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Contribution of the C1A and C1B Domains to the Membrane Interaction of Protein Kinase C†

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ABSTRACT: The hallmark for protein kinase C activation is its "translocation" to membranes following generation of lipid second messengers. This translocation is mediated by the C1 and C2 domains, two membrane-targeting modules, whose engagement on membranes provides the energy for an activating conformational change in which an autoinhibitory pseudosubstrate sequence is released from the active site. Novel and conventional protein kinase C isozymes contain a tandem repeat of C1 domains, the C1A and C1B, which each contain a binding pocket for phorbol esters/diacylglycerol. This study addresses the contribution of the C1A and C1B domains in the regulation of protein kinase C's membrane interaction using bisfunctional (dimeric) phorbol myristate acetate (PMA) molecules. We show that dimeric bisphorbols are an order of magnitude more effective at recruiting full-length PKC βII to membranes compared with monomeric PMA and that the effectiveness of the interaction depends on the nature and length of the cross-link between the PMA moieties. Most effective were dimeric phorbol 12-acetate 13-esters linked at the 13 position with a 14 carbon spacer. The increased potency of dimeric phorbol esters is reduced if either the C1A or C1B domains are mutated so that they are unable to bind PMA, if one moiety of the dimer contains a nonfunctional phorbol, or if the binding to the isolated C1B domain is measured. Thus, the increased potency of the dimeric phorbol esters results primarily from their ability to engage, to a limited extent, both C1 modules on the same molecule. Although dimeric phorbols were more potent than monomeric phorbol esters in recruiting protein kinase C to membranes, the magnitude of the increase was still several orders of magnitude lower than what would be predicted on the basis of the reduction in dimensionality that occurs when the first C1 domain is engaged on the membrane. Thus, engaging both domains can be forced but is highly unfavored. In summary, our data reveal that both C1 domains are oriented for potential membrane interaction but only one C1 domain binds ligand in a physiological context.

The reversible translocation from the cytosol to the membrane provides a mechanism to regulate the function of diverse signaling proteins. To effect high sensitivity and specificity in regulating this spatial redistribution, many signaling proteins take advantage of two membrane-targeting modules (*1*, *2*). Each module binds membranes with low affinity, but high-affinity binding is achieved when both domains are engaged on the membrane. Reversibility is achieved by having the membrane affinity of one of the modules depend on stimulus-dependent changes in cofactors (e.g., lipid mediators/Ca^{2+}) or protein structure (e.g., phosphorylation). Perhaps the best characterized example of this is the family of protein kinase C (PKC)[1] isozymes, in which most members are targeted to the plasma membrane in response to signals that promote phospholipid hydrolysis (*3*−*5*). This translocation is mediated by the engagement of membrane-targeting domains on the membrane providing the energy to release an autoinhibitory pseudosubstrate sequence from the active site of PKC, thus allowing substrate binding and phosphorylation (*1*).

PKC family members comprise a carboxyl-terminal kinase domain and amino-terminal regulatory moiety that contains an autoinhibitory sequence and one or two membrane-targeting modules (*4*, *5*). The membrane-targeting modules are of two types, a C1 and a C2 domain, with a ligand-binding and a non-ligand-binding variant of each (*2*, *6*). The C1 domain binds diacylglycerol and its functional analogues, phorbol esters; the C2 domain binds Ca^{2+}. It is the composition of the membrane-targeting modules in this regulatory moiety that defines the three subclasses of isozymes. Conventional isozymes (α, γ, and the alternatively spliced βI and βII) and novel (δ, ϵ, η/L, θ) PKC isozymes contain a tandem repeat of two ligand-binding C1 domains (C1A and C1B) and a Ca^{2+}-binding (conventional isozymes) or non-Ca^{2+}-binding (novel isozymes) C2 domain. Atypical

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[1] Abbreviations: PKC, protein kinase C; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; PDBu, phorbol 12,13-dibutyrate; EtOAc, ethyl acetate.

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isozymes (ζ and ι/λ) contain one C1 domain that is not competent to bind ligand.

The C1B domain is a relatively small (8 kDa) globular domain that is stabilized by two tightly bound Zn^{2+} ions (*7*−*9*). It contains a binding pocket for diacylglycerol (or phorbol esters) formed by two pulled-apart β sheets. This domain also selectively binds the anionic phospholipid, phosphatidylserine (*10*). The C2 domain of conventional PKCs is a globular domain (16 kDa) with an Asp-lined Ca^{2+}-binding site formed by two loops comprising sequences at opposite ends of the primary structure (*11*). The C2 domain of novel PKCs is a topological variant of the conventional C2 domain; it has the same overall fold except that three Ca^{2+}-coordinating Asp residues are absent and the domain is unable to bind Ca^{2+} (*12*). Whether this domain still functions as a membrane-targeting module has not been established.

The C1 and C2 domains of conventional isozymes enable reversible interaction with the membrane. Each domain has a relatively low membrane affinity on its own, but high-affinity binding to membranes is achieved when both domains are engaged on the membrane (*10*). Metabolism of diacylglycerol in the cell disengages the C1 domain. This releases PKC from the membrane since the affinity of the C2 domain alone is not sufficient to retain the kinase at the membrane. The C1 and C2 domains are found in a number of other proteins, including diacylglycerol kinase and Raf, which both contain a C1 domain, and phospholipase C and synaptotagmin, which contain a C2 domain (*13*, *14*).

Conventional and novel PKCs contain two C1 domains. A number of studies have addressed whether one or both of the two C1 domains are involved in regulating the function of PKC. In vitro binding measurements with conventional PKCs are consistent with 1 mol of phorbol ester binding per mole of PKC (*15*−*18*), although studies with a fluorescent analogue of phorbol ester have led Stubbs and coworkers to suggest that, in the case of PKC α, two ligands bind per PKC (*19*). In vivo studies suggest that one or both domains can mediate membrane binding depending on the isozyme. In the case of PKC δ, it is the C1B domain that mediates membrane targeting in vivo: mutation of the C1A domain to impair phorbol ester binding has little effect on the phorbol ester-dependent translocation of this isozyme in NIH 3T3 cells, whereas mutation of the C1B impairs translocation (*20*). In contrast, both domains play equivalent roles in causing PKC α translocation in vivo (*21*). Studies with isolated C1A and C1B domains have shown that only the C1B domain binds phorbol esters in the case of PKC δ (*22*); however, both the C1A and C1B bind phorbol esters in vitro in the case of PKC γ (*23*, *24*). Thus, phorbol esters may regulate PKC by either C1 domain (or both simultaneously) for some isozymes (e.g., PKC α) but only through the C1B domain in other isozymes (e.g., PKC δ).

This study uses bisfunctional (dimeric) PMA molecules to address the relative orientation and contribution of the C1A and C1B domains to the membrane binding of PKC β, γ, and δ. We show that, for each isozyme, the two domains can be simultaneously engaged on the membrane by phorbols connected by linkers of 14 carbons, indicating that the two domains are oriented with their ligand-binding pockets facing the membrane. However, the increased potency of the dimeric phorbol esters in recruiting PKC to membranes is considerably less than predicted on the basis of the reduction in dimensionality that accompanies the engaging of the first C1 domain on the membrane, indicating that this is an inefficient binding event. These data suggest that only one C1 domain engages the membrane in normal PKC activation.

MATERIALS AND METHODS

Materials

Phospholipids were purchased from Avanti Polar Lipids. Phorbol myristate acetate (PMA), protamine sulfate, and ATP were from Sigma. Radioisotopes were from NEN Life Sciences. Electrophoresis reagents were from Calbiochem or Bio-Rad. PKC δ constructs were kindly provided by Dr. Peter Blumberg. Antibody to PKC δ was from Cell Signaling Technology. PKC was purified to homogeneity from the baculovirus expression system as described previously (*25*). A GST-C1B construct was expressed in BL21(DE3) cells and purified using glutathione−Sepharose beads, and the tag was cleaved as described previously (*10*). All other chemicals were of reagent grade.

Methods

Sucrose-Loaded Vesicles. Mixtures of phospholipids in chloroform−methanol (2:1) were dried under a stream of nitrogen followed by vacuum, then resuspended in sucrose buffer (0.170 M sucrose in 5 mM $MgCl_2$ and 20 mM Hepes, pH 7.5), and subjected to five freeze−thaw cycles. Vesicles were extruded through two 0.1 μm polycarbonate filters in a microextruder (Avestin Inc.). The vesicles were suspended in 100 mM KCl and 20 mM Hepes, pH 7.5, to dilute the sucrose and centrifuged at 100 000*g* for 30 min. The pelleted sucrose-loaded vesicles (SLVs) were resuspended in buffer to a final concentration of 1.0 mM lipid. Trace amounts of ^{3}H-labeled dipalmitoylphosphatidylcholine were included in vesicles to determine the lipid concentration following centrigutation. The vesicle composition was 30 mol % 1-palmitoyl-2-oleoylphosphatidylserine and 70 mol % 1-palmitoyl-2-oleoylphosphatidylcholine.

Incorporation of PMA or Bisphorbol Analogue into Sucrose-Loaded Vesicles (SLVs). PMA or analogue was dissolved in DMSO and added to a solution of SLVs while vortexing to yield a final concentration of 10^{-5}−10^{1} mol % phorbol ester. The vesicles were incubated for 30 min to ensure full incorporation of PMA or analogue into the vesicles.

Synthesis of Bisphorbol Analogues. (A) 13−*13-Linked Diphorbol 12-Acetate 20-Trityl Ether.* Phorbol 12-acetate 20-trityl ether (0.1 mmol), the diacid (0.1 mmol), and DMAP (0.5 mmol) were dissolved in CH_2Cl_2 and cooled to −78 °C. EDCI (5 equiv) was then added. After being warmed to room temperature and stirred for 16 h, the reaction was filtered through neutral alumina, concentrated in vacuo, and purified by chromatography on silica gel using hexanes−EtOAc. The diphorbol product was characterized by ^{1}H NMR, ^{13}C NMR, HRMS, and IR. Detritylation was performed by treating the phorbol product with 10% trifluoroacetic acid in methylene chloride at 0 °C for 5 min. The reaction mixture was quenched with excess $NaHCO_3$, concentrated in vacuo, and purified by chromatography on silica gel using hexanes−EtOAc.

(B) 12−*12-Linked Diphorbol 13-Acetate 20-Trityl Ether.* Phorbol 13-acetate 20-trityl ether (0.1 mmol), the diacid (0.1

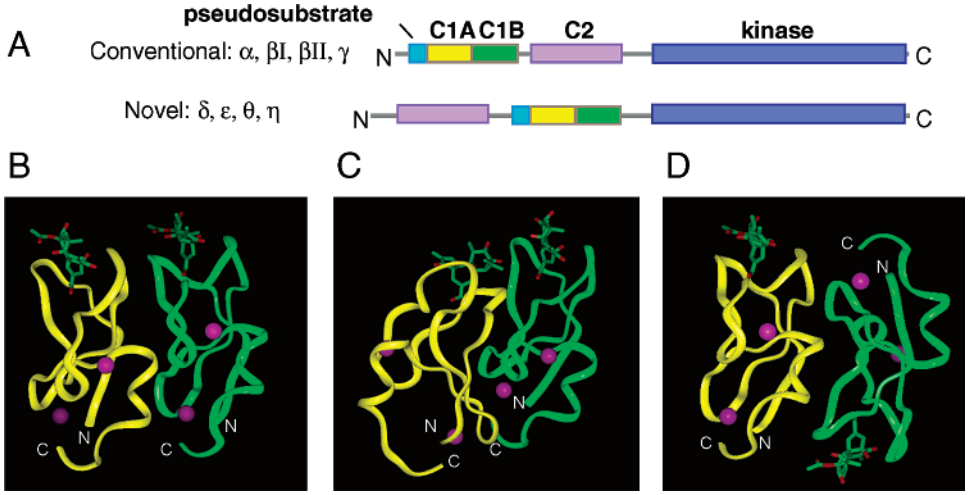

FIGURE 1: (A) Domain structures of conventional and novel PKC isozymes used in this study. Both contain a tandem repeat of C1A and C1B domains, shown in yellow and green, which correspond to the structures shown in panels B−D. (B−D) Three possible orientations of the C1A domain of PKC βII relative to the C1B domain. Domains were modeled against the known structure of the C1B domain of PKC δ and docked using the DOT program as described in the Methods section.

mmol), and DMAP (0.5 mmol) were dissolved in CH_2Cl_2 and cooled to −78 °C. EDCI (5 equiv) was then added. After being warmed to room temperature and stirred for 16 h, the reaction was filtered through neutral alumina, concentrated in vacuo, and purified by chromatography on silica gel using hexanes−EtOAc. The diphorbol product was characterized by ^{1}H NMR, ^{13}C NMR, HRMS, and IR. Detritylation was performed by treating the phorbol product with 10% trifluoroacetic acid in methylene chloride at 0 °C for 5 min. The reaction mixture was quenched with excess $NaHCO_3$, concentrated in vacuo, and purified by chromatography on silica gel using hexanes−EtOAc.

Expression of PKC δ. pcDNA II-SAI containing wild-type PKC δ, a P169G mutant, a P241G mutant, or a P169G/P241G double mutant were kindly provided by Peter Blumberg. PKC was cut out of pcDNA II and cloned into pcDNA 3.1(−) (Invitrogen) using the *Xho*I and *Hin*dIII sites. The constructs were transiently transfected into COS 7 cells with Superfect (Qiagen) according to manufacturer's protocol. Cells were lysed after 48 h, and soluble material was separated from the insoluble pellet by centrifugation at 50000*g* for 30 min at 4 °C. The supernatant obtained was used in binding assays. PKC δ or its mutants were the only bands visible on a western blot using phospho-PKC δ Thr505 (Cell Signaling Technology) as the primary antibody. No bands were seen in untransfected or vector-transfected controls.

Vesicle-Binding Assay for PKC β and γ. PKC binding to sucrose-loaded vesicles was measured as described previously (*26*). PKC was incubated for 10 min with 100 μM SLVs containing increasing amounts of phorbol esters in buffer containing 100 mM KCl, 5 mM $MgCl_2$, 20 mM Hepes (pH 7.5), 200 μM Ca^{2+}, and 0.3 mg/mL BSA, in a total volume of 525 μL. This was centrifuged at 100000*g* for 30 min at 25 °C to separate free from vesicle-bound PKC. The percentage of PKC bound to vesicles in the pellet was determined by assaying both the supernatant and pellet fractions for PKC activity under identical conditions, using protamine sulfate as a substrate. Vesicles were added to the supernatant fraction to keep the total amount of lipid in both fractions equal.

The percentage of membrane-bound enzyme was determined using the formula:

$$\% \text{ bound} = A_v/(A_b + A_t)$$

where A_v is the vesicle-associated kinase activity, A_b is the activity of the bottom fraction (in cpm), and A_t is the activity of the top fraction (in cpm). The vesicle-associated activity was calculated as

$$A_v = \frac{\beta A_b + (\beta - 1)A_t}{\alpha + \beta - 1}$$

where α is the fraction of sedimented vesicles and β is the fraction of kinase activity found in the top fraction in the absence of vesicles.

Vesicle-Binding Assay for PKC δ. The binding assay for PKC δ was a slight modification of that used for PKC β and γ. The incubation of PKC with vesicles and separation of supernatant and pellet fractions were carried out in the same manner. However, instead of performing a kinase assay on these fractions to determine the amount of PKC present, top and bottom fractions were run on a gel and analyzed by western blotting using an antibody specific for PKC δ (phospho-PKC δ Thr505 from Cell Signaling). Bands were quantified by densitometry and used in place of "activity" values in the above equation. Controls were performed with PKC βII. Binding curves were obtained with purified enzyme which was analyzed by the kinase assay, as well as with PKC which was overexpressed in a COS 7 cell lysate and analyzed by western blot. Similar binding curves were obtained regardless of the method used.

Vesicle-Binding Assay for Isolated C1B Domains. The assay was performed as above for PKC δ, but samples were analyzed by silver stain instead of western blot.

RESULTS

Figure 1A shows the domain structure of the two subclasses of PKC family members used in this study. To examine the contribution of the C1A and C1B domains in the membrane recruitment of PKC, we first addressed the

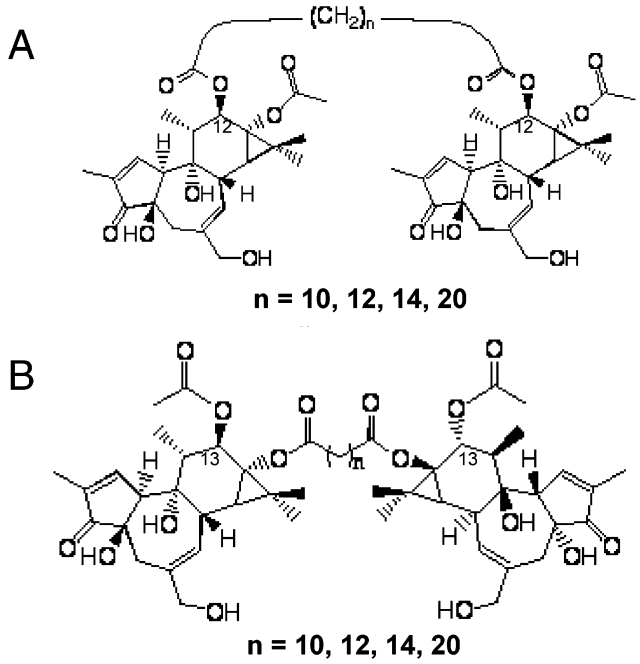

FIGURE 2: Structures of bisfunctional phorbol analogues used in this study. (A) Dimeric phorbol esters linked through C12 of phorbol (12−12 linked). (B) Dimeric phorbol esters linked through C13 of phorbol (13−13 linked).

potential orientation of these two domains relative to one another. The structures of the C1A and C1B domains of PKC βII were modeled on the basis of the structure of the C1B domain of PKC δ (*8*). The optimal docking of these domains was then obtained by analysis using the program DOT (*33*), which predicts protein−protein interfaces by searching the whole protein and taking into account both electrostatic and van der Waals interactions. Figure 1B−D shows the three lowest energy orientations: the two domains oriented side by side (B), the two domains oriented with their ligand-binding face on the same side but rotated 180° relative to each other (C), and the two domains oriented with their ligand-binding pockets on opposite sides of the complex (D). In models B and C the two ligand-binding pockets are on the same face of the complex and thus would be the only models in which both ligand-binding pockets could be engaged on the membrane simultaneously; pockets are separated by a distance of about 15 Å. In model D, the phorbol-binding sites are separated by a distance of about 36 Å.

To test whether both C1A and C1B can be bound simultaneously to ligand, a series of dimeric phorbol esters were synthesized. These were linked either by their C12 or C13 positions or by the C12 of one phorbol and the C13 of the other; linkages varied from 10 to 20 methylene units. The structures are shown in Figure 2.

Figure 3 compares the ability of a series of bisphorbol derviatives to recruit PKC βII to membranes with that of phorbol myristate acetate (PMA). Sucrose-loaded vesicles containing PS (30 mol %), PC (bulk lipid), and increasing amounts of PMA or bisphorbol derivatives (0−5 mol %, corresponding to 0−5 μM PMA or bisphorbol in the incubation mixture) were incubated with PKC, the vesicles were sedimented, and the percentage of PKC bound to the vesicles was analyzed as described in Methods. Half-maximal binding to vesicles containing 30 mol % PS was effected by

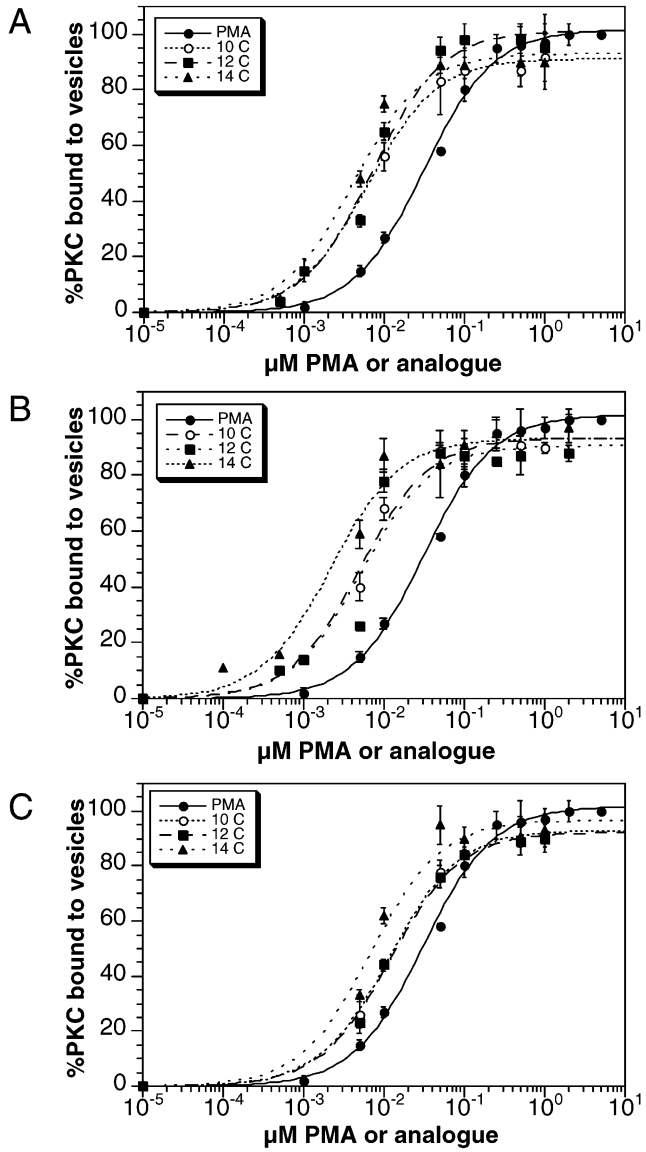

FIGURE 3: Binding of PKC βII to bisfunctional phorbol analogues linked at different positions and as a function of spacer length. (A) 12−12 linked analogues. (B) 13−13 linked analogues. (C) 12−13 linked analogues. Spacer lengths are 10 carbons (○), 12 carbons (■), or 14 carbons (▲). The binding to PMA is also shown (●). The binding of PKC βII to sucrose-loaded vesicles containing increased amounts of phorbol esters was measured as described in Methods. The total lipid concentration was 100 μM in each case, so that 1 μM PMA corresponds to 1 mol % PMA. Curves were fitted using Kaleidagraph. Data represent the average ± standard deviation of triplicate measurements. K_D values (listed in Table 1) were determined by taking the average of the K_Ds obtained from three independent binding curves in triplicate for PMA and the 13−13 linked 14C analogue and two independent binding curves in triplicate for all others.

25 ± 4 nM PMA (Figure 3, filled circles, and Table 1). Bisfunctional analogues were consistently more potent than PMA in recruiting PKC to vesicles. Their potency depended both on the length of the carbon chain connecting the two functional groups and on the position of the linkage. Specifically, phorbols linked by a 14 carbon chain were approximately 2-fold more potent than ones linked with 12C and 10C chains, which were of similar potency (14 carbon > 12C = 10C). Analogues with 20C linkers were no more effective than PMA at recruiting PKC to membranes (data not shown). Bisphorbols linked through the C13 position

Table 1: Apparent Binding Constants (K_a) for the Interaction of Various PKC Isozymes with PMA or Dimer Analogues[a]

		[analogue] (nM)	K_a (1/M)	*x*-fold increase	*n*
PKC βII					
PMA		25 ± 4	4.0×10^7	1.0	3
12−12 linked	10C	5 ± 1	2.0×10^8	5.0	2
12−12 linked	12C	6 ± 1	1.7×10^8	4.2	2
12−12 linked	14C	3 ± 1	3.3×10^8	8.3	2
12−12 linked	deoxy 14C	20 ± 5	5.0×10^8	1.3	2
13−13 linked	10C	4.0 ± 0.7	2.5×10^8	6.3	2
13−13 linked	12C	4 ± 1	2.5×10^8	6.3	2
13−13 linked	14C	2.5 ± 0.4	4.0×10^8	10	3
12−13 linked	10C	9 ± 1	1.1×10^8	2.8	2
12−13 linked	12C	7.9 ± 0.6	1.3×10^8	3.2	2
12−13 linked	14C	4 ± 1	2.5×10^8	6.3	2
C1B domain of PKC βII					
PMA		2000 ± 300	5.0×10^5	1	2
13−13 linked	14C	700 ± 100	1.4×10^6	2.9	2
PKC γ					
PMA		13 ± 1	7.7×10^7	1.0	1
12−12 linked	12C	1.7 ± 0.3	5.9×10^8	7.6	1
12−12 linked	14C	2.3 ± 0.3	4.3×10^8	5.7	1
13−13 linked	12C	1.9 ± 0.3	5.3×10^8	6.8	1
13−13 linked	14C	1.4 ± 0.2	7.1×10^8	9.3	1
PKC δ					
wild type					
PMA		160 ± 20	6.3×10^6	1.0	5
13−13 linked	14C	13 ± 1	7.7×10^7	12.3	5
C1A mutant					
PMA		190 ± 30	5.3×10^6	1.0	3
	14C	54 ± 9	1.9×10^7	3.5	3
C1B mutant					
PMA		1900 ± 300	5.3×10^5	1.0	3
	14C	600 ± 200	1.7×10^6	3.2	3

[a] Midpoints of each binding curve, K_a, *x*-fold increase over PMA, and number of independent binding curves used to obtain K_a are shown for all analogues. See Results section for explanation of the three values.

were slightly more potent than those linked through the C12 position and approximately twice as potent as those linked through the C13 position of one phorbol and the C12 position of the other (13−13 > 12−12 > 12−13). The most potent analogue (13−13 link, 14 carbon linker; Figure 3B, triangles) was 10-fold more potent than PMA in recruiting PKC βII to vesicles.

Figure 4 shows binding curves for PKC γ to membranes containing phorbol analogues linked through either the C12 or C13 position of phorbol. Trends were qualitatively similar to those observed with PKC βII for the 13−13 linked dimers, in that the analogues with a 14 carbon linker were more potent than those with a 12C linker (half-maximal binding occurring with 1.9 nM 12C phorbol compared with 1.4 nM 14C phorbol). However, the potency of the 12−12 linked dimers was not sensitive to the linker lengths studied. The 14 carbon chain analogue was actually slightly less effective than the 12C chain. Interestingly, the affinity of PKC γ for membranes containing PMA was almost two times stronger than that of PKC βII.

Similar results were obtained for PKC δ. Figure 5A shows that 14C 13−13 linked dimers were 12-fold more potent at recruiting wild-type PKC δ to the membrane than PMA. As with β and γ, 14C linkers were more effective than 12C linkers (data not shown for 12C for clarity of graph).

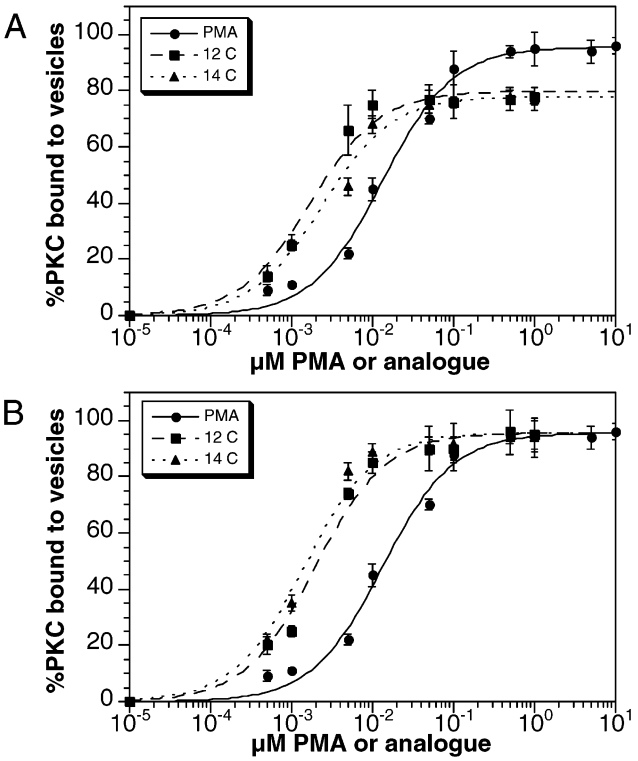

FIGURE 4: Binding of PKC γ to phorbol analogues linked at different positions and as a function of spacer length. (A) 12−12 linked analogues. (B) 13−13 linked analogues. Spacer lengths are 12 carbons (■) or 14 carbons (▲). The binding to PMA is also shown (●). The binding of PKC γ to sucrose-loaded vesicles containing increased amounts of phorbol esters was measured as described in Methods. The total lipid concentration was 100 μM in each case, so that 1 μM PMA corresponds to 1 mol % PMA. Curves were fitted using Kaleidagraph. Data represent the average ± standard deviation of triplicate measurements, and the K_D value was determined directly from the binding curves shown.

The above data revealed that bisphorbol derivatives were more potent than PMA in recruiting PKC to membranes. To test if this resulted from the bisphorbol molecules simultaneously engaging the C1A and C1B domains of the same molecule of PKC, we examined whether this increased potency was retained on molecules of PKC in which one or the other C1 domain was impaired so that it could no longer bind ligand. Specifically, we examined the binding of bisphorbol derivatives to constructs of PKC δ in which prolines 169 in the C1A domain and 241 in the C1B domain were mutated either individually or together to glycine; this point mutation reduces phorbol binding by 125-fold in the isolated domain (*20*).

Figure 5 shows the binding of wild-type and mutant PKC δ to membranes containing bisphorbol analogues linked at position 13 by a 14 carbon spacer. Wild-type PKC δ (panel A) and PKC δ with an impaired C1A domain (panel B) bound PMA with comparable affinity (filled circles); half-maximal binding was mediated by 160 ± 20 and 190 ± 30 nM PMA, respectively. PKC δ impaired in the C1B domain (panel C) bound PMA with approximately 12-fold lower affinity (1.9 ± 0.3 μM PMA), and constructs of PKC δ impaired in both the C1A and C1B domain had no significant binding to PMA-containing membranes (data not shown). These data are consistent with those of Blumberg and co-workers showing that the C1B domain of PKC δ is primarily responsible for membrane recruitment of this isozyme (*20*).

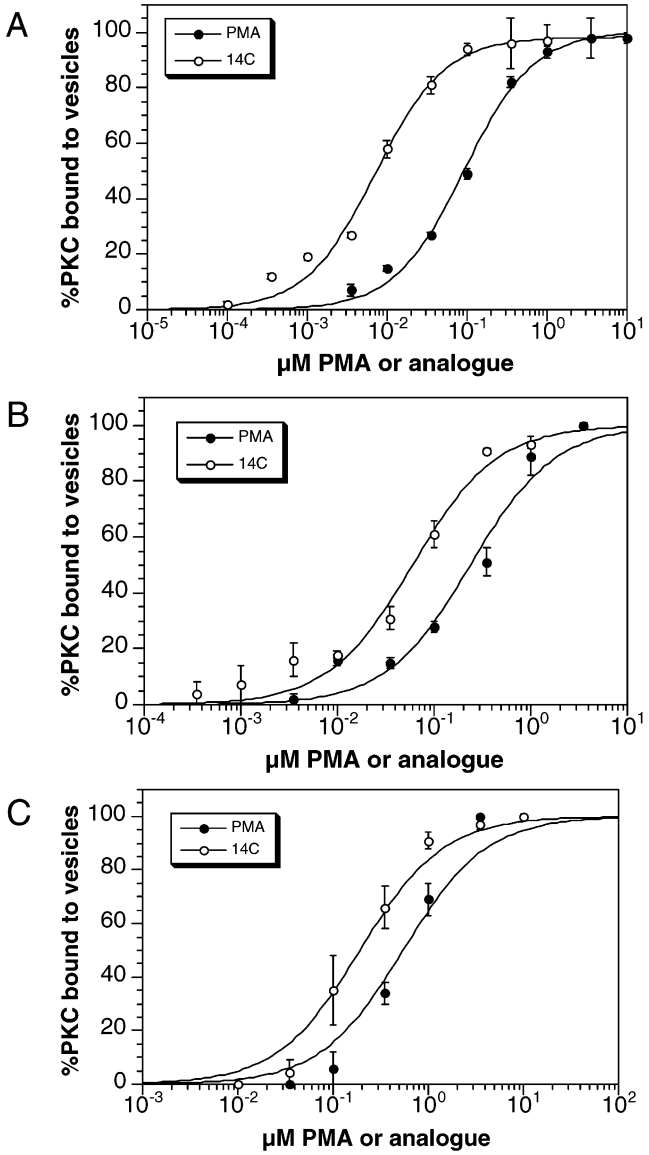

FIGURE 5: Binding of PKC δ constructs to 13−13 linked analogues: (A) wild-type PKC δ, (B) PKC δ with a P169G mutation in the C1A domain; (C) PKC δ with a P241G mutation in the C1B domain. The binding to the 13−13 linked, 14 carbon spaced analogue (○) or to PMA (●) is shown. Binding assays were performed as described for PKC δ in the Methods section. The total lipid concentration was 100 μM in each case, with the indicated amount of bisphorbol. Curves were fitted using Kaleidagraph. Data represent the average and range of duplicate measurements. K_D values (Table 1) were determined by taking the average of the K_Ds obtained from five independent binding curves in duplicate for wild-type PKC (panel A) and three independent binding curves in duplicate for the C1A and C1B mutants (panels B and C).

Importantly, the bisphorbol derivatives were 12 times more effective than the monophorbol, PMA, in recruiting wild-type PKC δ to membranes but only approximately 3 times more effective in recruiting PKC δ with only one functional C1 domain (see Table 1). These data reveal that the increased potency of the bisfunctional phorbols arises in part from the ability to engage both the C1A and C1B domains on membranes.

To further examine whether the increased potency of the bisfunctional analogues results from both functional groups engaging PKC, we tested the binding of a bisfunctional analogue containing the biologically active phorbol linked to the biologically inert 4α-deoxyphorbol. This analogue containing one active and one inactive phorbol was linked at the 12 position by a 14 carbon linker. Figure 6 shows that this compound recruits PKC βII to membranes with the same potency as the monofunctional phorbol, PMA. Thus, the increased potency of the bisphorbol derivatives results from both functional moieties engaging PKC on the membrane and not because of steric or conformational advantages conferred to one phorbol of the bisfunctional analogue.

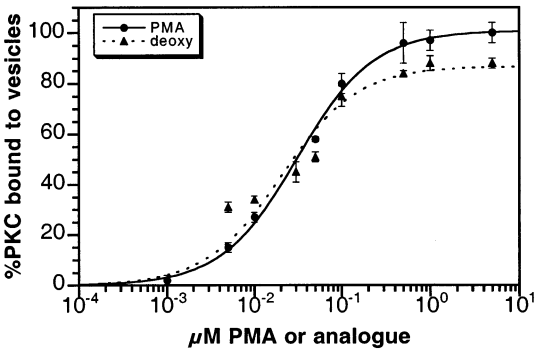

FIGURE 6: Binding of PKC βII to the bisfunctional phorbol analogue containing one biologically active phorbol linked to the biologically inactive 4α-deoxyphorbol. The phorbol analogue was linked through the C12 position by a 14 carbon chain (▲); the binding to PMA is also shown (●). Binding assays were performed as described for PKC βII in the Methods section. The total lipid concentration was 100 μM in each case, with the indicated amount of bisphorbol. Curves were fitted using Kaleidagraph. Data represent the average of two independent experiments in triplicate ± standard deviation. K_D values (Table 1) were determined directly from the curves shown.

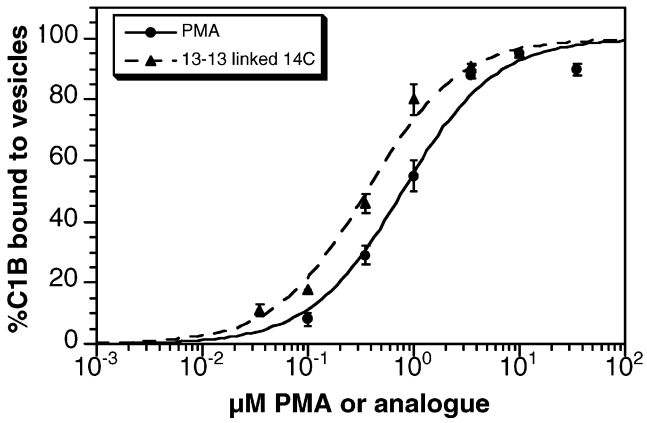

FIGURE 7: Bisfunctional analogues only modestly increase the affinity of the isolated C1B domain for membranes relative to PMA. The binding of the isolated C1B domain of PKC βII to sucrose-loaded vesicles containing increasing amounts of either the 13−13 linked, 14 carbon spaced phorbol analogue (▲) or PMA (●) was measured as described in the Methods section. The total lipid concentration was 100 μM in each case. Curves were fitted using Kaleidagraph. Data represent the average and range of duplicate measurements. K_D values (listed in Table 1) were obtained by taking the average of the K_Ds obtained from two independent binding curves.

As an additional measure of whether the increased potency of the bisfunctional analogues results from engaging both C1 domains in a single PKC molecule, we compared the binding of the isolated C1B domain to membranes containing PMA or the 13−13 linked, 14 carbon spacer bisfunctional analogue. Figure 7 shows that the bisfunctional analogue was 3 times more potent at recruiting the C1B domain of PKC

βII to membranes than the monofunctional PMA. Under the same conditions, the bisfunctional analogue was 10 times more potent than PMA in recruiting full-length PKC βII to membranes (Figure 3 and Table 1). This is consistent with the finding that impairing one of the C1 domains markedly reduces the ability of bisfunctional analogues to promote membrane binding, making them more comparable in potency to PMA. The 3-fold increase in affinity that the bisphorbols maintain when only one C1 domain is functional, or when the binding to the isolated C1 domain is observed, suggests that there may be a minor contribution of domain dimerization or that the bisfunctional analogues are more optimally oriented in the membrane for binding to the C1 domain. The latter possibility is less likely given that bisfunctional analogues with one inert phorbol have the same potency as PMA.

DISCUSSION

The foregoing data suggest that the two tandem C1 domains in conventional and novel PKC isozymes are oriented side by side with both ligand-binding pockets membrane-accessible. However, PKC is much more likely to bind phorbol through only one domain rather than through both. Engagement of both ligand-binding sites can be forced with bisfunctional analogues, but this "forced" engagement is inefficient. There is at most a 1 order of magnitude increase in binding efficiency for bisfuntional compared to monofunctional analogues.

Relative Orientation of Tandem C1 Domains. The modest ability of bisphorbol analogues to engage both C1 domains on the membrane reveals that they are likely to both be oriented with their ligand-binding pockets facing the membrane. Analysis of possible orientations using the docking program "DOT" reveals three favored orientations: both domains aligned side to side (Figure 1B), both domains oriented with ligand-binding pockets on the same side and rotated 180° relative to each other (Figure 1C), or both domains oriented with ligand-binding pockets on opposite sides (Figure 1D). Of the three possible orientations, only orientations B and C are oriented with both the C1A and C1B binding sites accessible to the membrane. The distance between binding sites on the C1A and C1B domains exceeds what can be reached by a 14 carbon chain in orientation C. Thus, the simplest model which is compatible with the binding data is one in which the C1A and C1B domains are oriented side by side in the context of full-length PKC.

Phorbol Esters Engage One C1 Domain. A number of studies have addressed the binding of individual C1 domains to phorbol esters, and the consensus appears to be that isolated C1A and C1B domains bind phorbol esters with comparable affinity for some isozymes and with different affinities for other isozymes. Specifically, studies using either bacterially expressed or chemically synthesized C1 domains of PKC γ reveal that the C1A and C1B domains of this isozyme bind phorbol esters with comparable affinity (*23*, *24*). In contrast, studies with bacterially expressed domains of PKC δ (*22*) and chemically synthesized domains of PKC δ and PKC θ reveal that only the C1B domain binds phorbol esters with high affinity for these novel isozymes (*24*, *27*). It should be noted that the C1A domain is notoriously insoluble and this insolubility has precluded analysis of ligand binding to the C1A domain of most other isozymes (*27*). In vivo studies with full-length protein support in vitro findings. In the case of PKC δ, the C1A and C1B domains have nonequivalent roles in the context of the full-length protein: mutation of the C1B domain, but not C1A domain, to disrupt phorbol binding significantly reduces the phorbol ester-mediated translocation of PKC δ to membranes in NIH 3T3 cells (*20*). In contrast, mutation of either the C1A or C1B domain in PKC α has equivalent effects on disrupting membrane translocation. These data suggest that, for conventional isozymes of PKC, such as PKC α and γ, either C1 domain can promote membrane translocation, whereas for novel isozymes, such as PKC δ and PKC θ, the C1B domain binds ligand.

Numerous studies addressing the stoichiometry of binding of phorbol esters to PKC support the model of 1 mol of phorbol binding to 1 mol of PKC (*15*−*18*). This result is consistent with only one of the C1 domains engaging on the membrane. However, Stubbs and co-workers have suggested that both the C1A and C1B domains can engage on the membrane in the presence of the fluorescent phorbol derivative sapinotoxin (*19*, *28*) and have hypothesized, furthermore, that, in the case of PKC α, the C1A domain selectively binds diacylglycerol and the C1B domain selectively binds phorbol esters (*29*). The data in our current study support the model in which only one C1 domain is engaged on the membrane but suggest that, under the appropriate circumstances, the second C1 domain can engage the membrane. In our study, this engagement was promoted by the use of bisfunctional phorbol esters.

A recent study using similar 12−12 linked bisphorbols of various chain lengths found that the dimers were effective competitive inhibitors of phorbol dibutyrate (PDBu) binding to rat brain PKC (*30*), with K_Is up to an order of magitude lower than the K_D for PDBu binding. Although this study did not address whether one dimer was engaging both C1 domains, the results are consistent with those reported in our study.

The increased potency of bisphorbol analogues relative to monofunctional PMA in recruiting PKC to membranes suggests that these analogues simultaneously engage both C1 domains of PKC on the membrane. If each PKC molecule were binding through only one domain or the other, the affinity for the dimers for PKC would be expected to be only twice that of PMA, rather than the 10-fold increase seen with the 14 carbon chain dimers. However, this increased potency is significantly lower than expected, suggesting that engaging the second C1 domain is highly unfavored. Specifically, the reduction in dimensionality that follows binding of the first C1 domain to the membrane-embedded bisfunctional phorbol results in a several orders of magnitude greater probability of the second C1 domain binding phorbol.[2]

[2] In solution, the bulk lipid concentration that PKC is exposed to is 100 μM. However, once the enzyme is bound to the membrane, the local lipid concentration increases to on the order of 1 M (this is derived by taking a 10 nm × 10 nm × 10 nm cube around the membrane-bound PKC; this corresponds to on the order of 10^{23} lipids/L). Thus, the probability of binding a molecule in the membrane is about 4 orders of magnitude greater when PKC is prebound to the membrane compared to when it encounters the molecule from solution.

A recent study (*34*) reported that PKC α is able to dimerize through interactions between the C1 and C2 domain of separate molecules. To investigate whether the synergistic effects of bisfunctional phorbols resulted from binding a potential PKC dimer, we tested the concentration dependence of the synergism observed with the 13−13 linked, 14C analogue compared to PMA. Specifically, we asked whether the affinity of the analogues for PKC depended on bimolecular collisions with other PKC molecules. We found that the relative potency of the 13−13 linked, 14C analogue compared to PMA was consistent over a 10-fold range of PKC concentration (data not shown). Therefore, it is unlikely that the effects of the analogues on PKC binding are a result of PKC dimerization.

Isozyme Specificity. Comparison of the binding of bisphorbol analogues to different PKC isozymes reveals that both conventional and novel PKCs bind these compounds similarly (Table 1). Specifically, they bind the 13−13 linked, 14 carbon spaced bisphorbol analogues with about a 10-fold higher affinity than PMA. Of particular interest is the finding that PKCs whose isolated C1A and C1B domains have comparable affinities for phorbol esters have the same preference for bisphorbol analogues as do PKCs in which one C1 domain favors phorbol ester binding. For example, PKC γ, whose C1A and C1B domains are competent to bind phorbol esters, binds the bisphorbol analogue with 9-fold higher affinity than PMA, and PKC δ, whose C1B domain preferentially binds phorbol esters, binds bisphorbol binds PKC δ 12 times better than PMA. This finding suggests that the primary determinant in engaging the second C1 domain in full-length PKC is not driven by the affinity of this domain for phorbol esters but more likely by conformational or other constraints. The possibility that the second C1 domain is hampered from binding membranes once the first domain is engaged is supported by a study of the C1 domains of PKC γ: the affinities of isolated C1A and C1B domains of PKC γ domains were equal; however, the affinity of the C1A domain was higher than that of the C1B domain in a double C1A−C1B construct (*23*). These results are consistent with one C1 domain as the primary anchor to the membrane even in isozymes where both C1A and C1B domains have a high intrinsic affinity for phorbol esters.

Various values have been reported for the dissociation constants of different full-length PKC isozymes for binding to phorbol dibuturate (PDBu). Dimitrijevic et al. found K_D values of 9.5, 18, and 4.0 nM for PKC βII, γ, and δ, respectively (*31*). Kazanietz et al. found values of 0.14, 0.37, and 0.71 nM for the same isozymes (*32*). While these absolute values cannot be compared to ours, which represent the apparent association constant to vesicles containing 30 mol % PS, the relative values for each isozyme can be compared. In the case of PDBu, the strongest binder of the three isozymes found by Dimitrijevic et al. was δ, while Kazanietz found it to be βII. In our study, PKC γ had the highest apparent affinity for PMA. These differences likely arise from the method of presenting PMA to PKC, a possibility supported by the finding of Quest and Bell that the relative affinity of phorbol esters for various constructs of PKC γ depended on whether they used lipid vesicles or not (*23*).

CONCLUSION

Our data support a model in which both the C1A and C1B domains are positioned with their ligand-binding pockets accessible to the membrane. However, only one domain is actually engaged on the membrane, even for isozymes where the intrinsic affinity of each domain for phorbol esters is comparable. In addition, our data show that the potency of PMA can be increased by an order of magnitude by chemical cross-linking to form a bisfunctional analogue. These bisfunctional analogues can promote the engagement of both C1 domains on the membrane, thus increasing PKC's membrane affinity.

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