The activity of membrane-associated protein kinase C (PKC) has previously been shown to be regulated by two discrete high and low affinity binding regions for diacylglycerols and phorbol esters (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, 4827–4831). PKC is also known to interact with both cytoskeletal and nuclear proteins; however, less is known concerning the mode of activation of this non-membrane form of PKC. By using the fluorescent phorbol ester, sapintoxin D (SAPD), PKCo, alone, was found to possess both low and high affinity phorbol ester-binding sites, showing that interaction with these sites does not require association with the membrane. Importantly, a fusion protein containing the isolated C1A/C1B (C1) domain of PKC also bound SAPD with low and high affinity, indicating that the sites may be confined to this domain rather than residing elsewhere on the enzyme molecule. Both high and low affinity interactions with native PKC were enhanced by protamine sulfate, which activates the enzyme without requiring Ca\textsuperscript{2+} or membrane lipids. However, this “non-membrane” PKC activity was inhibited by the phorbol ester 4β-12-O-tetradecanoylphorbol-13-acetate (TPA) and also by the fluorescent analog, SAPD, opposite to its effect on membrane-associated PKCo. Bryostatin-1 and the soluble diacylglycerol, 1-oleoyl-2-acetylglcerol, both potent activators of membrane-associated PKC, also competed for both low and high affinity SAPD binding and inhibited protamine sulfate-induced activity. Furthermore, the inactive phorbol ester analog 4α-TPA (4α-12-O-tetradecanoylphorbol-13-acetate) also inhibited non-membrane-associated PKC. In keeping with these observations, although TPA could displace high affinity SAPD binding from both forms of the enzyme, 4α-TPA was only effective at displacing high affinity SAPD binding from non-membrane-associated PKC. 4α-TPA also displaced SAPD from the isolated C1 domain. These results show that although high and low affinity phorbol ester-binding sites are found on non-membrane-associated PKC, the phorbol ester binding properties change significantly upon association with membranes.

Protein kinase C (PKC) constitutes a group of isozymes that are central in cellular signaling pathways that regulate numerous cellular processes, including cell growth, differentiation, and metabolism (1). Each isozyme can be classified into one of three major classes according to the cofactor and activator requirements. The “conventional” PKCα, -β, -γ, and -ε isoforms are Ca\textsuperscript{2+}- and anionic phospholipid-dependent, whereas the “novel” PKCδ, -θ, -η, and -ι and “atypical” PKCζ and -λ isoforms retain a phospholipid dependence but lack a Ca\textsuperscript{2+} requirement (2). In addition, the activities of all PKC isoforms, except atypical PKC, are potentiated by the lipid second messenger, diacylglycerol, derived from the receptor-G-protein and phospholipase-catalyzed hydrolysis of phosphatidylinositides and phosphatidylcholines (3) and also by the potent tumor-promoting phorbol esters (4).

The Ca\textsuperscript{2+} and phospholipid requirements for PKC activity differ according to the lysine and arginine content of the substrate (5). Thus, the PKC-catalyzed phosphorylation of the lysine-rich protein, histone H1, requires the presence of both Ca\textsuperscript{2+} and phospholipid, whereas the phosphorylation of the arginine-rich protein, protamine sulfate, requires neither cofactor (6–10). The mechanism of activation by protamine sulfate, which also acts as a substrate, has been suggested to involve a binding site(s) for arginine-rich proteins on the PKC molecule, separate from the active center of the enzyme (6, 11). Similar to that which occurs upon membrane association induced by Ca\textsuperscript{2+} and diacylglycerol or phorbol esters; interaction with protamine sulfate has been suggested to mediate an allosteric activating conformational change resulting in the removal of a pseudosubstrate region from the active site which, in the inactive state, blocks substrate binding (12). Therefore, use of protamine sulfate provides a useful model for the activation of PKC in the absence of lipids by interaction with other proteins. The question of the mechanism by which PKC activity induced by protein-protein interactions is regulated has become an urgent concern, since it has become apparent that there are a large number of non-membrane protein targets for the enzyme, such as, for example, cytoskeletal and nuclear elements (e.g. Refs. 13–21).

Although interaction with arginine-rich proteins such as protamine sulfate relieves the Ca\textsuperscript{2+} and phospholipid requirements for PKC activity, it is not known whether this non-membrane PKC activity is modulated by phorbol esters and/or diacylglycerols in a similar manner to the membrane-associated enzyme. Evidence supporting this possibility was provided recently by the finding that PKCδ is capable of binding phorbol esters with low affinity in the absence of membrane lipids (22) and that these compounds can induce binding of PKCe to fila-

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12-O-tetradecanoylphorbol-13-acetate; BPS, bovine brain phosphatidylcholine; GST, glutathione S-transferase; OAG, 1-oleoyl-2-acetglycerol; POPC, 1-palmitoyl-2-phosphatidylcholine; RET, resonance energy transfer; SAPD, sapintoxin-D; TPA, 4β-12-O-tetradecanoylphorbol-13-acetate; PDBu, phorbol 12,13-dibutyrate; PS, phosphatidylyserine.
mentous actin (F-actin) (23). Although F-actin itself was not found to be a substrate for this isoform, this may lead to enhanced phosphorylation of other protein targets. PKC\(\beta\)II has also been shown to bind F-actin which is reported to be a substrate for this isoform (24). In this respect, F-actin can be considered to be an example of a number of specific intracellular proteins that bind the activated form of PKC, termed “receptors for activated C-kinase” (RACKs) (20, 21), which allow precise targeting of PKC isoforms to specific cellular locations. However, the non-membrane, phorbol-induced interaction of PKC with F-actin departs from the original definition of RACKs that were described as proteins that bind PKC that has been activated by membrane association (25).

For membrane-associated PKC, activation by diacylglycerol proceeds by two parallel mechanisms. The first involves an interaction with diacylglycerol so that the two activator types initially appeared to share a single site or two identical sites of interaction on the enzyme (4, 26, 27). Second, interaction with diacylglycerol and phosphatidylinerine induces an activating conformational change that results in the folding out of an N-terminal pseudosubstrate region (12, 28–31). Phorbol esters have been suggested to potentiate PKC activity in a similar manner to diacylglycerol so that the two activator types similarly interacted with a single or two identical sites of interaction with diacylglycerol and phorbol esters interact with differing affinities with two activator binding sites on the enzyme molecule (34–36). Furthermore, it was shown that the site with low affinity for phorbol esters binds diacylglycerol with a relatively higher affinity, suggesting that the specificity of this site may differ from the high affinity phorbol ester-binding site (35). Interaction of diacylglycerol with this low affinity phorbol ester-binding site was found to lead to an enhancement in the level of high affinity phorbol ester binding and consequently to a potentiated level of PKC activity. By contrast to diacylglycerol, the potent PKC activator and anti-tumor agent, bryostatin-1, was found to compete more effectively for high affinity phorbol ester binding and did not potentiate the level of phorbol ester-induced PKC activity. Based on these results, a model for PKC activation was proposed in which interaction of an activator with the low affinity for phorbol ester-binding site leads to an enhanced level of binding of either the same or a second activator to the high affinity phorbol ester-binding site and consequently to an elevated level of PKC activity (35).

The activating site with relatively high affinity for phorbol esters likely resides within the C1 domain, which consists of two cystine-rich zinc finger motifs, termed C1A and C1B (37). Interaction of phorbol esters with these subdomains has been extensively characterized by truncation/deletion and mutagenesis studies (38–42) along with both crystal (43) and solution-state structural determinations (44, 45). By using glutathione S-transferase (GST) fusion proteins containing either the C1A or C1B subdomains, it has been shown that both are capable of binding phorbol esters (39, 42, 46). However, the phorbol ester binding affinities of the two subdomains may differ for each PKC isoform. For example, whereas PKC\(\gamma\) C1A and C1B appear to bind phorbol esters with similar affinities, in the case of the novel PKCs, PKC\(\gamma\) and PKC\(\delta\) C1B bind phorbol esters with higher affinity than C1B (47, 48). Recent studies have indicated that these isoform-specific differences in phorbol ester binding to C1A and C1B may be carried over into the native enzyme. While for PKC\(\delta\) C1B has been identified as being a high affinity phorbol ester-binding site, due to its role in phorbol ester-induced intracellular translocation (49), for PKC\(\gamma\) the high affinity site may correspond to C1A (46).

The first aim of this study was to determine if the high and low affinity activator binding sites, previously identified on membrane-associated PKCa (35), similarly exist on the non-membrane form of this isoform. Second, the effects of phorbol esters and other activators of membrane-associated PKC on lipid-independent activity induced by protein-protein interactions with proteamine sulfate were determined. Finally, while the high affinity phorbol ester-binding site clearly resides with the C1 domain, whether the low affinity binding site is similarly located was also investigated. The results indicate that both high and low affinity phorbol ester-binding sites on non-membrane PKCa pre-exist in the absence of membrane lipids and that both sites are confined within the C1 domain of this isoform. However, phorbol esters, diacylglycerol, and bryostatin-1, although clearly described as being potent activators of membrane-associated PKCa, are shown in the present study to be potent inhibitors of non-membrane PKCa activity induced by proteamine sulfate.

**EXPERIMENTAL PROCEDURES**

*Materials—*SAPD was from Calbiochem, 4\(\beta\)-12-O-Tetradeacnoylphorbol-13-acetate (TPA), 4\(\alpha\)-12-O-tetradeacnoylphorbol-13-acetate (4\(\alpha\)-TPA), and proteamine sulfate were from Sigma. Bryostatin-1 was obtained from Alexis Biochemicals, Inc. (San Diego, CA). Bovine brain phosphatidylinerine (BPS), 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), and 1-oleoyl-2-acetyl-glycerol (OAG) were from Avanti Polar Lipids (Alabaster, AL). Adenosine 5’-triphosphate (ATP) was from Boehringer Mannheim, and [\(\gamma\)-\(\alpha\)P]ATP was from NEN Life Science Products. All other chemicals were of analytical grade and obtained from Fisher.

*Preparation of Intact PKCa—the* recombinant conventional PKC isoform, PKCa (rat brain), was prepared using the baculovirus Spodoptera frugiperda (Sf9) insect cell expression system (50) and purified to homogeneity, as described previously (51). The specific activity of the PKCa preparation was typically ~1 nmol min \(^{-1}\) mg \(^{-1}\).

*Preparation of GST-C1-(His)\(_6\), Fusion Protein—*Based on evidence that GST stabilizes the isolated C1 domain and to ensure the production of only full-length peptides, the fusion protein, GST-C1-(His)\(_6\), was constructed. The nucleotide consensus sequence assigned to the C1 domain of PKCa was described previously (52). This was amplified by PCR with Pfu polymerase (Stratagene, La Jolla, CA) using full-length rat cDNA as the template. Primers were designed so that EcoRI and HpaI restriction sites were inserted at the 5’ and 3’ ends of the domain, respectively, to facilitate insertion into pGEX-5X-2 (Amersham Pharmacia Biotech). The reverse primer also encoded a blunt restriction site after the last amino acid of the domain which was utilized to insert a (His)\(_6\) sequence (5’-CAT CAC CAT CAC CAT CAC TGA-3’). The derived DNA fragment was subcloned into pGEX-5X-2 yielding a plasmid, the sequence of which was confirmed by dyeoxy sequencing (Nucleic Acid Facility, Thomas Jefferson University), that encoded GST-C1-(His)\(_6\), a protein-tagged N terminus with GST and C terminus with (His)\(_6\). Along with the GST-C1-(His)\(_6\), a fusion protein lacking the C1 domain was prepared by insertion of the (His)\(_6\) sequence alone into pGEX-5X-2 (GST-(His)\(_6\)).

*Escherichia coli* BL21 cells harboring the expression plasmids for GST-C1-(His)\(_6\), or GST-(His)\(_6\), were grown in LB medium containing 100 \(\mu\)g/ml ampicillin until the absorbance at 600 nm (\(A\)\(_{600}\)) was ~1. Expression of the fusion proteins was induced at room temperature with 0.1 mM isopropylthio-galactoside for 4 h, after which the cells were pelleted, washed once with phosphate-buffered saline, re-pelleted, and stored at \(-80^\circ\)C. The fusion proteins were purified from the frozen *E. coli* pellets as described previously (52). Briefly, frozen cell pellets were homogenized at 4°C in buffer A (50 mM Hepes, pH 8.0, 10 mM ethylene glycol, 1% v/v Triton X-100, 0.5 mg/ml lysozyme, 320 units of benzonase, 1 mM dithiothreitol, 10 mM benzamidase, 4 mM/ml peptatin A, 4 mM/ml apro tin, 10 \(\mu\)g/ml leupeptin). The homogenate was placed on ice for 20 min and then centrifuged at 30,000 \(\times\) g for 30 min at 4°C. The supernatant was aliquoted to the clarified lysate to yield a final concentration of 1 mM which was then loaded slowly onto a 1-mL column containing glutathione agarose (Sigma) previously equilibrated with buffer A. The eluate was re-applied to the column two times followed by extensive washing with 1 mM sodium phosphate buffer, pH 7.3, containing 15 mM NaCl, 0.5% v/v Triton X-100. The fusion proteins were eluted in a pH 8.0 buffer containing 50 mM Hepes, 10% v/v ethylene glycol.
glycol, 15 mM reduced glutathione, and 0.4% w/v sucrose monolaurate. In order to isolate full-length peptides the crude preparation was further purified by metal affinity chromatography using TALON resin (CLONTECH, Palo Alto, CA) according to the manufacturer's procedures. Fractons containing the purified fusion protein (detected by SDS-PAGE and gel electrophoresis followed by Coomassie Blue staining) were pooled and dialyzed extensively against 50 mM Hepes, pH 8.0, containing 10% v/v ethylene glycol. The resultant product was finally concentrated by dialysis against the same buffer saturated with polyethylene glycol and stored at −80 °C in the presence of 20% v/v glycerol.

Preparation of PKCζ-(His)₆—To facilitate isolation and purification, a (His)₆ affinity tag was added to the C terminus of PKCζ. Briefly, the last 1100 base pairs were amplified by PCR using pBu polymerase (Stratagene, La Jolla, CA) and using PKCζ cDNA as a template, which was cloned in this laboratory. The reverse primer was designed so that the stop codon was eliminated, and the (His)₆ sequence (CATCACCATCACCATCAGTA) followed by a stop codon and an HpaI restriction site was inserted in frame with the coding sequence. The PCR product was gel-purified on 1% agarose, A-tailed using T4 polymerase and dATP, and subcloned into pCR 2.1 (Invitrogen, Carlsbad, CA). The nucleotide sequence was confirmed by dyeoxy sequencing (Nucleic Acid Facility, Thomas Jefferson University). The first 1203 nucleotides of PKCζ were excised from PKCζ/pCR2.1 using EcoRI/BstUI, and the last 576 nucleotides including the (His)₆ tag were excised from PKCζ/pCR2.1 using BglII/HpaI. The fragments were gel-purified, ligated into the EcoRI/StuI site of pFastBac1 (Life Technologies, Inc.), and transfomed into DH5α E. coli. A clone containing the full-length PKCζ coding sequence including the (His)₆ tag was selected by restriction analysis of plasmid DNA. A recombinant baculovirus containing the PKCζ-(His)₆ sequence was generated using the Bac-to-Bac Baculovirus Expression System (Life Technologies, Inc.). Sf9 cells were infected with the recombinant baculovirus at a multiplicity of infection of 10, incubated for 3 days at 27 °C, harvested by centrifugation, and pellets containing PKCζ-(His)₆ were isolated by centrifugation, and pellets stored at −80 °C.

Preparation of PKCε—PKCε was gel-purified on 1% agarose, A-tailed using T4 polymerase and dATP, and subcloned into pCR 2.1 (Invitrogen, Carlsbad, CA) according to the manufacturer's procedures. Fractons containing PKCε-(His)₆ were pooled and concentrated by dialysis against 20 mM Tris/HCl, pH 7.4, 150 mM NaCl, 0.5 mM EDTA, 0.5 mM EGTA, and 10 mM β-mercaptoethanol saturated with polyethylene glycol at 4 °C. The concentrated pool was then loaded onto a Superdex 200 gel filtration XK 16/70 column (Amersham Pharmacia Biotech), connected to a fast protein liquid chromatography system, and equilibrated with 20 mM Tris/HCl, pH 7.4, 150 mM NaCl, 0.5 mM EDTA, 0.5 mM EGTA, 0.5 mM β-mercaptoethanol. The column was developed at 0.2 ml/min overnight. Fractons containing PKCε-(His)₆ were pooled and dialyzed against 20 mM Tris/HCl, pH 7.4, 100 mM NaCl, 0.5 mM EDTA, 0.5 mM β-mercaptoethanol, 10 mM β-mercaptoethanol and stored at −80 °C in the presence of 20% glycerol.

PKCa Activity—PKCa activity was determined in the presence of protamine sulfate, which acts both as a phosphate acceptor and an activator of the enzyme, as described previously (34). Briefly, the assay consisted of 50 mM Tris/HCl, pH 7.4, PKCs (0.04 ng/ml or 0.52 nM final), and protamine sulfate (0.4 mg/ml). To this was added either TPA, 4α,TPA, OAG, or bryostatin-1 at the required concentration. After incubation for 10 min at 30 °C in a stirred quartz cuvette, SAPD was titrated from stock solutions of the required concentration prepared from a Me₂SO solution of the phorbol ester. The fluorescence intensities were recorded for each phorbol ester addition after allowing equilibration. To isolate the fluorescence signal resulting from RET, the observed fluorescence intensities at each SAPD concentration were corrected for volume changes and turbidity prior to subtraction of the direct excitation of the SAPD fluorophore according to the following:

\[
F_{	ext{RET,obs}} = F_{	ext{RET,calc}} - F_{	ext{calc,SAPD}} - F_{	ext{calc,RET}} - F_{	ext{calc,protamine}}
\]

where, \(F_{\text{RET,obs}}\) is the observed fluorescence intensity, \(F_{\text{RET,calc}}\) the calculated fluorescence intensity, \(F_{\text{calc,SAPD}}\) the fluorescence intensity due to SAPD, and \(F_{\text{calc,RET}}\) and \(F_{\text{calc,protamine}}\) the fluorescence intensities due toprotamine sulfate in the absence of SAPD and in the presence and absence of PKCa, respectively. The resultant data were fitted by nonlinear least squares analysis to a modified Hill equation, assuming a model involving two independent SAPD-binding sites (35) as shown in Equation 2.

\[
\log \left( \frac{F_{\text{max}}}{{F_0} + F_{\text{max}}} \right) = \log \left( \frac{F_{\text{max}}}{F_{\text{max-L}} + \frac{F_{\text{max-L}}}{\left( \frac{[\text{SAPD}]}{K_{L}} + 1 \right)} + \frac{K_{H}}{\left( \frac{[\text{SAPD}]}{K_{H}} + 1 \right)} \right)
\]

where \(F_{\text{max-L}}\), \(F_{\text{max-H}}\), \(F_{\text{max-L,calc}}\), and \(F_{\text{max-H,calc}}\) are the minimum and maximum fluorescence intensities (i.e. the corrected RET signal for saturation of the high and low affinity phorbol ester-binding sites by SAPD, addition of further SAPD not further increasing the signal); \(K_{H}\) and \(K_{L}\) are binding constants (defined as the SAPD concentration corresponding a half-maximal fluorescence intensity increase); and \(n_H\) and \(n_L\) are the Hill coefficients for high and low affinity binding, respectively. Upon activation by protamine sulfate there was small decrease in the (nonmembrane) PKCa tetratranopeptide fluorescence fluoroscope (Photon Technology International, Inc., South Brunswick, NJ) at 332 and 425 nm, corresponding to the emission maxima of tryptophan and SAPD, respectively. The binding assay system (2 ml) consisted of 50 mM Tris/HCl, pH 7.4, 5 μg/ml (or 0.12 μM) PKCa, PKCζ-(His)₆, or GST-C1-(His)₆ and protamine sulfate at the indicated concentrations. To this was added TPA, 4α,TPA, OAG, or bryostatin-1 at the required concentration. After incubation for 10 min at 30 °C in a stirred quartz cuvette SAPD was titrated from stock solutions of the required concentration prepared from a Me₂SO solution of the phorbol ester. The fluorescence intensities were recorded for each phorbol ester addition after allowing equilibration. To isolate the fluorescence signal resulting from RET, the observed fluorescence intensities at each SAPD concentration were corrected for volume changes and turbidity prior to subtraction of the direct excitation of the SAPD fluorophore according to the following:

\[
F_{\text{RET,obs}} = F_{\text{RET,calc}} - F_{\text{calc,SAPD}} - F_{\text{calc,RET}} - F_{\text{calc,protamine}}
\]

where, \(F_{\text{RET,obs}}\) is the observed fluorescence intensity, \(F_{\text{RET,calc}}\) the calculated fluorescence intensity, \(F_{\text{calc,SAPD}}\) the fluorescence intensity due to SAPD, and \(F_{\text{calc,RET}}\) and \(F_{\text{calc,protamine}}\) the fluorescence intensities due toprotamine sulfate in the absence of SAPD, in the presence and absence of PKCa, respectively, and \(F_{\text{calc,RET}}\) and \(F_{\text{calc,protamine}}\) are the fluorescence intensities measured in the absence of SAPD, in the presence and absence of PKCa, respectively. The resultant data were fitted by nonlinear least squares analysis to a modified Hill equation, assuming a model involving two independent SAPD-binding sites (35) as shown in Equation 2.

\[
\log \left( \frac{F_{\text{max}}}{{F_0} + F_{\text{max}}} \right) = \log \left( \frac{F_{\text{max}}}{F_{\text{max-L}} + \frac{F_{\text{max-L}}}{\left( \frac{[\text{SAPD}]}{K_{L}} + 1 \right)} + \frac{K_{H}}{\left( \frac{[\text{SAPD}]}{K_{H}} + 1 \right)} \right)
\]

Results

Previous studies from this laboratory demonstrated the existence of high and low affinity phorbol ester-binding sites on membrane-associated PKCa (34, 35). In order to determine if these binding site(s) “pre-exist” on lipid-independent PKCαs or whether they are exposed upon membrane association, phorbol ester binding to non-membrane PKCs was studied. The effects of TPA, SAPD, OAG, and bryostatin-1 on non-membrane PKCs were studied. The assay was quenched by addition of protamine sulfate which was also determined. To control for potential nonspecific interactions of these compounds with non-membrane PKCαs, phorbol ester binding to the isolated C1 domain of this isoform, and also to the atypical isoform, PKCζ, which is incapable of binding phorbol esters (55, 56), was determined. The binding assay used was based on the increase in fluorescence intensity due to RET from tryptophans.

* Frank J. Taddeo, Mark D. Yeager, and Christopher D. Stubbis, manuscript in preparation.
Effect of PKC Activators on Lipid-independent Enzyme Activity

Summary of binding constants (Kh and KL) and Hill coefficients (nH and nL), generated from fits of RET as a function of SAPD concentration data obtained in the presence of increasing protamine sulfate concentration, shown in Fig. 1B, to Equation 2

Errors are reported as ± S.D. Regression coefficients were >0.99 for each data set. For details see “Experimental Procedures.”

| Protamine sulfate | nH | Kh | nL | KL |
|-------------------|----|----|----|----|
| μ                |    |    |    |    |
| 0                | 0.9 ± 0.2 | 580 ± 15 | 1.2 ± 0.2 | 3450 ± 190 |
| 10^{-2}          | 1.1 ± 0.2 | 116 ± 25 | 1.5 ± 0.3 | 3500 ± 120 |
| 10^{-4}          | 1.0 ± 0.1 | 760 ± 76 | 1.2 ± 0.2 | 4609 ± 256 |
| 10^{-6}          | 0.9 ± 0.1 | 515 ± 20 | 0.9 ± 0.1 | 3940 ± 420 |
| 10^{-2}          | 1.2 ± 0.3 | 732 ± 45 | 1.1 ± 0.1 | 4620 ± 340 |
| 10^{-4}          | 1.1 ± 0.3 | 530 ± 35 | 1.3 ± 0.3 | 4020 ± 120 |
| 10^{-6}          | 0.9 ± 0.1 | 388 ± 45 | 1.5 ± 0.2 | 3260 ± 110 |
| 10^{-4}          | 1.2 ± 0.1 | 375 ± 23 | 0.9 ± 0.1 | 3490 ± 200 |
| 10^{-3}          | 1.0 ± 0.1 | 226 ± 18 | 1.1 ± 0.1 | 2710 ± 150 |

 breaches the high affinity site, in the absence of lipids, led to a 5-fold decrease in Rs, sufficient to saturate the high affinity SAPD binding site. The resulting dual sigmoidal SAPD binding curve was not significantly influenced by this small correction.

Binding isotherms obtained for the interaction of SAPD with non-membrane PKCa in the presence of increasing concentrations of protamine sulfate were again dual sigmoidal (Fig. 1B), further indicating the existence of low and high affinity phorbol ester-binding sites on the non-membrane-associated enzyme. The effect of increasing protamine sulfate concentration, shown in Table I, was to increase the resonance energy transfer signal proportionately with increasing SAPD concentration. However, neither the affinities (Kh and KL) nor the Hill coefficients (nH and nL) for high and low affinity SAPD binding were found to be affected by interaction with protamine sulfate (Table I).

Effects of the Phorbol Ester, TPA, and Its “Inactive” Eipmer, 4α-TPA, the Diglyceride OAG, and Bryostatin-1 on SAPD Binding to Non-membrane PKCa in the Presence of Protamine Sulfate—In order to determine the specificity of low and high affinity phorbol ester binding to non-membrane PKCa associated with protamine sulfate, the effects of TPA and the inactive 4α-oh eipmer, 4α-TPA, on SAPD binding were first determined. This was accomplished by utilizing the increase in fluorescence anisotropy of SAPD as it binds to the phorbol ester-binding site, reflecting a more restricted motion compared with membrane-associated or free SAPD. Addition of SAPD (1 μM, sufficient to saturate the high affinity site), in the absence of lipids, led to a marked increase in Rs, consistent with binding to the non-membrane enzyme (Fig. 2A). Addition of TPA (1 μM) resulted in a decrease in Rs, due to the displacement of bound SAPD. The
The binding association of the enzyme, as observed for membrane-associated PKC, was determined from fits of binding data to Equation 2 by nonlinear regression analysis. Other methods were as described under “Experimental Procedures.”

Fig. 3B shows that the presence of OAG resulted in an inhibition of both high and low affinity SAPD binding to protamine sulfate-activated PKCα. This result again contrasts with previously observed effects of diacylglycerol on SAPD binding to membrane-associated PKCα, where it was found that while low affinity SAPD binding was inhibited, the level of high affinity binding was enhanced (35).

Along with phorbol esters and diacylglycerols, bryostatin-1 has also been commonly described as a potent "activator" of PKC (57). However, despite this similarity, bryostatin and phorbol esters have been shown to have dramatically different effects on PKC-regulated cellular processes. Indeed, it appears that bryostatin may antagonize many of the cellular effects induced by phorbol esters, such as tumor promotion (58–62). It was therefore of interest to determine whether the effects of bryostatin on non-membrane PKCα activity induced by protamine sulfate in the absence of lipids also differed from those on the membrane-associated enzyme. Fig. 3C shows that protamine sulfate-activated PKCα was also inhibited by bryostatin-1. These effects contrast with those on binding to membrane-associated PKCα, where high affinity SAPD binding was found to be inhibited by bryostatin-1, whereas the low affinity SAPD interaction was relatively unaffected (35).

Interaction of SAPD with the Isolated C1 Domain of PKCα (GST-C1-(His)₆) and with PKCζ. To address the possibility that the compounds studied may interact “nonspecifically” with a site(s) outside of the C1 domain, binding of SAPD to the isolated C1 domain of PKCα was determined. The fusion pro-
that the level of RET from the tryptophans of GST-(His)6 was
plotted on a linear abscissa. Other methods were as described under
"Experimental Procedures."
The Hill coefficient for this process was ~1 again indicating at least one inhibitory site, as found for SAPD, TPA, 4α-TPA, and OAG (see Table II). The potency of inhibition of non-membrane, protamine sulfate-induced PKCa activity (IC50) by bryostatin-1 was similar to the EC50 for the activation of the membrane-associated enzyme (see Table II).

DISCUSSION

In the present study, it is shown that the low and high affinity phorbol ester-binding sites, previously shown to exist on membrane-associated PKCa (35), also exist on soluble PKCa. The results show that although membrane-association is not required to expose these sites, the affinities and specificities of the sites are modified by this interaction. Furthermore, evidence is also presented that both high and low affinity phorbol ester-binding sites may be confined within the C1 domain of the enzyme. However, binding of phorbol esters, which are commonly classed as activators of membrane-associated PKC, resulted in a potent inhibition of lipid-independent enzyme activity induced by protein-protein interactions with the arginine-rich protein, protamine sulfate. Furthermore, two other important activators of membrane-associated PKC, diacylglycerol and bryostatin-1, competed for both high and low affinity phorbol ester binding and also inhibited protamine sulfate-induced activity.

The observation that PKCa alone bound SAPD in the absence of protamine sulfate (or membrane lipids) is consistent with membrane association not being an absolute requirement for phorbol ester binding, as observed in several other studies. For example, it has been previously observed that phorbol esters are capable of inducing a low level of PKC activity in the absence of membranes or protein elements, providing evidence for a lipid-independent interaction of phorbol esters with PKC (63). Finally, it has been shown that the novel Ca2+-independent isofrom, PKCβ, and also a peptide corresponding to the C1B region, bound phorbol ester in the absence of lipids (22). In the present study, binding of phorbol ester to non-membrane PKCa utilized SAPD as a probe for low and high affinity binding sites, as previously observed to exist on the membrane-associated enzyme.
enzyme (35). Phorbol ester binding to PKC is commonly determined using phorbol 12,13-dibutyrate (PDBu) which, being relatively hydrophilic compared with TPA (or SAPD), minimizes nonspecific interactions and enables the physical separation of bound from free ligand. However, this precludes the detection of low affinity binding. By contrast, the SAPD binding assay used in the present study does not require physical separation of bound from free ligand, and the affinity of SAPD for binding to PKCα is ≈10-fold greater than that of PDBu (64), which allows for the detection low affinity phorbol ester binding. The results show that both the high and low affinity phorbol ester-binding sites, previously shown to be present on the membrane-associated enzyme (35), pre-exist on non-membrane PKCα. Furthermore, the observation that the isolated C1 domain of PKCα also bound SAPD with high and low affinities provides evidence that, as for the high affinity site, the low affinity phorbol ester-binding sites may also be contained within this domain, rather than residing elsewhere on the PKC molecule. This is also apparent from the observation that PKCζ failed to bind SAPD, in keeping with the inability of this isoform to bind other phorbol esters. However, further experiments are required to determine the nature of the low affinity phorbol ester-binding site within the C1 domain and whether this site corresponds to either the C1A or B subdomains.

Comparison of isotherms corresponding to SAPD binding to membrane-associated PKCα with that obtained for binding to the soluble isozyme (Fig. 1A) revealed that interaction with membrane lipid vesicles containing PS results in an ≈5-fold increase in the strength of high affinity SAPD binding. This is in keeping with the results of studies by Kazanietz and co-workers (22), who showed that the structure-activity relationship for phorbol ester binding to soluble, non-membrane PKCζ, differed markedly compared with that for binding to the membrane-associated isoform. For example, although it was shown that the affinity of PDBu for binding to PKCζ in the presence of phosphatidylserine (PS) was enhanced 80-fold compared with the soluble isozyme, the binding affinity of phorbol-12-decanoyl ester was found to be only enhanced 6-fold.

In the present study it was shown that 4α-TPA, TPA, OAG, and bryostatin-1 each displaced both high and low affinity SAPD binding to native PKCα and to the isolated C1 domain, suggesting that both sites on the non-membrane enzyme may have similar binding specificities. By contrast, the low and high affinity phorbol ester-binding sites on membrane-associated PKCα have previously been shown to have differing specificities (35). In particular, the observation that 4α-TPA inhibited high affinity SAPD binding to non-membrane PKCα while having negligible effects on high affinity SAPD binding to the membrane-associated enzyme (Fig. 2) suggests that this site on non-membrane PKCα may have a reduced specificity for the orientation of the 4-OH and/or the A and B rings of SAPD, compared with that on the membrane-associated enzyme. Furthermore, whereas bryostatin-1 appears to compete for low affinity SAPD binding to non-membrane PKCα and to the isolated C1 domain, this compound failed to compete for low affinity SAPD binding to the membrane-associated enzyme, suggesting that the specificities of the low affinity sites on non-membrane and membrane-associated PKCα may also differ. In keeping with this was the finding that the ratios of the values of IC_{50} for the inhibition of non-membrane PKCα to the corresponding values of EC_{50} for the activation of the membrane-associated enzyme differed for the compounds studied (see Table II).

The mechanism of activation of PKC by arginine- and lysine-rich proteins has been suggested to involve the formation of high order aggregates (6, 7). Also consistent with the formation of an aggregate was the observation that the specific activity of protamine sulfate phosphorylation displayed positive cooperativity with respect to protamine sulfate concentration, as observed previously (6). Importantly, the observation that neither the Hill coefficient nor the mid-point of the specific activity against protamine sulfate curve was affected by the presence of SAPD argues against the possibility that the inhibition of protamine sulfate activity resulted from an effect on PKCα-protamine sulfate interactions. Rather, the apparent decrease in the maximal reaction velocity suggests that the inhibitory effect involves an attenuation of the activating conformational change induced by interaction with the arginine-rich protein. This conformational change could provide a basis for the observed increase in RET induced by protamine sulfate, since the molecular rearrangement would result in a change in the average distance between participating PKCα tryptophan and SAPD fluorophores.

The finding that the value of the IC_{50} for the inhibition of protamine sulfate activity by SAPD was close to the phorbol ester concentration required for half-maximal binding to the high affinity site on protamine sulfate-associated PKCα strongly suggests that the inhibitory effect may be mediated by interaction with this site. However, based on the present data, it is not possible to determine whether, similar to SAPD, the inhibitory effect of these compounds is mediated specifically by interaction with the non-membrane high affinity phorbol ester-binding site per se. Only direct measurements of binding of these compounds to the PKCα would resolve this issue, which is technically difficult to achieve at the sensitivity required. Interaction of phorbol esters, diacylglycerol, and bryostatin with the SAPD-binding sites on PKCα appears to inhibit the protamine sulfate-induced activating conformational change, based on the observation that the inhibitory effect of SAPD on protamine sulfate-induced activity corresponded to a reduction in the maximal reaction velocity (V_{max}), rather than an effect on the PKCα-protamine sulfate interactions. Conversely, the protamine sulfate-induced activating conformational change does not appear to impact on phorbol ester binding, based on the observation that neither the binding constants nor the Hill coefficients for high and low affinity SAPD binding changed significantly in the presence of increasing protamine sulfate concentrations. These observations suggest that the inhibition of activity by phorbol esters (and other activators) may proceed by an "uncompetitive" type mechanism in which the effect of SAPD binding is to prevent rather than reverse the activating conformational change.

Finally, the observed differences in the specificities of the low and high affinity phorbol ester-binding sites on non-membrane and membrane-associated PKCα may partially contribute to the distinct effects of bryostatin, compared with phorbol esters, on PKC-regulated cellular processes adding to differences in down-regulation, isoform-selectivity, and cellular location.

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