM ATP, 0.3 μCi of [γ-^{32}P]ATP (3000 Ci/mmol), and 5 mM Mg^{2+} in 50 mM Tris-HCl (pH 7.40).

Determination of Ca^{2+}-Induced cPKC Membrane Association Based on cPKC Tryptophan to Dansyl-PE RET. The interaction of cPKC isoforms with vesicles containing TPA, DAG, or both activators together was quantified using RET, as previously described (*42*−*45*). The increase in dansyl-PE fluorescence and quenching of cPKC tryptophan fluorescence, which resulted after excitation at a wavelength corresponding to the excitation maximum of the tryptophans (290 nm), was measured at wavelengths corresponding to the emission maxima for dansyl-PE (520 nm) using a PTI Alphascan dual-emission fluorescence spectrofluorimeter in T-format (Photon Technology International, Inc., Princeton, NJ). The assay components were Tris-HCl (50 mM, pH 7.40), 5 mM Mg^{2+}, 0.1 mM EGTA, LUV comprised of POPC, BPS, and dansyl-PE (3.75:1:0.25 molar ratio) at a total lipid concentration of 150 μM, and cPKC (0.1 μM) in 2 mL in a quartz cuvette. Also included were 0.3 mol % TPA [added from a 0.5 mM dimethyl sulfoxide (Me_2SO) stock] and DAG (4 mol % of the total lipid concentration), added either separately or together in the same assay. After thermal equilibration was allowed (30 °C), Ca^{2+} was titrated into the assay system as $CaCl_2$ from standard solutions so that each Ca^{2+} addition yielded the required calculated free Ca^{2+} concentration when buffered by 0.1 mM EGTA (*41*). After each Ca^{2+} addition, equilibrium was again established and the emission fluorescence intensity at 520 nm was recorded. The contribution of RET to the observed signal was determined by subtracting the fluorescence intensity measured in the presence of all assay components, except Ca^{2+}. Potential effects of Ca^{2+} on the dansyl-PE to cPKC tryptophan RET signal arising from the restructuring of membrane lipids were ruled out by determining the effect of Ca^{2+} on RET from dansyl-PE to TOE. This compound was assumed to be located at the surface of the membrane and randomly distributed within the bilayer, allowing its presence in the same lipid domains as the cPKC isoforms. While assays of cPKC activity contained ATP and MBP_{4-14}, the omission of these substrates from this and other assays of membrane association affected neither the affinities nor the maximal extent of Ca^{2+}-induced association with vesicles (results not shown).

Determination of Ca^{2+}-Dependent SAPD Binding to cPKC. Phorbol ester binding to cPKC was quantified on the basis of RET from cPKC tryptophans to the 2-(*N*-methylamino)-benzoyl fluorophore of the phorbol ester, SAPD, as previously described (*3*). Briefly, the fluorescence intensities at the emission maxima of PKC tryptophans and SAPD (340 and 425 nm, respectively), obtained upon excitation of the tryptophan fluorophore at 290 nm, were determined using a PTI Alphascan spectrofluorimeter. The assay consisted of 50 mM Tris-HCl (pH 7.40), 0.1 mM EGTA, 150 μM BPS/POPC LUV (1:4 molar ratio), 5 mM Mg^{2+}, 1 μM SAPD added from a 0.5 mM Me_2SO stock, and cPKC (0.1 μM) in a total volume of 2 mL in a quartz cuvette. After thermal equilibration was allowed (30 °C), Ca^{2+} was titrated into the assay system, as described above for dansyl-PE RET measurements, and after equilibrium was attained, the emission fluorescence intensities at 425 and 340 nm were measured. The contribution of RET to the observed signal was isolated by subtracting the fluorescence intensity measured in the presence of all assay components, except Ca^{2+}.

Determination of Ca^{2+}-Induced cPKC Membrane Association Based on Dansyl-PE Steady State Anisotropy. Interaction of cPKC isoforms with vesicles was also determined on the basis of the measurements of the increase in dansyl-PE anisotropy, which results from the hindrance of the motional freedom of the headgroup fluorophore due to the proximity of membrane-associated cPKC. Values of dansyl-PE anisotropy were determined according to a previously described method (*46*). The extent of membrane association of cPKC induced by TPA or DAG alone, or by both activators in combination, was determined by measuring dansyl-PE anisotropy as a function of Ca^{2+} concentration using an assay system identical to that described for RET determinations (see above).

Determination of Ca^{2+}-Induced cPKC Membrane Association Based on a Centrifugation Method. The extent of association of cPKC isozymes with membranes was also determined by centrifugal separation of free cPKC from that bound to sucrose-loaded vesicles (SLV) essentially as described previously but with minor modifications (*29*, *47*). Briefly, SLV were prepared by evaporating the required volumes of chloroform solutions of BPS and POPC to dryness under nitrogen to give a lipid molar ratio of 1:4 and a total lipid concentration of 3 mM. Following this, 1 mL of Tris-HCl buffer (50 mM, pH 7.40) containing 170 mM sucrose was placed on top of the dried lipid film, and after hydration was allowed for 10 min at room temperature, the mixture was vortexed for 1 min to form multilamellar vesicles. To promote the incorporation of sucrose into these

vesicles, the preparation was first frozen in liquid nitrogen and then thawed to room temperature and the process repeated five times. SLV were produced by extrusion through 100 nm filters using a Liposofast extruder (Avestin, Inc., Ottawa, ON), as previously described (*40*). To remove excess sucrose external to the SLV, the vesicles were diluted 5-fold with Tris-HCl (50 mM, pH 7.40) and centrifuged for 30 min at 100000*g*. The supernatant was then discarded and the SLV pellet resuspended in 2 mL of Tris-HCl buffer (50 mM, pH 7.40) to give a final lipid concentration of 750 μM (assuming 100% lipid recovery). To determine losses of lipid during the manufacturing of SLV, the fluorescent phospholipid, *N*-(lissamine rhodamine-B-sulfonyl)dipalmitoyl-L-α-phosphatidylethanolamine (N-Rho-PE), was codispersed with the lipids at a level of 0.25 mol % of the total lipid concentration. Corrections required for losses of lipid were calculated on the basis of a comparison of the N-Rho-PE emission fluorescence intensity, measured at 530 nm upon excitation at 480 nm, for the phospholipid dispersion formed prior to extrusion with that of the final product.

The assay components used for determinations of cPKC binding to SLV were identical to those used for RET measurements (see above), except that the assay volume was 100 μL. Briefly, cPKC (0.1 μM) was preincubated in the presence of the required free Ca^{2+} concentration (buffered by EGTA) with the remaining assay components at 30 °C for 15 min. This was followed by centrifugation for 30 min at 100000*g*. Following this, 10 μL of the supernatant was removed and the cPKC activity determined in the presence of protamine sulfate according to a previously described method (*5*). The concentration of free cPKC in each supernatant was expressed as a specific activity.

Determination of Ca^{2+}-Induced cPKC Membrane Association Based on Changes in the Susceptibility to Proteolysis. The extent of interaction of cPKC with membranes was determined on the basis of the corresponding changes in the susceptibility to trypsin-catalyzed proteolysis, as previously described (*44*, *48*, *49*). The assay system used was like that described above for the RET measurements, except that the cPKC concentration was 40 nM and the total volume was 80 μL. After incubation of the assay at 30 °C for 5 min, proteolysis was initiated by the addition of 0.016 unit of trypsin. After 10 min, a 45 μL aliquot was removed and proteolysis halted by addition to 30 μL of 0.6 M Tris-HCl (pH 6.8) containing 50% (w/v) sucrose, 20% sodium dodecyl sulfate (SDS), 0.5% bromophenol blue, and 250 mM dithiothreitol (DTT). After being placed in a boiling water bath for 5 min followed by cooling, aliquots were loaded onto a 10% polyacrylamide gel and the proteins visualized by silver staining. PKC was quantified by measuring band densities using a Pharmacia Image Master 1D system (Pharmacia LKB, Piscataway, NJ).

Determinations of Conformational Differences in cPKC. Different PKCα and PKCβI conformers resulting from interaction with TPA or DAG individually or both together were distinguished by measuring the intrinsic stability of each cPKC conformer to thermal denaturation. The activity of cPKC induced by TPA (0.3 mol %), DAG (4 mol %), or the activator pair was measured as a function of time at either 30 or 45 °C in an assay system identical to that described above for determinations of Ca^{2+}-dependent activity. Loss of cPKC due to thermal denaturation was determined from measurements of activity at each time point.

Conformational differences in cPKC isoforms induced by 0.3 mol % TPA, 4 mol % DAG, or both activators together were also determined from measurements of steady-state tryptophan anisotropy, as described elsewhere (*46*) in an assay system identical to that used for determinations of activity except that the enzyme concentration was 0.1 μM and the total volume was 2 mL.

RESULTS

The aim of this study was to investigate the role of Ca^{2+}-dependent and Ca^{2+}-independent membrane-binding events and conformational changes in the synergistic enhancement of cPKC activity that results from coaddition of phorbol esters and DAG (*2*, *3*). To investigate this, the Ca^{2+}-induced membrane association and activity of PKCα, PKCβI, and PKCγ were determined in the presence of TPA at concentrations which have been shown previously to be sufficient to induce a maximal level of activity in each case.

Effects of the Coaddition of DAG and TPA on the Ca^{2+} Dependence of cPKC Activity. Concentration−response curves for Ca^{2+}-induced PKCα, -βI, and -γ activity are shown in Figure 1. Increasing Ca^{2+} levels in the absence of either TPA or DAG resulted in marginal effects on activity (●). For each cPKC isoform, inclusion of either TPA (■) or DAG (▲) in the vesicles resulted in a decrease in the Ca^{2+} requirements for activity. The concentration of TPA used was chosen such that it saturated high-affinity phorbol ester binding (*2*, *3*, *8*). The presence of TPA, but not DAG, resulted in a small increase in the level of cPKC activity obtained in the absence of Ca^{2+}, in keeping with the previously reported resistance of phorbol ester-induced activity to Ca^{2+} chelation (*34*, *36*). The effects of adding TPA and DAG simultaneously in the same assay were both enhancement of the activities of each cPKC isoform in a Ca^{2+}-independent manner and reduction of the Ca^{2+} concentration required for half-maximal activation (EC_{50}) by ∼5-fold for PKCα and PKCβI and ∼10-fold for PKCγ (see the legend of Figure 1 for EC_{50} values). Importantly, the level of activity induced by coaddition of TPA and DAG in the presence of maximal Ca^{2+} levels (>100 μM) was found to exceed that induced by either activator separately, suggesting that the corresponding activating conformational changes may be distinct.

Effects of the Coaddition of DAG and TPA on cPKC−Membrane Interactions Based on a RET Binding Assay. Determination of RET between the tryptophans of proteins that associate with membranes and fluorophores attached to lipids has frequently been used as the basis for the measurement of protein−membrane binding (*42*, *43*, *45*, *50*). The Ca^{2+} dependence of the interaction of PKCα, -βI, and -γ with membranes containing TPA, quantified by measuring RET from cPKC tryptophans to dansyl-PE, is shown in Figure 2. Titration of Ca^{2+} in the absence of activators resulted in an increase in fluorescence intensity due to RET, although this was negligible except at very high Ca^{2+} levels (●). Thus, at low Ca^{2+} levels (≤1 μM), the extent of association of cPKC isoforms with vesicles in the absence of activators was marginal which is in keeping with the low level of Ca^{2+}-induced activity observed under similar condi-

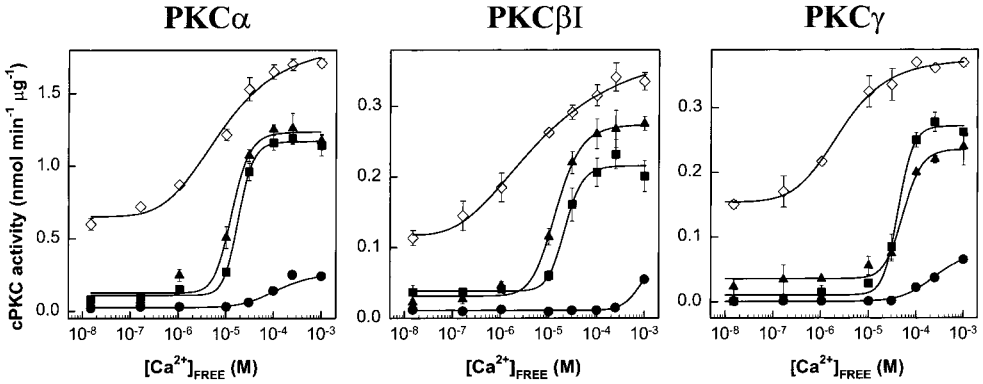

FIGURE 1: Ca^{2+} requirements for cPKC (α, βI, and γ) activity induced by TPA or DAG separately, or by both activators together. Specific activities of PKCα, -βI, and -γ isoforms were determined in the presence of POPC/BPS LUV (4:1 molar ratio, 150 μM total lipid concentration) as a function of free Ca^{2+} concentration in the absence of activator (●) or in the presence of 0.3 mol % TPA (■) or 4 mol % DAG (▲) separately, or with both activators together (◇). Solid curves correspond to fits of data to a modified Hill equation (*44*). The calculated values of EC_{50} (micromolar) were as follows for PKCα: 10 ± 2 (TPA), 17 ± 5 (DAG), and 2.0 ± 0.5 (TPA and DAG). They were as follows for PKCβI: 23 ± 12 (TPA), 18 ± 4 (DAG), and 2.1 ± 1.0 (TPA and DAG). They were as follows for PKCγ: 52 ± 13 (TPA), 49 ± 2 (DAG), and 15.2 ± 1 (TPA and DAG). Data represent the means of triplicate determinations ± standard deviation (SD). Other details are described in Materials and Methods.

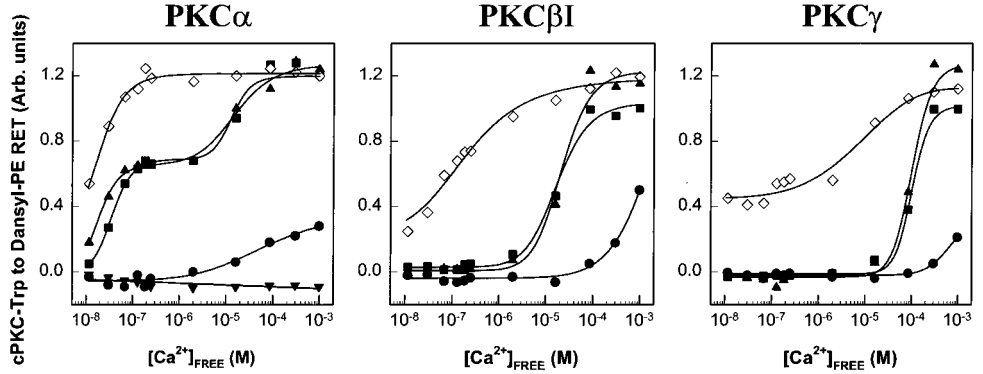

FIGURE 2: Ca^{2+} requirements for membrane association of cPKC induced by TPA or DAG separately, or by both TPA and DAG together, determined from measurements of dansyl-PE RET. Fluorescence intensities corresponding to RET from cPKC tryptophans to dansyl-PE were measured as a function of free Ca^{2+} concentration in the presence of POPC/BPS LUV (4:1 molar ratio, 150 μM total lipid concentration) containing 5 mol % dansyl-PE in the absence of activators (●) with either 0.3 mol % TPA (■), 4 mol % DAG (▲), or both activators together (◇). Also shown are data corresponding to RET between TOE and dansyl-PE determined in the presence of POPC/BPS LUV containing 0.3 mol % TPA (▼). Solid curves correspond to fits of data to a modified Hill equation assuming a single class (*44*) or two classes of Ca^{2+}-binding sites (*3*), as required. The calculated values of EC_{50} ± SD (micromolar) were as follows for PKCα: 0.05 ± 0.10 and 14 ± 6 (TPA) and 0.04 ± 0.10 and 12 ± 5 (DAG). They were as follows for PKCβI: 25 ± 5 (TPA), 21 ± 5 (DAG), and 0.5 ± 0.2 (TPA and DAG). They were as follows for PKCγ: 95 ± 35 (TPA), 98 ± 32 (DAG), and 7 ± 5 (TPA and DAG). Data are representative of triplicate determinations. Other details are described in Materials and Methods.

tions. For PKCα, the presence of either 0.3 mol % TPA (■) or 4 mol % DAG (▲) separately resulted in two separate increases in RET. For both activators, these increases occurred within similar Ca^{2+} concentration ranges, indicating that the presence of 0.3 mol % TPA and 4 mol % DAG had equipotent effects on membrane binding affinity. The second rise in the RET signal occurred within Ca^{2+} concentration ranges similar to those required to induce activation. In contrast to that for PKCα, the Ca^{2+}-dependent RET curves for PKCβI and PKCγ obtained in the presence of TPA or DAG only showed an increase in RET at higher Ca^{2+} levels, corresponding again to the level of Ca^{2+} required for activation. For each cPKC isoform, the coaddition of TPA and DAG resulted in both an increase in the level of the RET signal observed in the absence of Ca^{2+} and also a decrease in the Ca^{2+} concentration requirement for the second rise in RET relative to that observed in the presence of TPA or DAG alone (◇). However, the maximal level of RET attained at the highest Ca^{2+} concentrations was unaffected by coaddition of the two activators. The possibility that the observed increases in tryptophan to dansyl-PE RET may have resulted from an effect of Ca^{2+} on the bulk distribution of dansyl-PE within the bilayer was ruled out by the finding that RET to dansyl-PE from the tryptophan of TOE was unaffected within the Ca^{2+} concentration range used (▼).

In a previous study from this laboratory, it was shown that the level of RET from PKC tryptophans to the fluorescent phorbol ester SAPD increases upon membrane association (*5*). The Ca^{2+} dependencies of the increases in the SAPD RET signal for each cPKC isoform, a function of the extent of membrane binding, are shown in Figure 3. The results confirm that PKCα associates with membranes at Ca^{2+} levels that are lower than those required for activity. Thus, similar to the results obtained from measurements of tryptophan to dansyl-PE RET shown in Figure 2 for PKCα, the PKCα tryptophan to SAPD RET data show that there are two Ca^{2+} binding events at distinct low and high Ca^{2+} levels. Further, as with the cPKC tryptophan to dansyl-PE RET measurements, the second increase in cPKC tryptophan to SAPD RET corresponded to the Ca^{2+} concentration

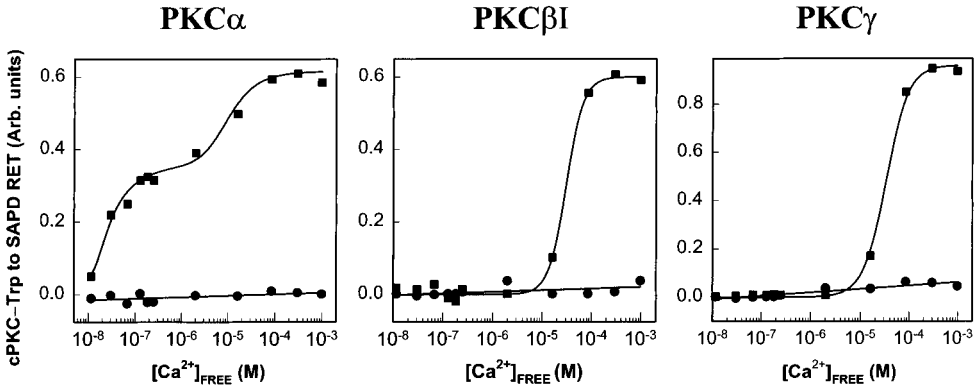

FIGURE 3: Extent of membrane association determined from the Ca^{2+} requirements for the interaction of the phorbol ester, SAPD, with cPKC isoforms. The level of binding of SAPD to cPKC, determined from the fluorescence intensity increase accompanying cPKC tryptophan to SAPD RET, was measured as a function of Ca^{2+} concentration in the presence of LUV comprised of POPC/BPS (4:1 molar ratio, 150 μM total lipid concentration). The effect of Ca^{2+} on the fluorescence signal was measured in the presence (■) or absence (●) of cPKC. Solid curves correspond to fits of data to a modified Hill equation assuming a single class (*44*) or two classes of Ca^{2+}-binding sites (*3*), as required. The calculated values of $EC_{50} \pm$ SD (micromolar) were as follows for PKCα: 0.03 ± 0.10 and 10 ± 2. They were as follows for PKCβI and PKCγ: 30 ± 15 and 35 ± 20, respectively. Data are representative of triplicate determinations. Other details are described in Materials and Methods.

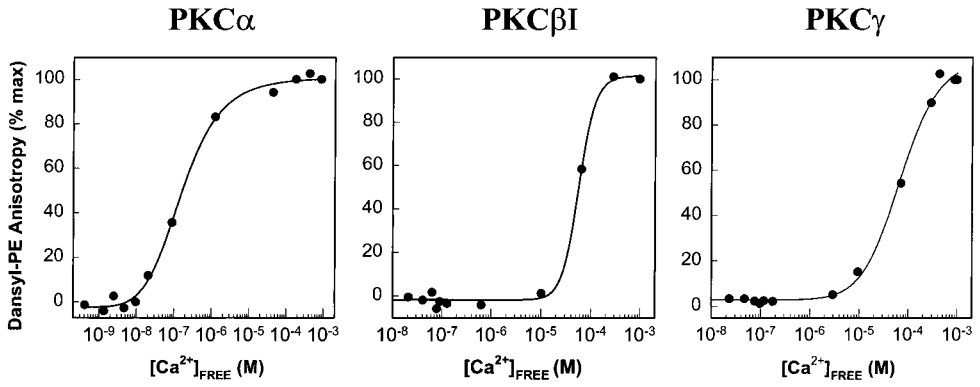

FIGURE 4: Ca^{2+} dependence of the interaction of cPKC isoforms with membranes in the presence of TPA determined from measurements of dansyl-PE steady-state anisotropy. Dansyl-PE steady-state anisotropy, which is proportional to the motional freedom of the dansyl fluorophore, was measured as a function of Ca^{2+} concentration in the presence of LUV identical to those used for tryptophan to dansyl-PE RET determinations (see the legend of Figure 2) incorporating 0.3 mol % TPA. Solid curves correspond to fits of data to a modified Hill equation (*44*). The calculated values of $EC_{50} \pm$ SD (micromolar) were as follows for PKCα, PKCβI, and PKCγ: 0.1 ± 0.1, 43 ± 16, and 50 ± 32, respectively. Data are representative of triplicate determinations. See Materials and Methods for other details.

requirement for activation, shown in Figure 1. By contrast, PKCβI and -γ did not appear to associate with membranes at low Ca^{2+} levels, corresponding to the lack of activity of these isoforms under similar conditions. The Ca^{2+} concentration−response curves for the interaction of SAPD with PKCβI and -γ were again similar to those for the Ca^{2+}-induced association of these isoforms with vesicles derived from measurements of RET from PKC tryptophans to the fluorophore of dansyl-PE (Figure 2). The SAPD fluorescence signal was unaffected by increasing concentrations of Ca^{2+} determined in the absence of cPKC isoforms, ruling out the possibility that either of the Ca^{2+}-induced events may have arisen from effects on SAPD fluorescence due to structural perturbations of the vesicles (Figure 3, ●).

Since RET is directly proportional to the distance between participating dansyl-PE or bound SAPD and cPKC tryptophan fluorophores, the magnitude of the observed increase in RET may have corresponded to both Ca^{2+}-induced membrane association and a conformational change in the membrane-bound form of the enzyme. To separate the contribution of membrane association from conformational changes to the observed RET signals, the Ca^{2+} concentration dependencies of the physical interaction of cPKC isoforms with vesicles were determined. This was achieved using measurements of dansyl-PE fluorescence anisotropy, changes in the susceptibility of cPKC isoforms to proteolysis (*44*, *48*, *49*), and centrifugal separation (*29*, *47*).

Determination of the Extent of Membrane Association from Measurements of Dansyl-PE Steady-State Anisotropy. The dansyl fluorophore of dansyl-PE is located within the headgroup region of the vesicle lipid bilayers used for RET determinations (see above), where its motional freedom is rendered susceptible to hindrance upon binding of cPKC to the bilayer surface. Measuring this effect in terms of a steady-state fluorescence anisotropy of the dansyl fluorophore as a function of Ca^{2+} concentration gives a further method for assessing cPKC membrane association, as shown in Figure 4. For PKCα, the midpoint of the Ca^{2+} concentration curve, obtained in the presence of TPA, was similar to that observed for the low-Ca^{2+} concentration-dependent increase in tryptophan to dansyl-PE or SAPD RET and was ~2 orders of magnitude lower than that required for activity (Figure 1). By contrast, for PKCβI and PKCγ, the midpoints of the anisotropy versus Ca^{2+} concentration curves obtained in the presence of TPA were similar to those obtained for the activation of these isoforms. These data also confirm that

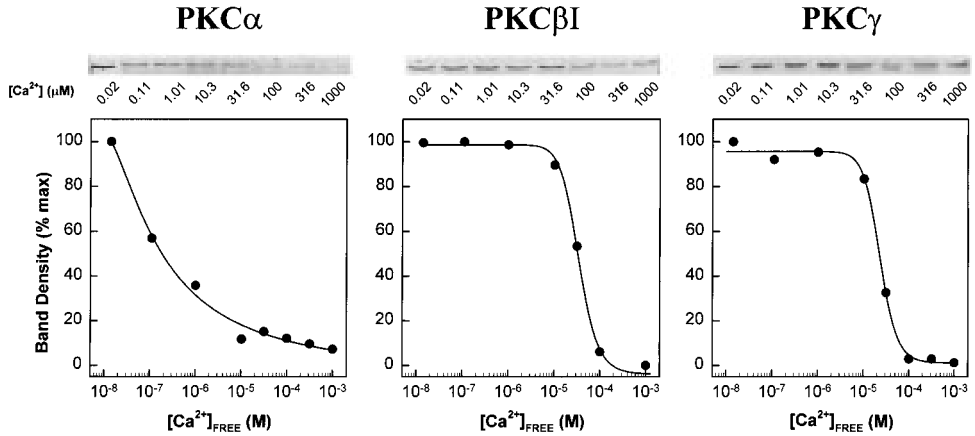

FIGURE 5: Ca^{2+} dependence of the interaction of cPKC isoforms with membranes in the presence of TPA determined from susceptibilities to proteolysis. The disappearance of full-length cPKC due to proteolysis, the susceptibility to which increases upon membrane association, was measured from the corresponding decrease in silver stain band density as a function of Ca^{2+} in the presence of POPC/BPS LUV (4:1 molar ratio, 150 μM total lipid concentration) incorporating 0.3 mol % TPA (see the representative gels). Solid curves correspond to fits of data to a modified Hill equation (*44*). The calculated values of $EC_{50} \pm$ SD (micromolar) were as follows for PKCα, PKCβI, and PKCγ: 0.2 ± 0.1, 21 ± 15, and 22 ± 15, respectively. Experiments were repeated in triplicate with essentially similar results. Other details are described in Materials and Methods.

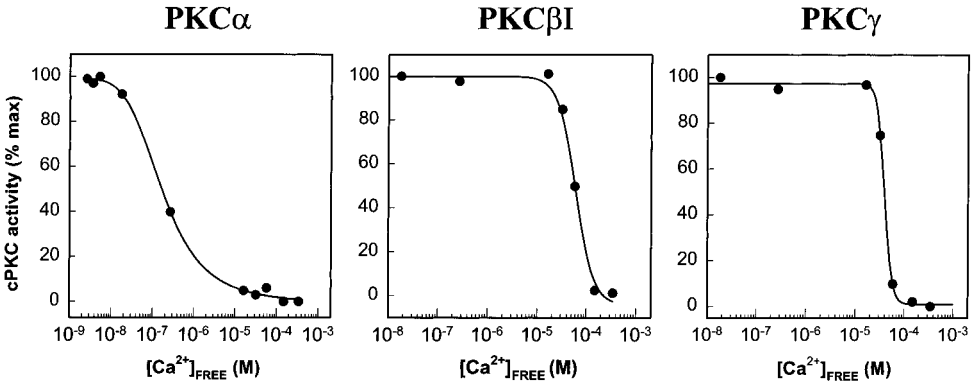

FIGURE 6: Ca^{2+} dependence of the interaction of cPKC isoforms with membranes in the presence of TPA determined from centrifugal separation. The association of cPKC isoforms with sucrose-loaded vesicles (SLV) composed of POPC/BPS (4:1 molar ratio, 150 μM total lipid concentration) incorporating 0.3 mol % TPA was measured as a function of free Ca^{2+} concentration by centrifugal separation of free enzyme from bound enzyme. The concentration of free cPKC is expressed as the specific activity of each isoform remaining in the supernatant. Solid curves correspond to fits of data to a modified Hill equation (*44*). The calculated values of $EC_{50} \pm$ SD (micromolar) were as follows for PKCα, PKCβI, and PKCγ: 0.1 ± 0.2, 46 ± 36, and 40 ± 30, respectively. Data are representative of triplicate determinations. Other details are described in Materials and Methods.

the Ca^{2+} levels required for the association of PKCβI and PKCγ with membranes are ~2 orders of magnitude higher than those required for the membrane association of PKCα.

Determination of the Extent of Membrane Association from Proteolysis. Proteolysis of PKC initially results in the formation of three major breakdown products corresponding to the catalytic and regulatory domains, arising from cleavage of the V3-hinge region and a "nicked" form of the enzyme resulting from the loss of the pseudosubstrate (*31*, *32*, *44*, *48*, *49*, *51*). Recent studies have shown that the association of PKC with Triton X-100 micelles containing phosphatidylserine and diacylglycerol results in a large increase in the susceptibility of the enzyme to proteolytic cleavage at both the hinge and pseudosubstrate regions (*31*). On the basis of these findings, the Ca^{2+} requirements for the membrane association of cPKC isoforms were determined by measuring the rate of disappearance of the intact enzyme resulting from proteolysis, as shown in Figure 5.

Increasing Ca^{2+} concentrations in the presence of TPA were found to result in an increase in the degree of proteolysis, determined from the decrease in the densities of bands corresponding to the full-length isozymes, which is consistent with Ca^{2+}-induced membrane association (Figure 5). Trypsin activity was unaffected by Ca^{2+} within the concentration range used, as shown in a separate experiment using *N*-benzoyl-L-arginine ethyl ester as a substrate (results not shown). Using this method, PKCα was found to associate with LUV containing TPA within a Ca^{2+} concentration range similar to that required for the low-Ca^{2+} concentration-dependent increase in tryptophan to dansyl-PE or SAPD RET and ~2 orders of magnitude lower than that required for activity (Figure 1). Further, membrane association of PKCα occurred within Ca^{2+} levels ~2 orders of magnitude lower than for the membrane association of either PKCβI or PKCγ.

Determination of the Extent of Membrane Association by Centrifugal Separation. To obtain further confirmation of the membrane association data, the extent of cPKC−membrane binding was also determined by direct separation of membrane-bound enzyme from free enzyme (Figure 6). In this case, sucrose loading of the vesicles (SLV) was required to facilitate separation of unbound from vesicle-

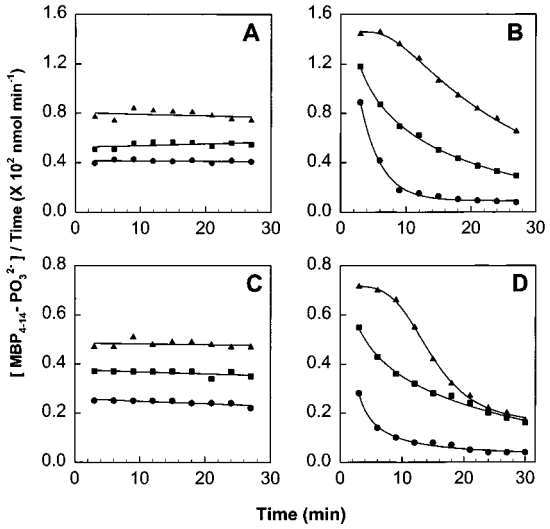

FIGURE 7: Coaddition of TPA and DAG induces the formation of PKCα and PKCβI conformers distinct from those induced by either activator separately. PKCα and PKCβI conformers induced by interaction with 4 mol % DAG (●), 0.3 mol % TPA (■), or both activators together (▲) in the presence of 0.1 mM Ca^{2+} were distinguished by measuring the corresponding decrease of activity ($[MBP_{4-14}-PO_3^{2-}]$/time) as a function of time due to thermal denaturation. For PKCα, activities were measured at 30 (A) and 45 °C (B), while PKCβI activities were determined at 30 (C) and 43 °C (D). The assay system used was as described for the determination of Ca^{2+}-dependent cPKC activity (see the legend of Figure 1). Data are representative of triplicate determinations. Other details are described in Materials and Methods.

bound cPKC by centrifugation. The specific activity of PKCα, -βI, and -γ remaining in the supernatant, determined using a protamine sulfate assay, was found to decrease as a function of Ca^{2+} concentration, consistent with the enzyme being bound to the SLV, as shown in Figure 6. Again, it was found that PKCα associated with SLV containing TPA at Ca^{2+} concentrations ~2 orders of magnitude lower than those required for the interaction of either PKCβI or PKCγ with these vesicles, which were similar in this respect. The activities of the cPKC isoforms were found to be unaffected by sucrose loading of vesicles (results not shown). Comparison of the curve obtained for PKCα with the binding isotherm obtained from tryptophan to dansyl-PE or SAPD RET measurements suggests that the association of this isoform with the membrane occurs within Ca^{2+} levels corresponding to the first increase in fluorescence observed in Figures 2 and 3. Furthermore, comparisons of these binding isotherms with those obtained for TPA-induced activity indicate that the Ca^{2+} levels required to induce PKCα activity were ~2 orders of magnitude greater than those required for interaction of this isoform with LUV. By contrast for PKCβI and PKCγ, the binding isotherms were found to coincide with the corresponding Ca^{2+}-induced activity curves (Figure 1).

Comparison of Ca^{2+}-Dependent Conformations of PKCα and PKCβI Induced by DAG or TPA Separately or Together. Differing conformers of PKCα and PKCβI induced by interaction with DAG and TPA together were distinguished from those induced by each activator separately on the basis of measurements of the thermal stability, shown in Figure 7, and the steady-state anisotropy of cPKC tryptophans, shown in Table 1. The concentrations of TPA (0.3 mol %), DAG (4 mol %), Ca^{2+} (0.1 mM), and PS (20 mol %) were chosen such that for both measurements of thermal stability and anisotropy, membrane association of each isoform was complete.

Table 1: Ca^{2+}-Dependent Effects of 4 mol % DAG and 0.3 mol % TPA in POPC/BPS LUV (4:1 molar ratio) on the Steady-State Anisotropy of PKCα Tryptophans[a]

$[Ca^{2+}]$ (μM)	basal	DAG	TPA	DAG and TPA
0	0.182 ± 0.004	0.196 ± 0.003	0.201 ± 0.003	0.198 ± 0.004
1	0.185 ± 0.002	0.211 ± 0.002	0.214 ± 0.003	0.232 ± 0.002
100	0.201 ± 0.005	0.256 ± 0.002	0.254 ± 0.002	0.267 ± 0.003

[a] See Materials and Methods for details. Data are averages of triplicate determinations ± SD.

Plots of activity as a function of time, determined at 30 °C for PKCα or PKCβI induced by either DAG or TPA alone or by both activators together, were linear and had negligible slope, indicating thermal stability (Figure 7A,C). By contrast, the corresponding curves obtained at elevated temperatures of 45 °C for PKCα and 43 °C for PKCβI deviated markedly from linearity, indicating a decrease in PKCα activity as a function time due to thermal denaturation (Figure 7B,D). Importantly, for both PKCα and PKCβI, the initial rate of loss of activity in the presence of DAG and TPA together was lower than that observed with either activator separately, suggesting that interaction with both activators may result in the formation of a distinct active cPKC conformer.

In the presence of DAG or TPA alone, the steady-state anisotropy of PKCα tryptophans shown in Table 1 was found to increase within a Ca^{2+} concentration range corresponding to the membrane association of this isoform (0−1 μM). This is consistent with a concomitant decrease in the average motional freedom of the PKCα tryptophans. The data also show that the tryptophans of membrane-bound but inactive PKCα (1 μM Ca^{2+}) are less hindered compared to those in the membrane-bound and active form of the enzyme (100 μM Ca^{2+}). These data are consistent with the second rise in PKCα tryptophan to dansyl-PE or SAPD RET induced by high Ca^{2+} concentrations for PKCα being due to a Ca^{2+}-dependent activating conformational change. The steady-state anisotropy of PKCα tryptophans were found to be increased upon coaddition of TPA and DAG to a higher value compared to that observed with either TPA or DAG separately, again suggesting a distinct enzyme conformation (Table 1). Interestingly, the anisotropy of PKCβI tryptophans was found to be unaffected by levels of Ca^{2+} sufficient for the membrane association and activation of this isoform (results not shown). This does not preclude a Ca^{2+}-dependent conformational change in PKCβI but may be either due to the tryptophan(s) which senses the conformational change in PKCα not being conserved in PKCβI or because the activating conformational changes in these two isoforms differ such that there is no net change in the environment of the PKCβI tryptophans.

Effects of the Coaddition of DAG and TPA on Ca^{2+}-Independent PKCα Activity and Membrane Interactions. Inspection of the data shown in Figure 1 indicates that coaddition of TPA and DAG results not only in a decrease in the Ca^{2+} concentration requirements for cPKC activation but also in an increase in the level of Ca^{2+}-independent activity. Phorbol esters have been reported to differ from DAG in inducing an increase in "chelator-resistant" activity

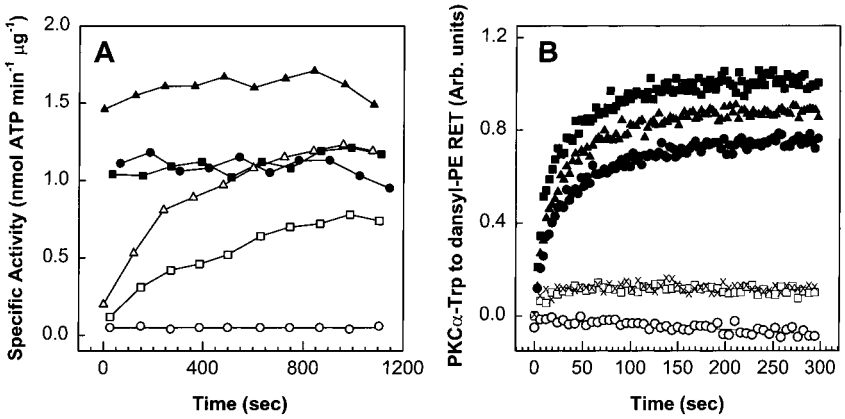

FIGURE 8: Effects of the coaddition of DAG and TPA on Ca^{2+}-independent PKCα activity and membrane interactions. (A) The rate of generation of Ca^{2+}-independent PKCα activity was determined in the presence of 0.1 mM EGTA after preincubation of the isozyme for fixed time intervals with POPC/BPS (4:1 molar ratio, 150 μM total lipid concentration) LUV containing 4 mol % DAG (○), 0.3 mol % TPA (□), or both activators together (△) in the presence of 0.1 mM EGTA. The corresponding Ca^{2+}-dependent activity induced by 4 mol % DAG (●), 0.3 mol % TPA (■), or DAG together with TPA (▲) was determined by an identical procedure, except that the enzyme was preincubated and assayed with 0.1 mM Ca^{2+}. (B) The time dependence of Ca^{2+}-independent association of PKCα with POPC/BPS LUV containing 4 mol % DAG (○), 0.3 mol % TPA (△), or both activators together (×) in the presence of 0.1 mM EGTA. The extent of Ca^{2+}-dependent association of PKCα with LUV induced by DAG (●), TPA (■), or both activators (▲) was determined in the presence of 0.1 mM Ca^{2+}. Other assay conditions were identical to those used for determinations of activity. Data are representative of triplicate determinations. For other details, see Materials and Methods.

which has been suggested to result from a time-dependent irreversible conformational change (*4*, *34*, *35*, *37*), or alternatively may be due to the relatively high-affinity interaction of PKC with membranes containing phorbol esters compared with those containing DAG (*24*, *36*). To investigate the contribution of these factors to the elevated level of Ca^{2+}-independent cPKC activity induced by coaddition of DAG and TPA, the time dependencies of the appearance of Ca^{2+}-independent activity induced by DAG or TPA separately or by both activators together were compared with the corresponding rates of membrane association (Figure 8). Consistent with the results of previous studies (*4*, *34*, *35*), preincubating PKCα, used as a representative cPKC isoform, with EGTA and vesicles containing TPA for increasing periods of time prior to measuring activity again in the presence of EGTA resulted in a time-dependent increase in Ca^{2+}-independent activity (Figure 8A). By contrast, PKCα activity induced by DAG remained completely Ca^{2+}-dependent irrespective of incubation time. The effect of the coaddition of DAG along with TPA was to increase both the rate and the maximal level of Ca^{2+}-independent activity compared to that obtained with TPA alone. By contrast to effects on Ca^{2+}-independent activity, Figure 8A shows that a maximal level of Ca^{2+}-dependent PKCα activity induced by TPA or DAG or both was rapidly attained (i.e., within the first time point), after which the level of activity remained independent of preincubation time. Also, the level of Ca^{2+}-dependent activity induced by either TPA alone or in combination with DAG was in each case greater than the corresponding level of Ca^{2+}-independent activity.

The rates of association of PKCα with vesicles containing DAG or TPA compared to those with both activators together, determined from measurements of PKCα tryptophan to dansyl-PE RET, are shown in Figure 8B. Addition of PKCα in the absence of Ca^{2+} to vesicles containing TPA resulted in a low level of membrane association relative to that observed in the presence of Ca^{2+}, while under similar conditions, the enzyme did not interact with vesicles containing DAG. The maximal rates of membrane association of PKCα, induced by TPA and DAG added separately or together, or in the presence or absence of Ca^{2+} were each similar. Importantly, the rate of Ca^{2+}-independent membrane association was found to be relatively fast compared with the slow evolution of Ca^{2+}-independent activity (Figure 8A).

DISCUSSION

In this study, the Ca^{2+} dependencies of membrane association and activation of cPKC isoforms have been determined as a function of the presence of TPA or DAG and in the presence of both activators together. It was found that membrane association of PKCα occurred at a much lower Ca^{2+} concentration compared to that required for activity, whereas for PKCβI and -γ, both membrane association and activation occurred at the same level of Ca^{2+} that was required for activation. The Ca^{2+} concentration-dependent membrane association and activation of cPKC were associated with distinct conformations of the enzyme. Furthermore, the additive levels of activation achieved by the coaddition of DAG and TPA resulted from Ca^{2+}-dependent and Ca^{2+}-independent conformational changes that led to the formation of cPKC conformers distinct from those induced by either activator alone.

The tryptophan to dansyl-PE or SAPD RET data for PKCα showed a biphasic Ca^{2+} concentration response curve. While the activity data shown in Figure 1 indicate that Ca^{2+} levels between 1 and 100 μM were required for activation by DAG or TPA, the first increase in RET was observed to occur ~2 orders of magnitude lower than this. Measurements of the increase in dansyl-PE anisotropy, sensitivity to proteolysis, and degree of centrifugal separation of free enzyme from membrane-bound enzyme as a function of the Ca^{2+} concentration indicated that membrane association was complete for PKCα at a Ca^{2+} level corresponding to that required to induce the first increase in the RET signal. This suggests that the second increase in RET was due to a conformational change in the membrane-associated enzyme that corresponds to activation. Other possibilities for the observed increase

in fluorescence, such as a change in the quantum yield of either fluorophore by increasing Ca^{2+} levels, were ruled out in separate measurements (results not shown), leaving a change in the average distance between the RET tryptophan donors and the dansyl acceptors as the likely explanation. This could only occur if the PKCα conformation was modified, and since enzyme activation was found to occur within the same Ca^{2+} concentration range, this process is suggested to correspond to an activating conformational change. However, since dansyl-PE anisotropy was observed to be a monophasic function of Ca^{2+} concentration, detecting only the association of the enzyme with the membrane, this conformational change apparently does not lead to a change in the average environment of dansyl-PE molecules that are located in vicinity of the PKCα molecule.

Support for a Ca^{2+}-induced conformational change in PKCα was provided by the finding that increasing the level of Ca^{2+} from 1 μM, sufficient for maximal TPA- or DAG-induced membrane association but lower than that required for activation, to 100 μM, sufficient for maximal activation, resulted in an increase in tryptophan anisotropy. Consistent with this, a recent mutagenesis study showed that one of two discrete Ca^{2+}-binding sites identified within the C2 domain of PKCα may mediate a conformational change that leads to the exposure of two tryptophan residues to the membrane bilayer interior (*33*). For PKCα, the Ca^{2+}-dependent conformational change that accompanies membrane association and which results in the exposure of the hinge region to proteolysis appears to be physically distinct from that resulting in activation, in keeping with other studies (*31*, *32*). On the basis of the results presented here, it is not possible to distinguish between the membrane association and activation of PKCβI and PKCγ since these processes were observed to occur within similar Ca^{2+} concentration ranges.

In a previous study from this laboratory, it was proposed that the synergistic enhancement of phorbol ester-induced PKCα activity results from an increase in the level of high-affinity phorbol ester binding induced by the interaction of DAG with the low-affinity phorbol ester binding site (*3*). On the basis of the observations presented here, it would appear that one of the effects of the interaction of DAG with the low-affinity phorbol ester binding site on cPKC is to decrease the Ca^{2+} concentration dependence for activity. The observation that at maximal Ca^{2+} concentrations (>100 μM) the level of cPKC activity induced by coaddition of phorbol ester and DAG remained greater than that which could be achieved with by either activator separately suggests that the interaction of DAG with the low-affinity phorbol ester binding site may induce the formation of cPKC conformers distinct from those induced by interaction with either activator separately. Support for this came from the observation that the activated forms of PKCα and PKCβI resulting from interaction with both DAG and TPA together displayed greater thermal stability compared to either activator alone. Further evidence for this was obtained for PKCα from the observation that the coaddition of DAG and TPA resulted in an increase in the steady-state anisotropy of PKCα tryptophans.

On the basis of the observations presented here, cPKC bound to membranes containing phorbol esters may become active either via a Ca^{2+}-dependent activating conformational change or via a time-dependent, Ca^{2+}-independent process, which is not available to cPKC associated with DAG alone, in keeping with previous findings (*4*, *34*, *35*, *37*). This chelator-resistant activity has been suggested to result from the relatively high-affinity interaction of the enzyme with membranes containing phorbol esters (*24*, *36*). The finding that the level of Ca^{2+}-independent PKCα activity induced by TPA increased over a relatively long time period compared to the rate of association with vesicles containing the same surface concentration of the phorbol ester is consistent with a rate-limiting phorbol ester-induced activating conformational change, as proposed to occur in a number of previous studies (*4*, *34*, *35*, *37*). However, the observation that the maximal level of Ca^{2+}-independent PKCα activity induced by TPA was lower than that attained in the presence of Ca^{2+}, and that level of Ca^{2+}-induced activity was unaffected by preincubation with TPA (Figure 8A), suggests that the corresponding Ca^{2+}-dependent and Ca^{2+}-independent activating conformational changes may be distinct.

It appears that occupation of the low-affinity phorbol ester binding site on PKCα by DAG results in a slight increase in the extent of Ca^{2+}-independent membrane binding and also a time-dependent, Ca^{2+}-independent activating conformational change, the rate and extent of which are enhanced compared to that induced by phorbol ester alone. Consistent with this, recent studies from this laboratory indicate that the activity of Ca^{2+}-independent recombinant PKCδ is also enhanced in the presence of TPA and DAG together compared to that induced by either activator separately, although the magnitude of this effect appears to be reduced compared to that with the cPKC isoforms (F. J. Taddeo, C. Ho, S. J. Slater, and C. D. Stubbs, unpublished observations). Also, Blumberg and co-workers proposed that the appearance of this chelator-resistant form of PKCα may be regulated by a distinct site on the enzyme with relatively low phorbol ester binding affinity compared to that which mediates the reversible, Ca^{2+}-dependent membrane interaction and activation (*4*).

The observation of two separate Ca^{2+}-induced increases in the level of the tryptophan to dansyl-PE or SAPD RET signal is consistent with the existence of two distinct "classes" of Ca^{2+}-binding sites on PKCα with high and low affinities, respectively. In keeping with this possibility, it was shown in a previous study that PKCα is capable of binding Gd^{3+}, commonly used as a probe for Ca^{2+} binding, with high and low levels of binding, respectively (*52*, *53*). Also, it was reported that PKCβ (I or II) differs from PKCα in containing only a single class of low-affinity Gd^{3+}-binding sites, consistent with the observation in this study that the Ca^{2+}-induced increase in RET for PKCβI was monophasic. This difference between PKCα and PKCβ (I or II) was also observed in another study where the activities of both isoforms, induced in the presence of short chain phospholipid micelles, were entirely Ca^{2+}- and phospholipid-dependent, while PKCα was shown to have an extra Ca^{2+} requirement that did not involve phospholipid interactions (*54*). However, the stoichiometry of Ca^{2+} binding to PKCβI remains unclear. Thus, while one study provided evidence for a single high-affinity Ca^{2+}-binding site within a GST fusion protein containing the entire regulatory region of PKCβI (*55*, *56*), another study reported that the isolated C2 domain of this isoform may cooperatively bind two Ca^{2+} ions (*57*). Multiple

Ca^{2+} binding sites have also been identified within the C2 domains of synaptotagmin (*58*), phospholipase Cγ1 (*59*, *60*), and cytosolic phospholipase A_2 (*61*−*63*), all of which are appreciably homologous with respect to sequence (*64*). On the basis of the observed similarity of the binding isotherms for PKCβI and PKCγ from the RET, dansyl-PE anisotropy, centrifugation, and proteolysis measurements of this study, the membrane association and activation of these isoforms may involve either a single class of Ca^{2+}-binding site or two classes of sites with similar Ca^{2+}-binding affinities.

In conclusion, this study provides evidence that the synergistic enhancement of cPKC activity induced by phorbol ester in combination with DAG proceeds by two distinct mechanisms, involving both Ca^{2+}-dependent and Ca^{2+}-independent membrane binding events and distinct activating conformational changes. (a) The cPKC isoforms, which are inactive in solution due to the occupation of the active site by the pseudosubstrate, may translocate to the membrane surface by interaction with Ca^{2+}. (b) The Ca^{2+}-dependent membrane association of PKCα apparently occurs at Ca^{2+} levels lower than those required for activation, resulting in an "inactive" membrane-bound form of this isozyme, whereas for PKCβI and PKCγ, translocation and the activating conformational change occur within similar Ca^{2+} concentration ranges. (c) Increasing the level of Ca^{2+} further results in an activating conformational change in each cPKC isoform (involving the release of the pseudosubstrate from the active site). (d) The combined interaction of DAG together with phorbol ester with cPKC results in a Ca^{2+}-dependent activating conformational change leading to a form of cPKC which is distinct from that induced by interaction with either phorbol ester or diacylglycerol alone. (e) Translocation of cPKC to membranes may also occur in the absence of Ca^{2+} by interaction with phorbol ester which results in an inactive cPKC form that may then undergo a time-dependent, chelator-resistant activating conformational change. (f) The interaction of DAG with the low-affinity phorbol ester binding site induces a time-dependent activating conformational change that again leads to a form of cPKC which is distinct from that induced by interaction with phorbol ester alone.

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