Regulation of PKCα Activity by C1-C2 Domain Interactions*

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In this study, the role of interdomain interactions involving the C1 and C2 domains in the mechanism of activation of PKC was investigated. Using an in vitro assay containing only purified recombinant proteins and the phosphor ester, 4β-12-O-tetradecanoylphorbol-13-acetate (TPA), but lacking lipids, it was found that PKCα bound specifically, and with high affinity, to a αC1A-C1B fusion protein of the same isozyme. The αC1A-C1B domain also potently activated the isozyme in a phorbol ester- and diacylglycerol-dependent manner. The level of this activity was comparable with that resulting from membrane association induced under maximally activating conditions. Furthermore, it was found that αC1A-C1B bound to a peptide containing the C2 domain of PKCe. The αC1A-C1B domain also activated conventional PKCβ, -βII, and -γ isoforms, but not novel PKCδ or -ε. PKCδ and -ε were each activated by their own C1 domains, whereas PKCα, -βII, -βIII, or -γ activities were unaffected by the C1 domain of PKCδ and only slightly activated by that of PKCe. PKCγ activity was unaffected by its own C1 domain and those of the other PKC isoforms. Based on these findings, it is proposed that the activating conformational change in PKCα results from the dissociation of intra-molecular interactions between the αC1A-C1B domain and the C2 domain. Furthermore, it is shown that PKCα forms dimers via intermolecular interactions between the C1 and C2 domains of two neighboring molecules. These mechanisms may also apply for the activation of the other conventional and novel PKC isoforms.

The 10 closely related isoforms that constitute the protein kinase C (PKC) family of serine/threonine kinases each occupy critical nodes in the complex cellular signal transduction networks that regulate diverse cellular processes, including: secretion, proliferation, differentiation, apoptosis, permeability, migration, and hypertrophy (1–7). In common with many signaling proteins, the structure of PKC is modular, consisting of a C-terminal catalytic region containing the active site, and a regulatory region with conserved domains that mediate membrane association and activation. PKC isoforms are classified according to the structural and functional differences in these conserved domains (8, 9). In the case of the “conventional” PKCα, -βII/βIII, and -γ isoforms, these include the activator-binding C1 domains, and the Ca²⁺-binding C2 domain. The C1 domains consist of a tandem C1A and C1B arrangement, each of which can potentially bind the endogenous activator, diacylglycerol and exogenous activators including phorbol esters. The “novel” PKCδ, -ε, -η, -θ, and -μ isoforms, contain C2 domains that lack Ca²⁺ binding ability, while retaining functional C1A and C1B domains. The “atypical” PKCζ, -ι, and -λ regulatory domains also lack a functional C2 domain and contain a single C1 domain that lacks the ability to bind activators, the function of which remains obscure. Each isozyme becomes catalytically competent by undergoing multiple serine/threonine and tyrosine phosphorylations that are either autocatalytic, or catalyzed by “PKC kinases” such as the phosphoinositide-dependent kinase, PDK1 (7, 10, 11).

The mechanism by which the conventional PKC isoforms become membrane associated, and thus activated, involves two sequential steps. The first involves an initial Ca²⁺- and anionic phospholipid-dependent interaction of the C2 domain with the membrane, which is then followed by binding of diacylglycerol or phorbol esters to the C1 domains (12–15). Mutagenesis studies have identified a number of critical hydrophobic residues within the C1 domains of PKCδ and PKCα that appear to have distinct roles in ligand binding and membrane association (16–18). Based on the x-ray crystallographic structure of the C1B domain of PKCδ, it has been suggested that activator binding to the C1 domain facilitates membrane-insertion by “capping” a hydrophilic groove to form a contiguous hydrophobic surface that can interact with the membrane interior (19). However, it would appear from our studies that the interaction of the phorbol ester induces a conformational change in the C1 domain, the extent of which is reflected in the activity of the enzyme, implying a more active role for phorbol ester-C1 domain interactions beyond that of presenting a hydrophobic surface to the interior of the membrane (20, 21). The interaction of diacylglycerol with the C1 domains also results in an increased stereo- and regiospecificity of both membrane association and activation for PS (13, 22–24). The combined interactions of the C1 and C2 domains with the membrane provides the free energy required for structural rearrangements that lead to the dissociation of the N-terminal pseudo-substrate from the active site to allow substrate binding (25–27). This process is thought to be further facilitated by a weak interaction of the released pseudo-substrate with anionic head groups at the membrane surface (28).

There is increasing evidence supporting the notion that the existence of two C1 domains affords a complex modulatory role in the regulation of PKC activity. Phorbol esters have been...
shown to interact with both of the C1A and C1B domains, with distinct low and high affinities (29–33). Furthermore, we have shown that diacylglycerol inhibits the low affinity phorbol ester interaction while
enhancing high affinity phorbol ester binding, indicating that the diacylglycerol has a higher affinity for the low affinity phorbol ester-binding site than does phorbol ester itself (30, 31). Additional support for the non-equivalence of the interaction of diacylglycerol, phorbol esters, and also other activators with the two C1 domains has been provided by other studies that have observed non-equivalent roles of the domains with respect to membrane association and activation (34–37). Studies from this laboratory showed that the increased level of phorbol ester binding that results from interaction of diacylglycerol with the low affinity phorbol ester-binding site on conventional PKC isozymes corresponded to an elevated level of enzyme activity that was greater than that induced by either phorbol ester or diacylglycerol alone (30, 31, 38). The above, and recent data suggesting that the C1A of PKCo is the diacylglycerol-binding site, while the C1B domain binds phorbol ester (16, 24), are compatible with the C1A and C1B domains containing the low and high affinity phorbol ester-binding sites, respectively.

Inter-domain interactions are important in PKC regulation, although detailed features of these interactions remain obscure. The conformational change that leads to the displacement of the pseudosubstrate of membrane-associated PKC isozymes involves pronounced rearrangements of the individual domains that constitute the enzyme molecule. In a recent study, it was shown that the rate of translocation of GFP-tagged PKCγ in RBL cells was markedly slower when compared with that of a truncation mutant lacking the N-terminal V1 region (14). Based on this, it was suggested that membrane association and activation resulting from binding of diacylglycerol to the C1 domains of this isozyme first requires the release of a "V1 clamp," which holds the C1 domain between the catalytic and V1 region, due to an interaction of the pseudosubstrate with the active site. In another study, the mutation of a single aspartate (Δasp58) residue in the C1A domain of PKCo was shown to result in a marked reduction in both the PS and Ca2+ concentration requirements for membrane association and activation, implicating a tethering of the C1A domain to a neighboring region within the PKCo molecule (15, 24). It was suggested that such an interaction might retain the isozyme in an inactive conformation by preventing the penetration of the C1A domain into the membrane and thus its interaction with diacylglycerol (24).

The aim of the present study was to determine the role of interdomain interactions in the mechanism of activation of PKCo. Using in vitro activity and binding assays, it was found that PKCo engaged in a phorbol ester-dependent, high affinity and specific interaction with a fusion protein that contained its own C1A and C1B domains (αC1A-C1B). The level of activity induced by interaction with the αC1A-C1B domain was found to be comparable with that resulting from membrane association induced under maximally activating conditions. Also, the αC1A-C1B domain interacted with a fusion protein containing the C2 domain of PKCo. Taken together, these findings provide evidence for the existence of an interdomain interaction between the C1 and C2 domains, the dissociation of which is a rate-determining step in the mechanism of activation of PKCo.

**EXPERIMENTAL PROCEDURES**

**Materials**—Adenosine 5′-triphosphate (ATP) was from Roche Molecular Biochemicals (Indianapolis, IN). γ-[32P]ATP was from PerkinElmer Life Science (Boston, MA). 1-Palmitoyl-2-oleoyl phosphatidylcholine (POPC) and bovine brain phosphatidylserine (BPS) were each from Avanti Polar Lipids, Inc. (Alabaster, AL). Peptide substrates were custom synthesized by the Kimmel Cancer Center peptide synthesis facility of Thomas Jefferson University. 4β-12-O-Tetradecanoylphorbol-13-acetate (TPA) and the soluble diacylglycerol, 1,2-di-octanoyl-sn-glycerol (DiC8), were each obtained from Sigma. A modified preparation of the catalytic subunits of the conventional PKC isozymes was prepared from Calbiochem (La Jolla, CA). All other chemicals were of analytical grade and obtained from Fisher Scientific (Pittsburgh, PA).

**Expression and Purification of PKC Isozymes, the D55A Mutant of PKCo, and C1/C2 Domains—Recombinant PKCo, βII, -H9251, γ, -H9254, and ε (rat brain) were prepared using the baculovirus Spodoptera frugiperda with yeast cell expression system as originally described (39), with modifications (40). Purification procedures were as previously described (30, 40). Baculovirus encoding the PKCo mutant, D55A, in which the aspartate 55 of the C1A domain was mutated to an alanine (24), was a kind gift from Dr. Wonhwa Cho, and was purified using the same procedure as that used for wild-type PKCo (30, 40). The isoforms PKCβ, PKCζ, and PKCγ were overexpressed in S99 cells as fusion proteins containing a (His)6 attached to the C terminus (40) and were purified as described (32, 40). Fusion proteins containing fragments of PKC encompassing the C1A, C1B, and C2 (αC1A-C1B-C2), the C1A and C1B (αC1A-C1B), the separate C1A (αC1A) and C1B (αC1B) domains, and also the C1A and C1B domains of PKCo (εC1A-C1B), were each prepared as described previously (32, 40) (and see Fig. 1). To provide structural stability, solubility, and to aid purification, fusion peptides containing glutathione S-transferase (GST) at the N-terminus and with (His)6 at the C terminus. An expression vector containing the GST-tagged C1A and C1B domains of PKCo (εC1A-C1B) was a kind gift from Dr. Peter M. Blumberg. The isolation and purification of each tagged protein was performed previously described (32, 40).

**Measurements of PKC Activity—**PKC isozyme activities were assayed by measuring the rate of phosphate incorporation into a peptide substrate as previously described (30). For the "conventional" PKC isoforms and their catalytic subunits, a peptide corresponding to the phosphorylation site domain of myelin basic protein (KRRPSQKSKYL, MBF4–5) was used as the substrate, whereas assays of novel PKC and atypical PKC activity used a peptide corresponding to the pseudosubstrate region of novel PKCo (e-peptide), in which the single alanine residue was replaced by serine (25, 41, 42). Briefly, the assay (75 μl) consisted of 50 mM Tris-HCl (pH 7.40), 0.1 mM EDTA or CaCl2, 50 μM MBF4–5, or 50 μM e-peptide, TPA (500 nM or as indicated) or varying levels of DiC8, and fusion proteins containing the appropriate PKC domains present at a fixed concentration of 10 nM unless otherwise indicated. Where added, POPC and BPS were present at a total concentration of 150 μM as large unilamellar vesicles. PKC activities were determined by scintillation counting.

**Measurements of PKC-C1-domain Binding Using Surface Plasmon Resonance (SPR)—**Binding of C1 domain peptides to PKCo isozymes was determined using SPR from measurements of the accompanying increase in refractive index as a function of time using a Biacore™ 2000 (Biacore, Inc., Piscataway, NJ). All measurements were performed at 25 °C. Initially, the αC1A-C1B or εC1A-C1B domains were captured through the respective (His)6 tag to the nickel-NTA surface of an NTA sensor chip, prepared according to the manufacturers instructions (Biacore, Inc., Piscataway, NJ), to a level of 75 response units. Solutions containing either PKCo isozymes or fusion peptides at the required concentrations, in the presence or absence of 500 nM TPA were then injected over this surface and the response was measured as a function of time. The surface was regenerated after each injection by two 10-s injections of 100 mM NaOH, followed by a single 10-s injection of 10 mM HCl. After subtraction of the contribution of bulk refractive index changes and nonspecific interactions of PKCo isozymes with the nickel-NTA surface, which were typically less than 1% of the total signal, the individual association (ka) and dissociation (kd) rate constants were obtained by global fitting of data to a 1:1 Langmuir binding model using Biacore Evaluation software (Biacore, Inc., Piscataway, NJ). These values were then used to calculate the dissociation constant (KD). The values of average squared residuals (χ2) obtained were not found to be significantly improved by fitting data to models that assumed bivalent or heterogeneous interactions between PKCo isozymes and C1 domain peptides. In a separate control experiment (results not shown), it was found that the contribution of mass transport to the observed values of KD was negligible.
sites. PKC
ometry of 1:1, assuming maximal occupancy of ligand-binding
absence of TPA was negligible. The value of
dependent since it was found that the level of binding in the
interaction was phorbol ester-
dependent manner (Fig. 2
A
as shown by the
domain (Fig. 2
), although with a markedly reduced affinity,
N-terminal end with (His) _6. See "Experimental Procedures"
indicated a high affinity interaction (Table I). Furthermore, the
protein containing the C2 domain of PKC
was also found to interact with the
may interact with residues within the C2 domain, and
PKC Activation Mechanism

RESULTS
To investigate the role of domain-domain intramolecular inter-
actions in the mechanism of activation of PKC, the assumption
was made that if such interactions occur within the
isozyme molecule, and are involved in the activating confor-
mational change, then peptides corresponding to the isolated
domains might compete for these interactions and modulate the
activity of the isozyme. To address this, various fusion proteins
containing regions of the regulatory domain spanning the C1A
and C1B domains were prepared, as shown in Fig. 1. Binding of
these domains to PKC, and the effects on PKC isozyme activ-
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results in Activation
PKC Results in Activation—PKCα was found to be activated by low
nanomolar levels of the αC1A-C1B domain in the presence of a
fixed concentration of TPA (500 nM) and in the absence of
membrane lipids (Fig. 3A, ○). This effect was phorbol ester-de-
dependent since the αC1A-C1B domain negligibly affected PKCα
activity in the absence of TPA (Fig. 3A, □). The concentration of
αC1A-C1B corresponding to a half-maximal increase in
PKCα activity was ∼1 nM, which is consistent with the value of
KD for the interaction of PKCα with the αC1A-C1B domain,
determined from the SPR binding data (Table I), and again
indicates that the activation resulted from a high-affinity in-
teraction. It should be noted that the maximal specific activity
of PKCα induced by the αC1A-C1B domain was comparable with
that determined for PKCα associated with membranes
composed of BPS and POPC in the presence of Ca²⁺ and TPA,
each activator being at saturating levels (Fig. 3A, *).

PKCα Binding Site in the αC1A-C1B Domain—Based on
measurements of SPR, it was found that, in the presence of
TPA, PKCα bound to an immobilized fusion protein containing
the isolated αC1A-C1B domain in a reversible, concentration-
dependent manner (Fig. 2A). The interaction was phorbol ester-
dependent since it was found that the level of binding in the
absence of TPA was negligible. The value of KD, calculated from
the ratio of the association and dissociation rate constants,
indicated a high affinity interaction (Table I). Furthermore, the
value of the maximal level of PKCα binding at equilibrium (R_max)
is consistent with a PKCα-αC1A-C1B binding stoichi-
ometry of 1:1, assuming maximal occupancy of ligand-binding
sites. PKCα was also found to interact with the eC1A-C1B
domain (Fig. 2B), although with a markedly reduced affinity,
as shown by the ∼500-fold increase in the value of KD (Table I).

It has been suggested in a previous study that the C1A of
PKCα may interact with residues within the C2 domain, and
thereby impede the activating conformational change (24).
Consistent with this, the results shown in Fig. 2C indicate that
in the presence of TPA, the αC1A-C1B domain binds to a fusion
protein containing the C2 domain of PKCα (αC1A-C1B-C2).

Similar to the interaction of PKCα with the αC1A-C1B, the
binding of the αC1A-C1B-C2 peptide to the αC1A-C1B domain
was found to be TPA-dependent. Note that in this experiment
the αC1A-C1B domain was initially bound to the sensor chip
surface through the (His)_6 tag to a level corresponding to sat-
uration. Under these conditions, any interactions of the αC1A-
C1B with itself were also saturated, ruling out the possibility
that the immobilized αC1A-C1B domain may have interacted
with the C1A-C1B portion of the αC1A-C1B-C2 fusion protein.
Importantly, the value of KD for the interaction was similar
to that calculated for the interaction of the αC1A-C1B domain
with intact PKCα (Table I). Furthermore, it was found that
αC1A-C1B-C2 bound to the immobilized eC1A-C1B (Fig. 2D),
determined with markedly reduced affinity (Table I).
Interaction of PKCα C1A and C1B Domains with PKCα

FIG. 1. Domains used in this study. Each domain was expressed
as a fusion protein tagged at the C-terminal end with GST and at the
N-terminal end with (His)_6. See "Experimental Procedures" for details.

Consistent with the reported independence of the activity of the
catalytic subunit from cofactor and activator requirements (44,
45), the specific activity of the preparation was found to be
similar to that observed for PKCa induced by association with BPS/POPC vesicles in the presence of Ca\textsuperscript{2+} and TPA (Fig. 3A, *). Whereas PKCa was activated by low nanomolar levels of the αC1A-C1B domain, the activity of the catalytic subunit preparation was found to be unaffected by the domain within a similar concentration range (Fig. 3A, ○). This is consistent with the site of interaction of PKCa with the αC1A-C1B domain that mediates the activation being contained within the regulatory domain. However, similar to the effects on intact PKCa activity, the presence of the αC1A-C1B domain concentrations greater than 10 nM also resulted in an inhibition of catalytic subunit activity. This observation indicates that the inhibitory effects of high levels of the αC1A-C1B domain may involve interactions with the catalytic domain, which is consistent with the findings of a recent study that showed that GST fusion proteins containing the regulatory domains of PKCa and PKCe each inhibited the activity of the catalytic subunit of PKCa (46). The possibility that the activation of PKCa by the αC1A-C1B domain may have involved GST or (His)\textsubscript{6} was ruled out by the finding that PKCe activity was unaffected by a fusion peptide containing these moieties alone (Fig. 3A, ▲).

The dependence of PKCa activity on the concentration of TPA, determined in the presence or absence of αC1A-C1B, is shown in Fig. 3B. In the absence of αC1A-C1B, a small increase in the level of PKCa activity was observed within a high TPA concentration range (●). This is consistent with the results of previous studies that have indicated that PKC isozymes bind phorbol esters in the absence of membranes, although with relatively low affinity, and that this results in partial activation (47–50). Similar to the effect of membrane association, the addition of the αC1A-C1B domain resulted in a >1000-fold
PKC Activation Mechanism

The concentration-dependent effects of the individual C1A and C1B domains on the activity of PKCs induced in the presence of a saturating level of TPA, are shown in Fig. 3C. Both the C1A (○) and C1B (■) domains activated PKCα with similar concentration dependence. However, the C1A and C1B concentrations required to induce a half-maximal increase in activity were in each case ~200-fold greater than the value obtained for the full-length C1A-C1B domain. Furthermore, although the C1A domain induced a greater level of activity than the C1B domain, this activity was ~3-fold lower than that induced by the C1A-C1B domain.

The Effect of C1A-C1B on the Activity of the PKCα C1A Domain Mutant, D55A—Recently, it was observed that the mutation of aspartate 55 to an alanine in the C1A domain of PKCα (D55A) resulted in an increase in membrane binding affinity and an increased level of Ca\(^{2+}\) and PS independent activity. From this, it was proposed that PKCα might, in part, be restrained in an inactive conformation by an inhibitory intramolecular interaction involving this residue (24). Here, we further examined the intrinsic activity of D55A, first in the absence of the C1A-C1B domain, membranes, Ca\(^{2+}\), and TPA, and showed it was higher than that of wild-type PKCα (Fig. 4), as reported previously (24). Consistent with the higher intrinsic activity of D55A, the addition of TPA alone resulted in significant D55A activity, relative to the small effect on wild-type PKCα activity. The concentration-response curves for TPA-induced activation of D55A and wild-type PKCα, shown in Fig. 4 (inset), indicate that this corresponds to a marked decrease in the TPA concentration range required for D55A activation. In the absence of TPA, the activities of D55A and PKCα were both negligibly affected by the addition of the C1A-C1B or C1A domain. Importantly, contrasting with the TPA-induced activation of wild-type PKCα by the C1A-C1B domain, neither domain activated D55A in the presence of TPA.

Effects of C1A-C1B Domains of PKCα, -δ, -ε, and -ζ on the Activities of a Panel of PKC Isoforms—To determine the isozyme specificity of the activating effect of the C1A-C1B domain, the concentration-dependent effects of C1A-C1B, and also the δC1A-C1B, εC1A-C1B, and ζC1 domains, on the activities of PKCα, -βI, -βII, -γ, -δ, -ε, and -ζ were each determined in the presence of TPA (Fig. 5). Consistent with the results shown in Fig. 3A, PKCα activity was potentiated ~100-fold by the C1A-C1B domain in a concentration-dependent manner (Fig. 5, ○). The C1A-C1B domain also activated conventional PKC/βI, -βII, and to reduced extent, PKC/γ. However, the activities of novel PKCδ or -ε were each unaffected. Consistent with the observation that the C1A-C1B domain bound to PKCα, -δ, -ε, and -ζ on the Activities of a Panel of PKC Isoforms. Solid curves represent fits of activity data to a modified Hill equation (31). Values are mean ± S.D. Other details are given under “Experimental Procedures.”

The concentration dependence for TPA induced activity, consistent with a dramatic increase in phorbol ester binding affinity (Fig. 3B, ▲). Thus, the calculated value of the TPA concentration corresponding to a half-maximal increase in PKCα activity of 5 ± 2 nM is within a phorbol ester concentration range shown previously to be sufficient to elicit activation of the membrane-associated isozyme (31). Similar to TPA, the soluble diacylglycerol, DiC\(_8\), also activated PKCα in the presence of the C1A-C1B domain, in a concentration-dependent manner (Fig. 3B, ▲). The concentration of DiC\(_8\) required to induce a half-maximal increase in PKCα activity (875 ± 55 nM) was ~150-fold greater than that observed for TPA, which is consistent with the reduced affinity of diacylglycerols for binding to PKC compared with phorbol esters (51, 52).

Fig. 3. Concentration-dependent activation of PKCs by the αC1A-C1B, αC1A, and αC1B domains. Panel A, the activity of intact PKCα was measured as a function of the concentration of the αC1A-C1B domain either with (●) or without (□) 500 nM TPA. To control for nonspecific interactions with the GST or (His)\(_6\) moieties of the αC1A-C1B domain peptide, the effects of a GST-(His)\(_6\) fusion protein on PKCα were determined in the presence of 500 nM TPA (▲). The activity of a mixed conventional PKC catalytic subunit preparation was determined in the absence of TPA (○). For comparison, the activities of each PKC isozyme induced by association with vesicles composed of BPS and POPC (1:4, molar) in the presence of 0.1 mM Ca\(^{2+}\) and 500 nM TPA (each concentration corresponding to one that induces a maximal level of activation), are shown (⁎). Panel B, the activity of PKCα was measured in the absence of membranes as a function of the concentration of TPA, either in the presence (■) or absence (●) of the αC1A-C1B domain, and DiC\(_8\), in the presence of the αC1A-C1B domain (▲). Panel C, PKCα activity was determined as a function of the concentration of the individual αC1A (●) and αC1B (■) domains. Solid curves represent fits of activity data to a modified Hill equation (31). Values are mean ± S.D. Other details are given under “Experimental Procedures.”

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**Figure 3:** Concentration-dependent activation of PKCs by the αC1A-C1B, αC1A, and αC1B domains. **Panel A,** the activity of intact PKCα was measured as a function of the concentration of the αC1A-C1B domain either with (●) or without (□) 500 nM TPA. To control for nonspecific interactions with the GST or (His)\(_6\) moieties of the αC1A-C1B domain peptide, the effects of a GST-(His)\(_6\) fusion protein on PKCα were determined in the presence of 500 nM TPA (▲). The activity of a mixed conventional PKC catalytic subunit preparation was determined in the absence of TPA (○). For comparison, the activities of each PKC isozyme induced by association with vesicles composed of BPS and POPC (1:4, molar) in the presence of 0.1 mM Ca\(^{2+}\) and 500 nM TPA (each concentration corresponding to one that induces a maximal level of activation), are shown (⁎). **Panel B,** the activity of PKCα was measured in the absence of membranes as a function of the concentration of TPA, either in the presence (■) or absence (●) of the αC1A-C1B domain, and DiC\(_8\), in the presence of the αC1A-C1B domain (▲). **Panel C,** PKCα activity was determined as a function of the concentration of the individual αC1A (●) and αC1B (■) domains. Solid curves represent fits of activity data to a modified Hill equation (31). Values are mean ± S.D. Other details are given under “Experimental Procedures.”
PKC Activation Mechanism

FIG. 4. Comparison of the effects of the αC1A-C1B domain and the individual αC1A domain on the activity of the PKCα mutant, D55A, with those on wild-type PKCα activity. The activities of D55A (solid bars) and wild-type PKCα (open bars) were measured under identical assay conditions either alone, or in the presence of 10 nM αC1A-C1B or 500 nM αC1A domain, with or without 500 nM TPA. Inset, the activity of D55A (■) or wild-type PKCα (○) was measured as a function of TPA concentration. Solid curves represent fits of activity data to a modified Hill equation (31). Data represent means of triplicate determinations (± S.D). For other details see “Experimental Procedures.”

albeit with lower affinity (Fig. 2B and see Table I), the εC1A-C1B domain was found to induce a ~10-fold increase in PKCα activity, while having negligible effects on the activities of PKCβII and -βIII (Fig. 5, ▲). This suggests that it may also share with the αC1A-C1B domain some limited ability to interact with PKCα. By contrast, each of the conventional PKCα, -βII, -βIII, -γII, and -y activities were unaffected by the δC1A-C1B domain (Fig. 5, ▲), and also by the C1 domain (Fig. 5, ▼). Similar to PKCα, the activities of PKCδ and -ε were each potentiated by their respective C1A-C1B domains, while being unaffected by the C1 domains of the other isozymes. Furthermore, as found for PKCα, the level of activity induced by the respective C1 domain was close to that induced by membrane association in the presence of TPA and Ca2+, each activator being present at a maximally activating concentration. By contrast to the conventional and novel PKC isozymes, the activity of atypical PKCγ was unaffected by its own C1 domain and those of the other PKC isozymes.

Intermolecular Interactions Involving the αC1A-C1B Domain Mediate in the Self-association of PKCα—The possibility exists that once disengaged from the intramolecular interaction, the C1 and C2 domains may participate in an intermolecular interaction with their counterparts on a neighboring PKCα molecule, which would therefore be expected to result in the formation of dimers. To address this possibility, the activity of PKCα was measured as a function of its concentration, as shown in Fig. 6. The double-log plot of PKCα activity against concentration, obtained in the presence of a fixed level of TPA but in the absence of membrane lipids (Fig. 6, ▲), contained an inflection at a PKCα concentration of ~4 nM, which corresponded to a change in gradient from 0.83 ± 0.5 to 2.23 ± 0.3. This result is consistent with a change from 1 to 2 active sites per active PKCα complex and suggests the isozyme undergoes a monomer-dimer equilibrium, the dimer having a relatively higher activity. Furthermore, consistent with the notion that the dimerization of PKCα may be mediated by intermolecular C1-C2 domain interactions, the presence of a fixed concentration of the αC1A-C1B domain (Fig. 6, ▲) yielded a linear relationship of slope 0.89 ± 0.3 within the same PKCα-concentration range. To address the question whether PKCα associated with membranes may also engage in a monomer-dimer equilibrium, the concentration dependence of PKCα activity was measured in the presence of BPS/POPC vesicles, Ca2+, and TPA (Fig. 6, ▲). The concentration of BPS (20 mol % of the total lipid concentration), Ca2+ (0.1 mM), and TPA (500 nM) used each corresponded to those previously shown to induce complete membrane association of PKCα and a maximal level of activation of the isozyme (38). It was found that the activity of membrane-associated PKCα again displayed positive cooperativity with respect to PKCα concentration. Thus, a double-log plot of PKCα activity against concentration again contained an inflection corresponding to a change in the slope of the regression line from 0.78 ± 0.6 to 2.02 ± 0.4, which is consistent with PKCα-PKCα dimerization. Furthermore, the PKCα concentration corresponding to the inflection point was ~10-fold lower than that observed for PKCα in the absence of membranes, indicating that in this case dimerization occurred at a lower PKCα concentration, consistent with an increased monomer-dimer association constant.

DISCUSSION

The activation of PKC isozymes by association with membranes ultimately hinges on a conformational change induced by diacylglycerol- or phorbol ester binding to the C1 domains and PS/Ca2+ binding to the C2 domain (12, 15), that results in the removal of the pseudosubstrate from the active site to allow binding of a substrate (25–27). This contribution provides evidence supporting the existence of an additional intramolecular interaction between the C1 and C2 domains of PKCα, the dissociation of which is a requirement for the activating conformational change to proceed.

The observation from SPR experiments, showing that the αC1A-C1B domain peptide interacted with both intact PKCα and with the C2 portion of the αC1A-C1B-C2 peptide, suggests a critical and functional interaction between the C1A, C1B, and C2 domains of the isozyme. This is further reinforced by the observation that the interaction of PKCα with the αC1A-C1B domain resulted in pronounced enzyme activity, consistent with the activating conformational change requiring the dissociation of a C1-C2 domain interaction that otherwise holds the enzyme in a “closed” inactive state. We therefore propose that the activating conformational change in PKCα corresponds to
The activities of conventional PKC isozymes are determined in the absence of membranes with 500 nM TPA, whereas the activities of novel and atypical isozymes are measured in the presence of 0.1 mM Ca^{2+} each PKC isozyme induced by association with vesicles composed of BPS and POPC (1:4, molar) with 0.1 mM Ca^{2+} (II). PKC activity associated with vesicles composed of BPS and POPC (1:4, molar) with 0.1 mM Ca^{2+} (II) was also nonlinear. Inset, the same data plotted on a linear axis. Data are representative of at least three independent experiments. For details see the “Experimental Procedures.”

The finding that phorbol ester or diacylglycerol was absolutely required for PKC activation by the αC1A-C1B domain (see Fig. 3B), suggests that a transient dissociation of the phorbol ester-binding sites within the C1 domains is engaged in a transient equilibrium between closed and open states. This would be consistent with the low level of PKC activity that is induced by phorbol ester alone in the absence of membranes (see Fig. 3B and Refs. 47–50). The observation that the interaction of PKCα with the αC1A-C1B resulted in a dramatic reduction in the TPA concentration dependence of activity (see Fig. 3B) is also consistent with the proposal that the phorbol ester-binding sites within the C1 domains are exposed by the formation of the open state.

In this model, the binding of the αC1A-C1B domain peptide to the C2 domain combined with phorbol ester or diacylglycerol binding to the C1 domains, would then “lock” the PKC molecule in an open state by blocking the formation of the C1-C2 interaction. This open state appears to correspond to the fully active conformation of PKCα, based on the observation that the level of PKCα activity induced by interaction with TPA and the αC1A-C1B domain approached that resulting from membrane association induced under maximally activating conditions. The finding that the interaction of the αC1A-C1B domain with the αC1A-C1B-C2 peptide was also TPA-dependent, suggests that the properties of the αC1A-C1B-C2 peptide may reflect those of the intact PKCα molecule, in that it may also be engaged in a transient equilibrium between closed and open states; the latter being stabilized by phorbol ester binding to its C1 domains.

The finding that the dose-response curves for the activation of PKCα by the C1A and C1B domains were each shifted to the right by −2 orders of magnitude relative to that for the full-length αC1A-C1B domain (compare Fig. 3A with C), suggests that residues in both C1A and C1B domains may participate in interdomain interactions with the C2 domain that stabilizes the closed conformation of PKCα. The observation that the activity of D55A was still to some extent potentiated by phorbol ester (see Fig. 4), although the C1A-C2 domain interaction is absent in this mutant, also indicates that the dissociation of

an equilibrium between closed and “open” states that is regulated by a C1-C2 interaction; the open active state being stabilized by interaction with a membrane surface, or with filamentous actin (53). This provides experimental evidence for an interaction involving the C2 domain that was suggested could occur in a recent study for PKCa (24), and also was alluded to in an earlier study for a novel PKC isozyme from Aplysia (54).

The finding that phorbol ester or diacylglycerol was absolutely required for PKCa activation by the αC1A-C1B domain (see Fig. 3A), suggests that a transient dissociation of the C1-C2 interaction may be initially required to expose the phorbol ester- or diacylglycerol-binding sites within the C1 domains. This would be consistent with the low level of PKC activity that is induced by phorbol ester alone in the absence of membranes (see Fig. 3B and Refs. 47–50).
PKC Activation Mechanism

other interactions in addition to that between the C1A and C2 domains may be involved.

With regard to isozyme specificity, the interaction with the αC1A-C1B domain and the ensuing activation appears to be confined to conventional PKCs, βI, βII, and to a lesser extent PKCγ, based on the observation that the activities of novel PKCs, -ε, and atypical PKCζ were unaffected by the domain (see Fig. 5). This apparent specificity was also suggested by the observation that the activities of PKCα, -βI, and -βII were, to a lesser degree, activated by the αC1A-C1B domain and were unaffected by the αC1A-B and C1 domains. The finding that the αC1A-C1B and αC1A-C1B domains also potentiated the activities of PKCδ and PKCe, respectively, suggests that intramolecular interactions involving the C1 domains of these isoforms may also mediate the activating conformational change. Although, it is possible that these interactions may be similar to those between the C1 and C2 domains of PKCα, the organization of these domains and also the pseudosubstrate within the structures of PKCδ and -ε differ markedly from that of PKCα.

The observation that measurements of PKCα activity as a function of PKCα yielded curves that contained an inflection point corresponding to a change in gradient from –1 to –2 indicates a change in the reaction kinetics from being first-order to second-order, with respect to the enzyme concentration. This is consistent with a concentration-dependent equilibrium between monomeric and dimeric forms of PKCα. Evidence supporting the notion that PKC may be active in PKC-PKC and/or PKC-substrate complexes has also been provided elsewhere (55–60). Furthermore, evidence that PKC may become active upon self-association in the cellular environment was presented recently based on the observation of PKCα dimers in lysates derived from murine B2L fibroblasts treated with calcium ionophore, phorbol ester, or epidermal growth factor (60). The self-association of PKCα might be mediated by the same intramolecular interactions between the C1 and C2 domains discussed above but now between different PKCα molecules, since the presence of the αC1A-C1B peptide restored a linear relationship between enzyme concentration and activity. The observation that the interaction of the αC1A-C1B domain was specific for PKCα, -βI, and to a lesser extent PKCγ, and that the activity of PKCα was relatively less affected by the C1 domains of PKCδ, -ε, and -ζ suggests that PKCα can dimerize with itself, and potentially also with other conventional PKC isoforms. However, in the cellular environment, whether PKC isoforms form homo- or heterodimers would depend on whether these isoforms are co-localized. The roles of intermolecular PKC-PKC interactions, and the balance between inter- and intramolecular C1-C2 interactions in the mechanism of activation of PKC remain to be investigated.

Conclusion—The existence of C1-C2 domain interactions that retain the PKCα molecule in an inactive conformation was directly demonstrated by the observation that the domains involved bind directly to one another and that this leads to activation. The current observations support a model in which PKCα activation corresponds to a transition from a closed inactive to an open active state that is governed by the dissociation of the C1-C2 interaction.

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