Low- and High-Affinity Phorbol Ester and Diglyceride Interactions with Protein Kinase C: 1-*O*-Alkyl-2-acyl-*sn*-glycerol Enhances Phorbol Ester- and Diacylglycerol-Induced Activity but Alone Does Not Induce Activity†

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ABSTRACT: Phorbol ester-induced conventional protein kinase C (PKCα, -βI/II, and -γ) isozyme activities are potentiated by 1,2-diacyl-*sn*-glycerol. This has been attributed to a "cooperative" interaction of the two activators with two discrete sites termed the low- and high-affinity phorbol ester binding sites, respectively [Slater, S. J., Milano, S. K., Stagliano, B. A., Gergich, K. J., Ho, C., Mazurek, A., Taddeo, F. J., Kelly, M. B., Yeager, M. D., and Stubbs, C. D. (1999) *Biochemistry 38*, 3804−3815]. Here, we report that the 1-*O*-alkyl ether diglyceride, 1-*O*-hexadecyl-2-acetyl-*sn*-glycerol (HAG), like its 1,2-diacyl counterpart, 1-oleoyl-2-acetyl-*sn*-glycerol (OAG), also potentiated PKCα, -βI/II, and -γ activities induced by the phorbol ester 4β-12-*O*-tetradecanoylphorbol-13-acetate (TPA). Similar to OAG, HAG was found to bind to the low-affinity phorbol ester binding site and to enhance high-affinity phorbol ester binding, and to decrease the level of Ca^{2+} required for phorbol ester-induced activity, while being without effect on the Ca^{2+} dependence of membrane association. Thus, similar to OAG, HAG may also potentiate phorbol ester-induced activity by interacting with the low-affinity phorbol ester binding site, leading to a reduced level of Ca^{2+} required for the activating conformational change. However, HAG was found not to behave like a 1,2-diacyl-*sn*-glycerol in that alone it did not induce PKC activity, and also in that it enhanced OAG-induced activity. The results reveal HAG to be a member of a new class of "nonactivating" compounds that modulate PKC activity by interacting with the low-affinity phorbol ester binding site.

The protein kinase C (PKC)[1] family of serine/threonine kinases plays a pivotal role in the transduction of intracellular signals that govern diverse processes that include, for example, cell growth, differentiation, metabolism, and apoptosis (*1*−*5*). The requisite tight regulation of PKC activity is achieved by the combined interaction of multiple activators and cofactors with conserved domains, the presence of which varies for each isoform. Thus, common to the "conventional" PKCα, -βI, -βII, and -γ (cPKC) isoforms is the presence of a C2 domain which confers a Ca^{2+} requirement for activity, whereas the "novel" PKCδ, -ε, -θ, -μ, and -η (nPKC) and the "atypical" PKCζ, -λ/ι (aPKC) isoforms each lack a functional C2 domain and are consequently Ca^{2+}-independent. Also present in all PKC isoforms is a C1 domain that, with the exception of aPKC, contains the sites of interaction of the natural activator 1,2-diacyl-*sn*-glycerol (DAG) and also the tumor-promoting phorbol esters.

The C1 domain of PKC consists of two cysteine-rich zinc finger like motifs termed C1A and C1B (*6*). Structural studies have been performed on the C1B domain of PKCδ, the C1A and -B domains of PKCγ (*7*), and the C1B domain of PKCα (*8*, *9*). Based on such studies, the essential features of the phorbol ester structure that are required to optimally interact with the C1 domain of PKCδ have been determined (*10*). The amino acid residues within this domain important for phorbol ester binding are highly conserved in each PKC isozyme (*10*−*12*). Despite this, PKC isoforms have been shown to differ markedly in their phorbol ester binding properties (*13*, *14*), pointing to a potential role for nonconserved residues in determining the specificity of the phorbol ester interaction. Experiments in which the phorbol ester binding abilities of the C1A or C1B domains of PKCδ were removed in turn revealed that the membrane translocation of the isozyme in NIH 3T3 cells is mediated primarily by phorbol ester binding to the C1B domain, with C1A making a relatively small contribution (*15*). However, it was also shown that the decrease in translocation resulting from removal of phorbol ester binding to the C1B domain was reinforced when binding to the C1A domain was also ablated.

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[1] Abbreviations: BPS, bovine brain phosphatidylserine; DAG, 1,2-diacyl-*sn*-glycerol; dansyl-PE, *N*-(5-dimethylaminonaphthalene-1-sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine; HAG, 1-hexadecyl-2-acetyl-*sn*-glycerol; LUV, large unilamellar vesicles; MBP_{4-14}, myelin basic protein peptide substrate; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol; peptide-ε, peptide corresponding to the pseudosubstrate of PKCε in which the single alanine was replaced with serine; aPKC, "atypical" PKC; cPKC, "conventional" PKC; nPKC, "novel" PKC; PKC, protein kinase C; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; RET, resonance energy transfer; SAPD, sapintoxin-D; TPA, 4β-12-*O*-tetradecanoylphorbol-13-acetate.

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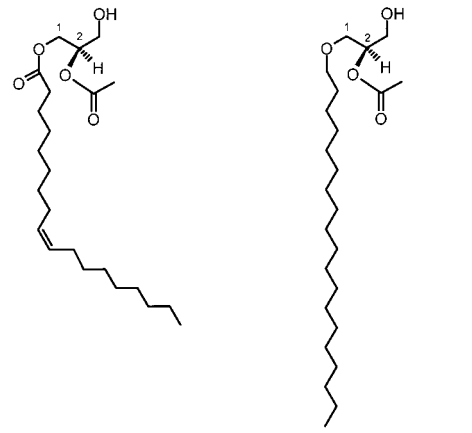
Based on these observations, it was concluded that the two domains do not play equivalent roles in membrane association, although there appears to be an interaction between them. Supporting this, it was recently concluded based on mutation studies with PKCα that the C1A but not the C1B domain inserts into the membrane and interacts with DAG, facilitating activation (*16*, *17*). Also, it has been shown that the C1A but not the C1B domain of PKCγ is translocated to the plasma membrane of cells when challenged with DAG (*18*). Interestingly, the same study also showed that in contrast to DAG, phorbol ester induced the translocation of *both* C1A *and* C1B domains to membranes, which is consistent with the notion that the two domains may have different binding affinities for phorbol ester compared to DAG.

Studies from this laboratory using the fluorescent phorbol ester sapintoxin-D (SAPD) have provided evidence for the existence of distinct low- and high-affinity phorbol ester binding sites on PKCα (*19*−*22*), both of which were shown to reside within its C1 domain (*23*). Other studies have lent support for such a notion of coexisting high- and low-affinity phorbol ester binding sites on PKC (*13*, *16*, *19*−*21*, *23*−*29*). In our previous study, we showed that the interaction of DAG with the low-affinity phorbol ester binding site induces an increase in the level of binding of phorbol ester to the high-affinity site (*19*). This increased level of binding was shown to be accompanied by an elevation of the level of enzyme activity beyond that achievable by either phorbol ester or DAG alone. Other compounds have also been found to interact with the two binding sites with affinities that differ from those of either phorbol ester or DAG. For example, the potent antitumor agent bryostatin-1 was found to compete with phorbol ester primarily for the high-affinity binding site (*19*), whereas low-affinity phorbol ester binding was found to be displaced by *n*-alkanols and anesthetics (*20*, *30*). Similar to DAG, *n*-alkanols and anesthetics were also found to *enhance* the level of high-affinity phorbol ester binding along with the level of phorbol ester-induced PKC activity.

The increased level of activity resulting from the combined interaction of DAG and phorbol ester compared to that induced by either activator alone corresponds, in part, to a shift in the Ca^{2+} dependence of activity to lower Ca^{2+} concentrations, and also to an increase in the level of Ca^{2+}-independent activity (*21*). This implies that both Ca^{2+}-dependent and Ca^{2+}-independent activating conformational changes can take place in the membrane-associated form of cPKC and that both these processes are subject to amplification by the combined interaction of phorbol ester and DAG (*21*). Furthermore, the active conformers of cPKC induced by either phorbol ester or DAG were shown to be distinct, based on differences in the stability of the phorbol ester-bound compared to the DAG-bound forms of the enzyme to elevated temperature (*21*).

The apparent differences in the affinities of DAG and phorbol ester for the two activator binding sites on PKC raise important implications. For example, the presence of multiple activators of PKC that interact differently with the enzyme is likely to occur quite commonly both in experimental and in physiological paradigms. However, an understanding of the mechanism and consequences of the interaction of DAG with the low-affinity phorbol ester binding site, combined with high-affinity phorbol ester binding, is impeded by the fact that DAG is itself an activator of PKC. In exploring possible candidates for compounds that may potentiate the level of PKC activity by interacting with the low-affinity phorbol ester binding site and enhancing high-affinity phorbol ester binding, while in isolation lacking the ability to activate the enzyme, we investigated the effects of the ether lipid 1-hexadecyl-2-acetyl-*sn*-glycerol (HAG) on PKC activity (see Figure 1).



1-Oleoyl-*2*-acetyl-*sn*-glycerol (OAG) *1*-O-Hexadecyl-*2*-acetyl-*sn*-glycerol (HAG)

FIGURE 1: Structures of OAG and HAG.

In the present study, using an in vitro assay system, we show first that phorbol ester- or DAG-induced conventional PKCα, -βI, -βII, and -γ activities were each enhanced by HAG, whereas novel PKCδ activity was unaffected. Second, like DAG, it is shown that HAG displaced low-affinity phorbol ester binding and increased high-affinity phorbol ester binding, resulting in the enhanced level of activity. However, unlike DAG, HAG alone was unable to induce PKC isozyme activity, suggesting that HAG may belong to a class of "nonactivating" compounds that can enhance phorbol ester- or DAG-induced PKC activity by interacting with the low-affinity phorbol ester binding site, which may provide unique tools for modulating PKC-mediated cellular functioning.

MATERIALS AND METHODS

Materials. Peptide substrates for PKC isoforms were custom-synthesized by the Jefferson Cancer Institute peptide synthesis facility. Lipids were from Avanti Polar Lipids, Inc. (Alabaster, AL), except for HAG which was purchased from Biomol (Plymouth Meeting, PA) and *N*-(5-dimethylamino-naphthalene-1-sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (dansyl-PE) which was from Molecular Probes (Eugene, OR). Sapintoxin-D (SAPD) was from Calbiochem (La Jolla, CA) or Alexis Biochemicals (San Diego, CA), and 4β-12-*O*-tetradecanoylphorbol-13-acetate (TPA) was from Sigma. ATP was from Boehringer Mannheim (Indianapolis, IN), and [γ-^{32}P]ATP was from New England Nuclear (Boston, MA). All other chemicals were of analytical grade and obtained from Fisher Scientific. Recombinant PKCα, -βI, -βII, -γ, and -δ (rat brain) were prepared using the baculovirus-insect cell expression system (*31*) and purified as previously described (*32*, *33*). To aid purification, PKCδ was tagged with 6×His at the N-terminus according to previously described methods (*33*).

Preparation of Vesicles. Large unilamellar vesicles (LUV) of 100 nm diameter and 750 μM total lipid concentration were prepared using the extrusion method as described elsewhere (*34*), and consisted of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and bovine brain phosphatidylserine (BPS) at a 4:1 molar ratio. When added, 1-oleoyl-2-acetyl-*sn*-glycerol (OAG) and HAG were co-dispersed with the lipids at the levels indicated in the figure legends (these being subtracted from the concentration of POPC). TPA was added to the assay, either separately or in combination with HAG or OAG, from a 0.5 mM Me_2SO stock to give a level of 0.3 mol % of the total lipid concentration.

Assay of PKC Activity. The activities of PKC isoforms were determined as previously described (*26*), using as a substrate either MBP_{4-14} (QKRPSQRSKYL**)** for cPKC, or a peptide corresponding to the pseudosubstrate motif of PKCϵ (peptide-ϵ, PRKRQGSVRRRVHQVNG) which contained a serine in place of an alanine residue for PKCδ (*35*−*37*). The assay system consisted of 50 mM Tris/HCl (pH 7.40) containing 0.1 mM Ca^{2+}, 50 μM MBP_{4-14}, and LUV (150 μM) in a total volume of 75 μL. In experiments where PKC activity was measured as a function of Ca^{2+} concentration, Ca^{2+} was added at a level calculated to yield the required free concentration when buffered by 0.1 mM EGTA (*38*). After thermal equilibration to 30 °C, assays were initiated by the simultaneous addition of PKC isoforms (0.3 nM) along with a solution containing 5 mM Mg^{2+}, 15 μM ATP, and 0.3 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) and terminated after 30 min with 100 μL of 175 mM phosphoric acid. Following this, a 100 μL aliquot was transferred to P81 filter papers which were then washed 3 times in 75 mM phosphoric acid. The phosphorylated peptide was determined by scintillation counting.

Determination of Ca^{2+}-Induced PKCα Membrane Association Based on Increased Dansyl-PE Steady-State Anisotropy. Interaction of PKCα with membrane lipid vesicles was determined from measurements of the increase in dansyl-PE anisotropy, which results from the hindrance of the motional freedom of the headgroup fluorophore due to the proximity of the membrane-associated isozyme, as described previously (*21*). The assay components consisted of Tris/HCl (50 mM, pH 7.40), 5 mM Mg^{2+}, 0.1 mM EGTA, along with LUV comprised of POPC, BPS, and dansyl-PE (3.75:1:0.25 molar ratio) at a total lipid concentration of 150 μM, and PKCα (0.1 μM) in a 2 mL quartz cuvette. TPA was added to the assay from a 0.5 mM Me_2SO stock (0.3 mol % of the total lipid concentration) and HAG (4 mol % of the total lipid concentration when co-dispersed with the other lipids). After allowing thermal equilibration at 30 °C, Ca^{2+} was titrated into the assay system as $CaCl_2$ from standard solutions so that each Ca^{2+} addition yielded the required calculated free Ca^{2+} concentration when buffered by 0.1 mM EGTA (*38*). After each Ca^{2+} addition, equilibrium was established, and values of dansyl-PE anisotropy were measured using a PTI Alphascan dual-emission fluorescence spectrofluorometer in the T-format (Photon Technology International, Inc., Princeton, NJ), as previously described (*39*). While assays of cPKC activity contained ATP and MBP_{4-14}, the omission of these substrates from this and other assays of membrane association affected neither the affinities nor the maximal extent of Ca^{2+}-induced PKCα association with vesicles (results not shown).

Determination of Phorbol Ester Binding to PKCα. Phorbol ester binding to PKC isozymes was quantified based on resonance energy transfer (RET) from PKC tryptophans to the 2-(*N*-methylamino)benzoyl fluorophore of the phorbol ester, SAPD, as previously described (*19*). Briefly, the fluorescence intensities at the emission maxima of PKC-tryptophans and SAPD (340 and 425 nm, respectively) were obtained upon excitation of the tryptophan fluorophore at 290 nm. The assay constituents consisted of 50 mM Tris/HCl (pH 7.40), 150 μM BPS/POPC LUV (1:4 molar ratio), 5 mM Mg^{2+}, 15 μM ATP, 0.1 mM Ca^{2+}, and PKC (0.1 μM) in a total volume of 2 mL. After allowing thermal equilibration at 30 °C, SAPD was titrated into the assay system, and after equilibrium was attained, the emission fluorescence intensity at 425 nm was measured. The contribution of RET to the observed signal was isolated by subtracting the fluorescence intensity measured in the presence of all assay components, except SAPD.

RESULTS

Effects of HAG and OAG Alone or in Combination with TPA on the Activity of Different PKC Isoforms. The effects of the soluble DAG, OAG, along with the 1-*O*-alkyl-2-acyl-*sn*-glycerol, HAG, and the phorbol ester, TPA, alone or in combination, on the membrane-associated activities of a panel of PKC isoforms are shown in Figure 2. Whereas the interaction of PKCα, -βI, -βII, and -γ with membrane lipid vesicles containing OAG or TPA resulted in activation, the interaction of these isoforms with membranes containing HAG did not result in a significant stimulation of activity. Similar to the cPKC isoforms, the activity of the Ca^{2+}-independent novel isozyme, PKCδ, was also increased by OAG and TPA while being unaffected by HAG. However, for this isozyme, neither of these compounds enhanced TPA-induced activity, contrasting with the effects on cPKC activities.

Concentration-Dependent Effects of OAG and HAG on PKCα, PKCβI, and PKCδ Activities in the Presence and Absence of TPA. The concentration-dependent effects of OAG and HAG on Ca^{2+}-induced, membrane-associated PKCα and -βI activities and on Ca^{2+}-independent, membrane-associated PKCδ activity, alone or in combination with TPA, are shown in Figure 3. The results obtained for PKCα and PKCβI were qualitatively similar. Thus, in both cases, increasing the mole fraction of OAG in BPS/POPC vesicles resulted in a concentration-dependent increase in the level of activity, as expected (Figure 3A,C, closed circles). The level of this OAG-induced activity was further potentiated in the presence of a fixed level of TPA (Figure 3A,C, squares), which is similar to the previously reported additive effects of 1,2-dioleoyl-*sn*-glycerol on TPA-induced PKCα activity (*19*, *26*). Similar to the effect of OAG, the level of TPA-induced PKCα and -βI activity was also enhanced by HAG in a concentration-dependent manner (Figure 3B,D, squares). Importantly, whereas OAG itself activated PKCα and -βI, the activities of neither isozyme were affected by the presence of HAG alone, even when it was present at high levels (Figure 3B,D, solid circles). Therefore, the interaction of HAG with PKCα and -βI is not equivalent to

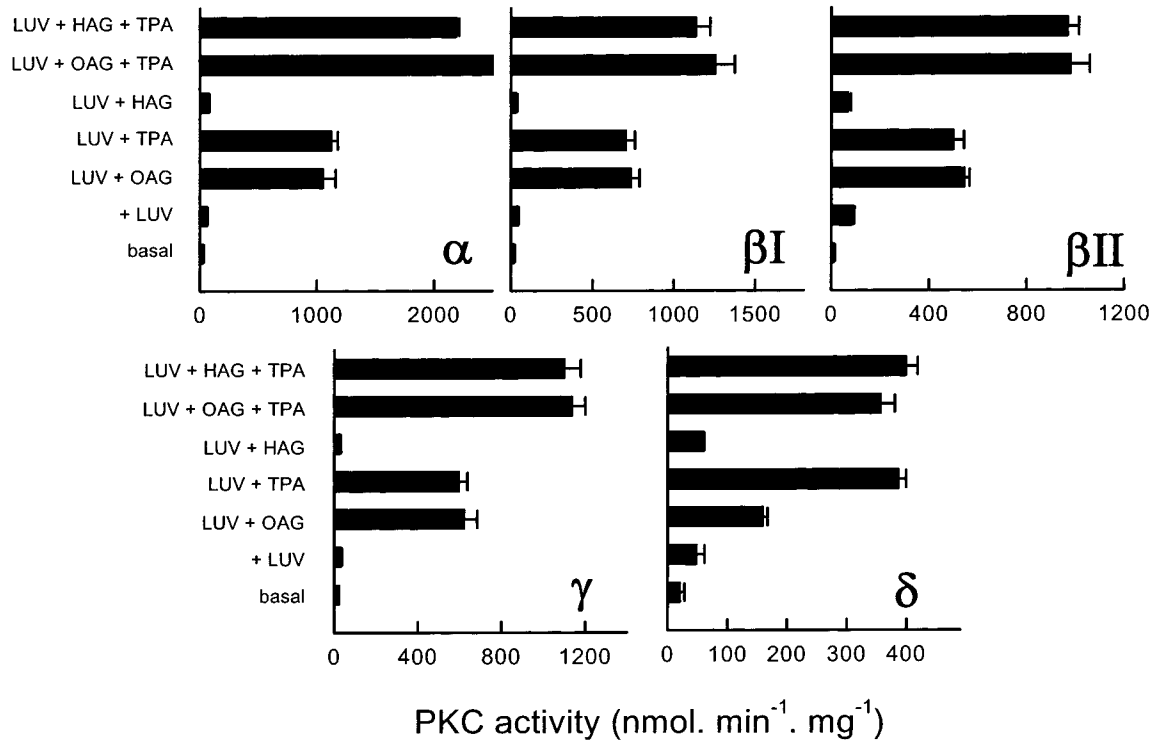


FIGURE 2: Effects of HAG and OAG alone or in combination with TPA on the activity of purified, recombinant PKCα, -βI, -βII, -γ, and -δ. PKC isoform specific activities were determined using an in vitro assay in the presence or absence of Ca^{2+} (0.1 mM) and 100 nm LUV composed of BPS and POPC (1:4 molar ratio), at a total lipid concentration of 150 μM, containing 0.3 mol % TPA, 4 mol % OAG, and 4 mol % HAG either separately or in combination. The mole fraction of OAG and HAG in vesicles was subtracted from the mole fraction of POPC. Data represent mean specific activities determined in triplicate (±SD), derived from experiments carried out at least 3 times. Other details are as described under Materials and Methods.

that of OAG. Further evidence that HAG differs from DAG in its effects on these isozymes was the finding that HAG also potentiated OAG-induced activity (Figure 3A,C, triangles). Consistent with this, the level of PKCα and -βI activity induced by a fixed concentration of OAG was also potentiated by HAG in a concentration-dependent manner (Figure 3B,D, triangles).

PKCδ activity was also enhanced by OAG with a concentration dependence similar to that observed for the conventional isozymes (Figure 3E, closed circles). Similar to PKCα and -βI, HAG alone did not induce PKCδ activation (Figure 3F, closed circles). However, contrasting with the potentiation of TPA-induced cPKC activities by OAG, the level of TPA-induced PKCδ activity was not affected by OAG (Figure 3E, squares) or HAG (Figure 3F, squares). Also, while HAG enhanced OAG-induced PKCα and -βI activities, it was found to slightly inhibit OAG-induced PKCδ activity (Figure 3E,F, triangles).

Ca^{2+} Concentration-Dependent Effects of OAG and HAG on TPA-Induced Activity and Membrane Association. The activation of conventional PKC isoforms proceeds by an initial Ca^{2+}-dependent association with the membrane followed by a Ca^{2+}-induced activating conformational change (*21*, *40*). To investigate whether the potentiation of TPA-induced cPKC activity by HAG may have involved an effect on the Ca^{2+} dependences of either or both of these processes, the effects of HAG on the Ca^{2+} concentration dependence of TPA-induced PKCα activity were compared with those on the Ca^{2+} concentration requirements for the association of this isozyme with TPA containing BPS/POPC membrane lipid vesicles (Figure 4).

In the absence of TPA, PKCα activity was unaffected by HAG, independent of the level of Ca^{2+} (Figure 4A, open squares), which is consistent with the lack of an effect of HAG on activity shown in Figure 3A. In the presence of TPA, the Ca^{2+} concentration required to induce a half-maximal increase in activity (EC_{50}) was 30 ± 2 μM, calculated by fitting the data shown in Figure 4A (solid circles) to a modified Hill equation (*21*). The presence of HAG resulted in a decrease in the value of EC_{50} to 8.0 ± 0.5 μM and also to an increase in the level of activity achieved at maximal levels of Ca^{2+} (Figure 4A, open circles). However, the low level of PKCα activity induced by TPA in the absence of Ca^{2+} was unaffected by HAG. Thus, the effects of HAG on TPA-induced activity are Ca^{2+}-dependent.

The Ca^{2+} concentration dependence of the association of PKCα with BPS/POPC vesicles in the presence of TPA was determined from measurements of the resultant increase in the steady-state fluorescence anisotropy of dansyl-PE (Figure 4B). The Ca^{2+} concentration corresponding to a half-maximal increase in the association of PKCα with BPS/POPC membrane lipid vesicles containing TPA was ~2 orders of magnitude less than the EC_{50} for Ca^{2+}-induced activity, measured under the same conditions (cf. Figure 4B with 4A, solid circles). HAG affected neither the midpoint of the Ca^{2+}-induced binding curve nor the level of membrane association achieved at maximal levels of Ca^{2+} (Figure 4B, open circles). Therefore, the effects of HAG on TPA-induced activity are not due to altered levels of membrane association.

Effects of HAG and OAG on Phorbol Ester Binding to Membrane-Associated PKCα, -βI, and -δ. In our previous study, it was found that the DAG 1,2-dioleoyl-*sn*-glycerol

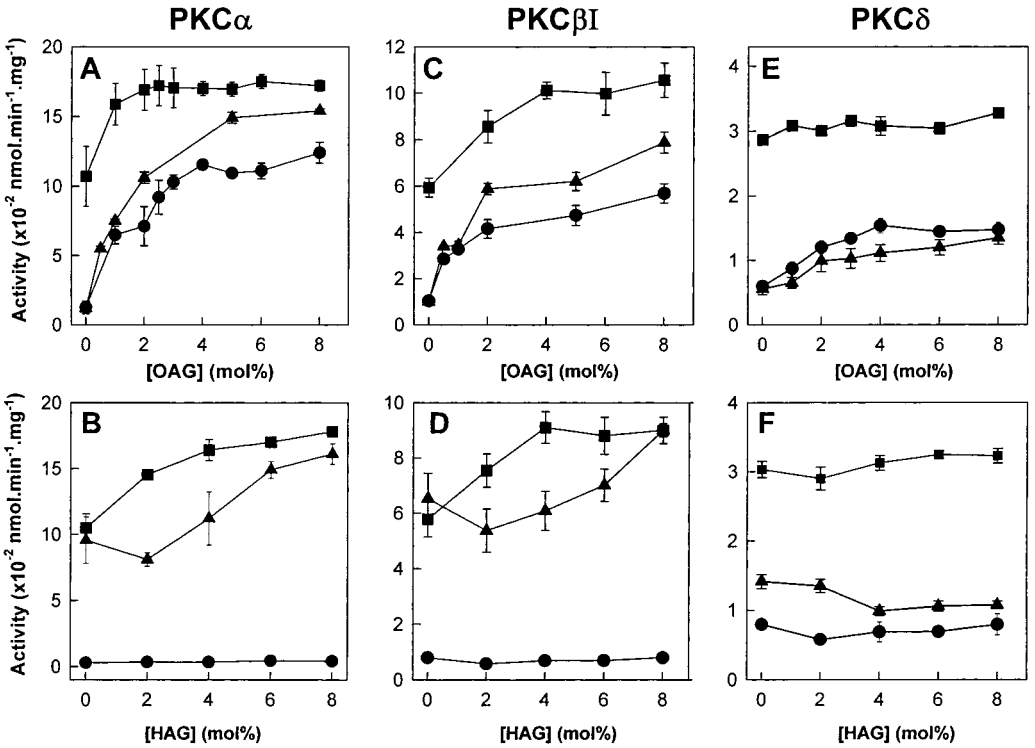


FIGURE 3: Concentration-dependent effects of OAG and HAG on conventional PKCα and -*β*I and novel PKC*δ* activities in the presence and absence of TPA. Specific activities were determined in the presence of 0.1 mM Ca^{2+} and LUV consisting of BPS and POPC (see legend to Figure 1). Panels A, C, and E: The effects of increasing mole fractions of OAG on the activities of PKCα, -*β*I, and -*δ*, respectively. OAG was present in LUV either alone (●) or in combination with 0.3 mol % TPA (■) or with 4 mol % HAG (▲). Panels B, D, and F: Effects of increasing levels of HAG in LUV on PKCα, -*β*I, and -*δ* activities, respectively, either alone (●) or in combination with 0.3 mol % TPA (■) or with 4 mol % OAG (▲). Data represent means of triplicate determinations of specific activities (±SD), derived from experiments carried out at least 3 times. Other details are as described under Materials and Methods.

displaced phorbol ester from the low-affinity binding site on PKCα, while enhancing the level of high-affinity phorbol ester binding (*19*). This provided a mechanistic basis for the potentiation of phorbol ester-induced PKCα activity by DAG.

Previously, we showed it was possible to clearly distinguish high- and low-affinity phorbol ester binding using the fluorescent phorbol ester SAPD, based on measurements of resonance energy transfer (RET) from PKC tryptophans to the 2-(*N*-methylamino)benzoyl fluorophore of SAPD (*19*). The commonly used binding assay based on the use of phorbol-12,13-dibutyrate (PDBu) only provides information on the high-affinity phorbol ester interaction. The dual-sigmoidal SAPD binding isotherm obtained for PKCα, corresponding to low- and high-affinity phorbol ester binding to this isozyme, is shown in Figure 4A (solid circles). The presence of *either* OAG (open squares) *or* HAG (solid squares) resulted in a decrease in fluorescence intensity due to RET within a SAPD concentration range corresponding to the low-affinity interaction, and an increase in RET arising from the high-affinity phorbol ester interaction, as was previously observed with the DAG 1,2-dioleoyl-*sn*-glycerol (*19*). The interaction of SAPD with PKC*β*I was also found to follow a dual-sigmoidal binding isotherm, indicating that, similar to PKCα, this isozyme also contains low- and high-affinity phorbol ester binding sites (Figure 4B, solid circles). The low-affinity interaction was again inhibited by both OAG (open squares) and HAG (solid squares), which in both cases resulted in an enhanced level of high-affinity phorbol ester binding. Thus, both HAG and OAG appear to interact similarly with a low-affinity phorbol ester binding site which is present on both PKCα and PKC*β*I.

The properties of SAPD binding to the *novel* isozyme PKC*δ* differed markedly from those observed for the conventional PKC isozymes, as shown in Figure 4C (solid circles). Thus, the binding isotherm obtained for PKC*δ* was found to be monophasic, suggestive of a single phorbol ester binding site, at least for SAPD. Consistent with the observed lack of an effect of HAG or OAG on TPA-induced activity (Figure 3E,F, solid squares), neither compound was found to affect the midpoint of the SAPD concentration−response curve or the maximal level of SAPD binding obtained (Figure 4C, open squares).

DISCUSSION

In this study, we show that phorbol ester- and DAG-induced conventional PKCα, -*β*I, -*β*II, and -*γ* activities were each enhanced by HAG and that this effect was Ca^{2+}-dependent and was not found for novel PKC*δ*. Unlike DAG, HAG alone does not induce PKC isozyme activity, suggesting that HAG may belong to a novel class of "nonactivating" compounds that can enhance phorbol ester- or DAG-induced PKC activity by interacting with the low-affinity phorbol ester binding site.

The 1,2-diacyl-*sn*-glycerols, formed by the specific phospholipase C catalyzed hydrolysis of 1,2-diacyl-*sn*-glycero-3-phosphoinositides and 1,2-diacyl-*sn*-glycero-3-phosphocholines, are well-known activators of PKC. The 1-*O*-alkyl derivatives of these lipids can also serve as substrates in the formation of 1-*O*-alkyl-2-acyl-*sn*-glycerols which mainly proceeds via the phospholipase D catalyzed hydrolysis of 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholines (*41*). An im-

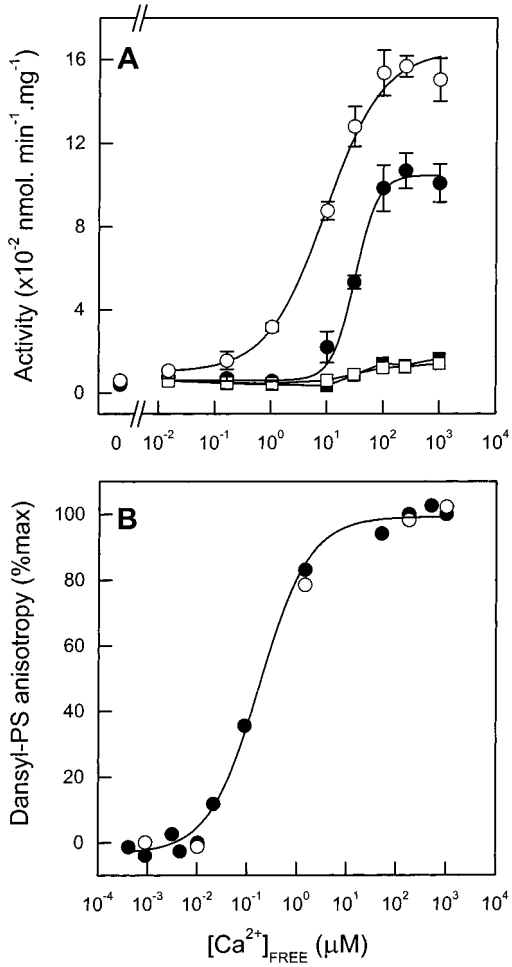


FIGURE 4: Effects of HAG on the Ca^{2+} concentration dependence of PKCα activation and membrane association induced by TPA. Panel A: The specific activity of PKCα was measured as a function of Ca^{2+} concentration in the presence of BPS/POPC LUV alone (■) or containing 4 mol % HAG (□), 0.3 mol % TPA (●), or 4 mol % HAG together with 0.3 mol % TPA (○). Data represent the mean of triplicate determinations of specific activities (±SD) derived from experiments carried out in triplicate. Panel B: The Ca^{2+} concentration dependence of the association of PKCα with BPS/POPC LUV, containing 0.3 mol % TPA alone (●) or with 4 mol % HAG (○), was determined from measurements of the accompanying increase in dansyl-PE anisotropy, as previously described (*21*). Solid curves represent fits of anisotropy data to a modified Hill equation (*21*). Data are means of triplicate determinations (±SD), from experiments each carried out at least twice. Values of SD for dansyl-PE anisotropy measurements were typically within ±1% of the mean. See Materials and Methods for further details.

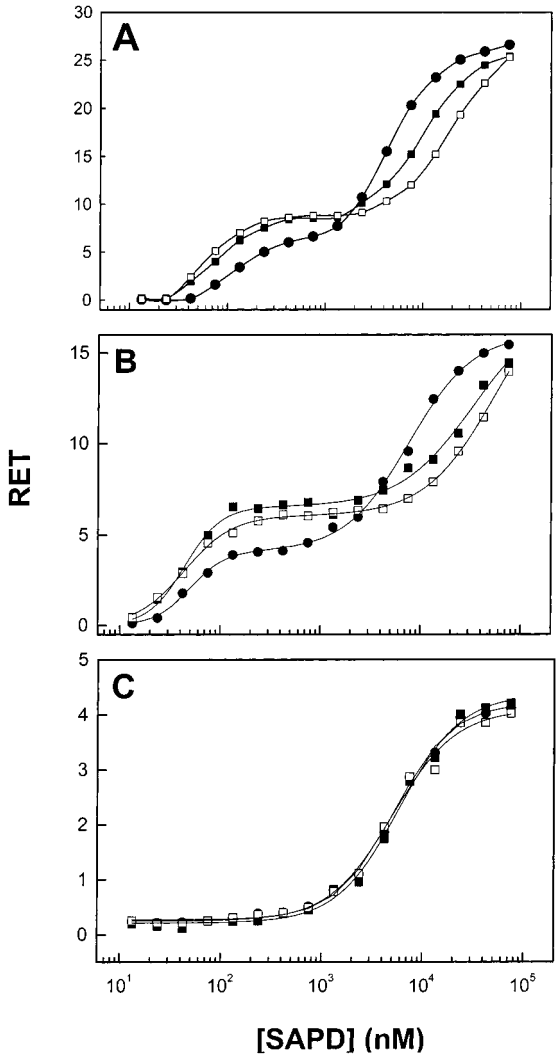


FIGURE 5: Effects of HAG and OAG on phorbol ester binding to membrane-associated PKCα, -βI, and -δ. Phorbol ester binding to PKCα (panel A), PKCβI (panel B), and PKCδ (panel C) associated with BPS/POPC LUV alone (●) or containing 4 mol % of either OAG (□) or HAG (■) was quantified in the presence of 0.1 mM Ca^{2+} based on resonance energy transfer (RET) from PKC tryptophans to the 2-(*N*-methylamino)benzoyl fluorophore of SAPD, according to a previously described method (*19*). Values of SD for fluorescence intensity measurements used in determinations of SAPD binding were typically within ±1% of the mean. Other details are as described under Materials and Methods.

portant example of such a 1-*O*-alkyl-2-acyl-*sn*-glycerol is HAG, which is a neutral ether lipid precursor in the de novo biosynthesis of platelet activating factor formed by the catalytic action of a specific phosphohydrolase (*42*, *43*). A number of biological properties of HAG have been described. For example, it stimulates Ca^{2+} influx into human bronchial epithelial HBE-16 cells (*44*), activates human neutrophil cytosolic phospholipase A_2 (*45*), accumulates during myocardial ischemia (*46*), and has effects on vascular smooth muscle cell proliferation (*47*). In HL-60 promyeloid leukemia cells, HAG was shown to attenuate stimulus−response coupling in tumor cells, thereby contributing to the mechanism by which the ether lipid 1-*O*-hexadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine exerts its anticancer effects (*48*). Therefore, apart from offering mechanistic insight into PKC regulation, the finding that HAG modulates PKC activity has significant physiological implications.

Whereas PKC is widely implicated in the effects of HAG, there is controversy in the literature concerning the nature of these effects on PKC activity. Thus, HAG has been described both as an inhibitor (*49*, *50*) and as an activator (*46*, *51*, *52*) of PKC, whereas other studies have suggested that PKC activity is unaffected by such 1-*O*-alkyl diglycerides (*53*). In the present study, we found that HAG has differing effects on PKC activity, depending on the activator and isozyme type. Thus, the presence of HAG alone in vesicles was found to have only negligible effects on the activities of each PKC isoform, whereas in the presence of either TPA or OAG, HAG *enhanced* conventional PKC isozyme activity but had no effect on PKCδ activity. The present results also provide a possible explanation for the

synergism that exists between phorbol ester and HAG effects on mast cell histamine release (*54*, *55*).

In previous work, using the fluorescent phorbol ester SAPD, we showed that the DAG 1,2-dioleoyl-*sn*-glycerol displaced low-affinity phorbol ester binding to PKCα, resulting in an increased level of high-affinity phorbol ester binding and an increased level of PKCα activity (*19*). It is shown here that, like DAG, HAG also competes for low-affinity SAPD binding to PKCα, while increasing high-affinity SAPD binding, along with the level of TPA-induced PKCα activity. Furthermore, the present results indicate that this mechanism is also applicable to PKC*β*I and possibly PKC*β*II and -*γ*. It would therefore appear that the affinity of HAG for binding to the low-affinity phorbol ester binding site on PKCα and -*β*I is in excess of that of either SAPD or TPA, but is not sufficient to cause the displacement of high-affinity phorbol ester binding. Furthermore, the observation that HAG not only potentiated TPA-induced PKCα and -*β*I activity but also OAG-induced activity, suggests that HAG and OAG do not interact equivalently with the low- and high-affinity phorbol ester binding sites. Providing evidence for this would require direct measurements of DAG binding which is experimentally not trivial. However, based on the observations of the present study, it can be speculated that the enhanced level of OAG-induced activity results from the displacement of OAG from the low-affinity site, leading to an enhanced level of DAG binding to the high-affinity phorbol ester binding site. In that case, the affinities for binding to the low-affinity phorbol ester binding site on PKCα and -*β*I would be in the order: HAG > OAG > phorbol ester, whereas the affinities for binding to the high-affinity phorbol ester binding site would be in the order: phorbol ester > OAG > HAG.

The comparisons of the effects of activator combinations on the activities of different PKC isozymes, shown in Figures 2 and 3, suggest that the observed interaction of OAG or HAG with the low-affinity phorbol ester binding site, leading to enhanced phorbol ester-induced activity, is a unique property of isozymes belonging to the cPKC group. Consistent with this is the observation that the low-affinity phorbol ester binding site present on PKCα and -*β*I may be absent in the structure of novel PKC*δ*, although this may also be due to differences in the binding properties of PKC*δ* compared to the cPKC isozymes for SAPD. In keeping with a single phorbol ester binding site is the observation that both HAG and OAG were unable to displace SAPD binding and failed to inhibit TPA-induced PKC*δ* activity under the assay conditions used. PKC*δ* activity induced by OAG was found to be inhibited by HAG, suggesting that the affinity of OAG for the phorbol ester binding site on PKC*δ* is lower than that of HAG, and again showing that these two compounds act in a nonequivalent manner.

The observation that the presence of HAG *alone* in vesicles was ineffective as an activator of PKCα, -*β*I, -*β*II, -*γ*, or -*δ* suggests that the *sn*-1 ester function of DAG is an absolute requirement for PKC activation, as was also concluded elsewhere (*56*). However, the finding that HAG competed for phorbol ester binding to the low-affinity phorbol ester binding site on PKCα and -*β*I suggests that this moiety is not required for the specific interaction with the low-affinity phorbol ester binding site on cPKC that leads to enhanced high-affinity phorbol ester binding. The interaction of HAG with the low-affinity site *alone* therefore appears to be sufficient neither to induce membrane association, as shown in Figure 4B, nor to induce the activating conformational change, both processes requiring occupation of the high-affinity phorbol ester binding site by either DAG or phorbol ester.

Previously, we showed that the Ca^{2+} concentration dependence of the activating conformational change in cPKC induced by DAG binding to the low-affinity phorbol ester binding site, in combination with high-affinity phorbol ester binding, was decreased relative to those for the activating conformational changes induced by interaction with either activator separately (*21*). The observation that the presence of HAG resulted in a decrease in the Ca^{2+} concentration dependence of TPA-induced PKCα activity while not affecting that for TPA-induced membrane association (see Figure 4) suggests that, similar to DAG, the interaction of HAG with the low-affinity phorbol ester binding site also results in a decrease in the Ca^{2+} dependence for the activating conformational change.

The present results suggest that HAG may have a unique modulatory role in PKC-mediated cellular events and suggest that the ensuing effects on PKC activity would differ according to the type of activator present (e.g., phorbol ester as compared to DAG) and also according to PKC isozyme type, although extrapolating an effect of HAG on PKC activity observed in vitro to one on a PKC-mediated cellular process requires caution. Apart from HAG, long-chain *n*-alkanols and general anesthetics also increase phorbol ester-induced activity while alone not being PKC activators (*20*, *57*). This suggests that each of these compounds may belong to a class of broadly similar PKC modulators. Finally, the observation that HAG *alone* failed to activate PKC isozymes while competing with phorbol ester for the low-affinity binding site, enhancing high-affinity phorbol ester binding and potentiating the level of activity, suggests that this compound may be useful tool for further investigation of the role of the low-affinity phorbol ester binding site on PKC in PKC-mediated cellular events.

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