0026-895X/90/020286-10$02.00/0

MOLECULAR PHARMACOLOGY, 37:286-295

Requirements for the Activation of Protein Kinase C: Comparison of the Molecular Geometries of Phorbol and Diacylglycerol

UBALDO LELI, GEORGE HAUSER, and MARK FROIMOWITZ

Department of Psychiatry, Harvard Medical School, and Ralph Lowell Laboratories (U.L., G.H.) and Alcohol and Drug Abuse Research Center (M.F.), McLean Hospital, Belmont, Massachusetts 02178

Received June 5, 1989; Accepted October 15, 1989

SUMMARY

MM2 calculations have been performed on a number of derivatives of phorbol and diacylglycerol (DAG) to establish the molecular features required for the activation of protein kinase C by a detailed comparison of the molecular geometries in these two classes of compounds. For DAG, a dihedral angle of about −60° appears to be required for the oxygens at C2 and C3 because that angle is fixed at this value in phorbols. There is good agreement between the computed Boltzmann distribution for the O1-C1-C2-O2 dihedral angle and NMR results for the same angle in phospholipids, as obtained by others. A conformer of DAG is identified with dihedral angles corresponding to those of β-phorbols. This conformer, however, is 3.2 kcal/mol above the global minimum found for DAG. The molecular geometry of this conformer is consistent with that of a number of active and inactive rigid analogues of DAG. The preferred conformation in β-phorbol diesters is found to be stabilized by an antiparallel stacking of the ester carbonyl groups. The lack of activity of α-phorbol esters appears to be due to differences in a portion of the molecule containing the five-membered/seven-membered rings, which are far from the DAG-like end of the phorbol molecule. It is proposed that some of the biological activities of phorbol diesters may be due to this portion of the β-phorbol molecule, which might represent a second active region, distinct from that resembling DAG.

Phorbol diesters, obtained from the seeds of the Euphorbia plant *Croton tiglium*, are polyfunctional derivatives of the tetracyclic hydrocarbon tigliane that have long been known for their biological activities as potent tumor promoters and as irritant and inflammatory agents (reviewed in Ref. 1). These effects are mediated by a major cell receptor (2) now demonstrated to be protein kinase C (3), which is a calcium- and phospholipid-dependent enzyme (4). The activity of this enzyme can be stimulated by DAG (5), generated by the hydrolysis of inositol phospholipids during the chain of events that follows agonist binding to receptors that are linked to intracellular calcium mobilization (reviewed in Ref. 6). Nishizuka (7) has proposed that phorbol diesters can substitute for DAG in activating this enzyme because molecular similarities exist between the two classes of compounds. Other authors have provided support for this hypothesis with competition binding studies, investigation of the mechanism of the direct activation of the enzyme by these two classes of compounds, and demonstration of similar biological effects caused by these molecules, including tumor promotion (8–15). However, modification of DAG, the phorbol molecule, or analogs synthesized on the basis of the pharmacophores predicted from molecular modeling have often yielded compounds devoid of most or all biological activity (16–19).

We report in this paper the results of a detailed quantitative study of the internal conformational flexibility of phorbol esters and the relationship of their preferred geometries to the preferred geometries of DAG. Phorbol esters are usually 3–4 orders of magnitude more potent ligands than DAG (13). This, and a reported heterogeneity of the receptor for phorbol esters (20, 21) has prompted us to compare the fine differences in the molecular structure of phorbol esters with DAG and to try to determine the geometric requirements of a pharmacophore that acts as a protein kinase C activator. For this purpose, we used a computational approach to determine the molecular geometries and steric energies of 1,2-diacetyl-*sn*-glycerol and 12,13-diacetyl-β-phorbol (Fig. 1), model compounds for DAG and phorbol diesters, using the molecular mechanics program MM2 (22). These model compounds differ from the more potent phorbol esters and DAG only in the length of the fatty chains at C12 and C13 but they conserve the ester bonds essential for biological activity (16, 17, 23, 24).

We also performed calculations on other compounds structurally related to DAG and phorbols to investigate other aspects of the molecular geometries of these compounds. Specifically, we analyzed 12,13-dideoxy-α- and -β-phorbols to elucidate the effects exerted on the overall geometry of the molecule by the

This work was supported by Grants NS06399 and NS19037 (G.H.) from the National Institutes of Health.

ABBREVIATION: DAG, diacylglycerol.

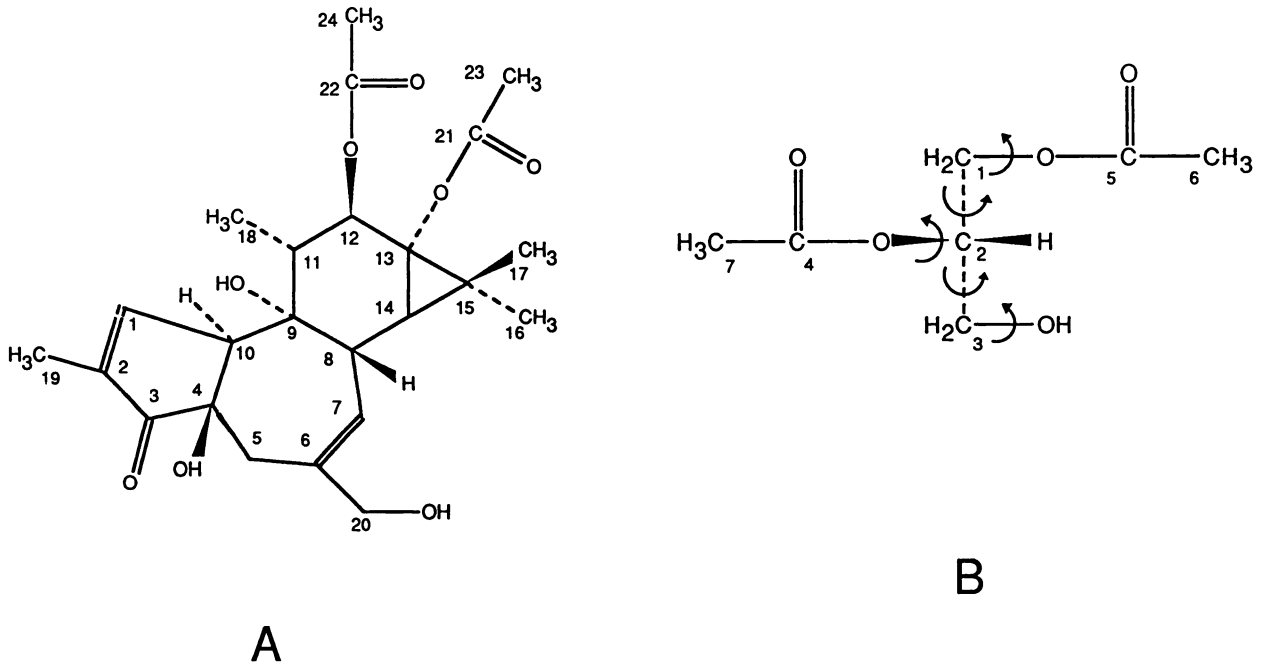


Fig. 1. Structures of 12,13-diacetyl-β-phorbol (A) and 1,2-diacetyl-*sn*-glycerol (B). The *arrows* indicate the bonds that were rotated in order to generate the conformers analyzed in this report.

stereochemistry of the hydroxyl group at C4. We also studied 12,13-dibutyryl-β-phorbol, a specific high affinity ligand for the phorbol receptor, to determine the effects of longer side chains on the geometry of the phorbol molecule; 1,2-diacetyl 3-methyl-*sn*-glycerol (Fig. 2), because one stereoisomer is completely inactive as a protein kinase C activator whereas the other still retains some residual activity (18); and the DAG analog 1,2-diacetyl-3-hydroxycyclohexane, which has the same geometry of the ester groups as phorbol diesters, but when synthesized with six carbon fatty acid residues, does not activate protein kinase C (17) (Fig. 3). Some of these results have been published in preliminary form (25).

Methods

All calculations were performed with the MM2-85 program and parameter set (22). Some stretching, bending, and torsional parameters for the ester groups adjacent to the cyclopropane ring were not available. The most notable of these are the torsional parameters for atom types 22-22-1-6 and 22-22-6-3. The missing parameters were approximated by substituting an sp^3 carbon atom (type 1) for the cyclopropane atom (type 22). This did not cause changes in the geometry of the cyclopropane and six-carbon atom ring area of the 12,13-diacetyl-β-phorbol molecule, as compared with the 12,13-dideoxy-β- and -α-phorbols for which all parameters were available. Energy minimizations were performed with respect to all internal coordinates. Where rotational barriers were computed, the dihedral angles were increased by 10° increments and the energies were minimized with respect to all other internal coordinates. The value of the dielectric constant used in all calculations was 1.5 D.

The published crystal coordinates of phorbol bromofuroate-chloroform solvate (26) were used to generate the initial geometries for the energy minimizations. The initial geometry of the phorbol moiety was used in the calculations on 12,13-dibutyryl-β-phorbol, with the acyl residues in the preferred all-*trans* conformation. The initial coordinates of all other compounds were generated with a previously described program (27). For each different rotational conformer, new sets of coordinates were generated and its energy was minimized.

Results

Phorbol Derivatives

12,13-Dideoxy-α- and -β-phorbol. Initial calculations were performed on 12,13-dideoxy-α- and -β-phorbols to determine the conformational properties of the multicyclic structure. For the β-compound (Fig. 4A), a boat conformation of the six-membered ring was found to be preferred by 0.9 kcal/mol over the chair conformation. The absence of the ester groups in positions 12 and 13 did not cause changes in the rest of the molecule compared with 12,13-diacetyl-β-phorbol (Table 1). There was also good agreement between the computed structure for β-phorbol and its X-ray crystallographic structure (Table 1). The preferred orientations of the hydroxyl hydrogens at C4 and C9 were determined by rotating the hydroxyl groups. Only a single minimum was observed for the hydroxyl at C4, with HO4-O4-C4-C10 in the vicinity of 160–170° (Fig. 5A), probably owing to steric factors. The more usual three energy minima were observed for the hydroxyl at C9 (Fig. 5B). A dihedral angle of −70° for HO9-O9-C9-C8 was found to be preferred by 0.5–0.7 kcal/mol.

α-Phorbol and its derivatives are biologically inactive (1). These differ from the β-isomer derivatives in that the hydroxyl at C4 is below the plane of the molecule rather than above. In order to gain quantitative information on the changes induced in the molecule by this difference, the energy of the structure of 12,13-dideoxy-α-phorbol was minimized (Fig. 4B). Although this compound also prefers the boat conformation for the six-membered ring like 12,13-dideoxy-β-phorbol, dramatic differences were observed in the overall geometry of the two molecules (Table 1). Interestingly, the major structural difference between α- and β-phorbol occurs in a region of the molecule that is absent in DAG, namely the five-membered ring, which is shifted above the plane of the molecule in α-phorbol (Fig. 4; Table 1). No significant difference in the geometry of the two

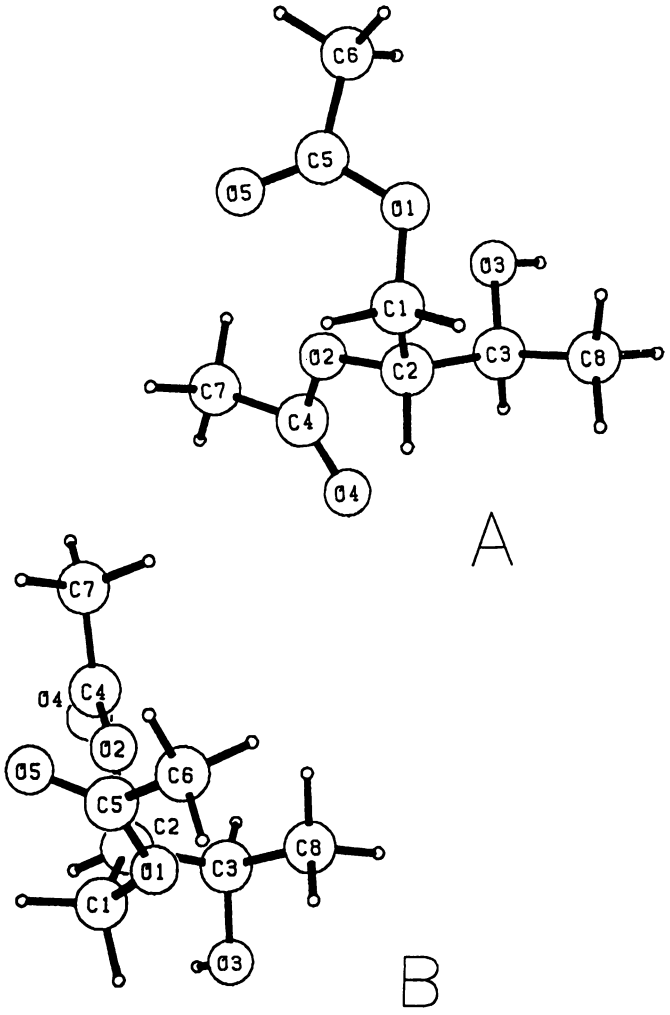


Fig. 2. 1,2-Diacetyl 3-methyl-*sn*-glycerol epimers. A, (2*R*)-acetyl-(3*S*)-hydroxylbutyl acetate; B, (2*R*)-acetyl-(3*R*)-hydroxylbutyl acetate. The drawings show the lowest energy conformers.

molecules was observed in the region that bears the diester bonds in 12,13-diacetyl-β-phorbol (Fig. 4; Table 2).

12,13-Diacetyl- and -dibutyryl-β-phorbols. As in 12,13-dideoxy-β-phorbol, the six-membered ring can assume a chair or a boat conformation when carbons 12 and 13 are substituted. However, in contrast to dideoxyphorbol, the chair conformation is favored by about 3.4 kcal/mol in the diacetyl compound. The rotation of the acyl residues around the C12-O12 and C13-O13 bonds can theoretically generate nine conformers each for the boat and the chair conformations, but only three for the former (steric energy = 61.4, 66.2, and 67.1 kcal/mol) and five for the latter (steric energy = 58.0, 60.9, 63.3, 63.7, and 65.3 kcal/mol) were found to be stable. The most favored conformer is shown in Fig. 6A. The cartesian coordinates of this conformer are reported in Table 3, and some dihedral angles relevant for the rings are reported in Table 1. The two acyl residues are in an *anti* conformation (C13-C12-O12-C22 = 159° and C14-C13-O13-C21 = 146°). This conformation is stabilized by an antiparallel stacking of the carbonyl dipoles, also present in the 12,13-dibutyryl-derivative (Fig. 7). In contrast to DAG, the bond between C12 and C13 cannot rotate freely in 12,13-diacetyl-β-phorbol because it is part of the six-membered ring, with a dihedral angle of −81° for O13-C13-C12-O12; this applies to all phorbol derivatives.

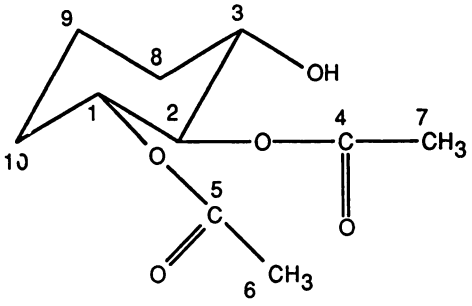
The introduction of the longer chain residues did not modify significantly the geometry either of the ring system or of the ester bonds (Tables 1 and 2) (O13-C13-C12-O12 = −93° in 12,13-dibutyryl-β-phorbol). It may be of interest that the fatty acid chains appear to be diverging in this conformer.

DAG and Derivatives

1,2-Diacetyl-*sn*-glycerol. 1,2-Diacetyl-*sn*-glycerol (Fig. 1B) was used as a model compound for DAG, because the interactions between the ester groups and the free hydroxyl at C3 are responsible for the conformation of DAG molecules. This molecule has considerable internal flexibility because most of its bonds can rotate freely. There are seven conformationally relevant dihedral angles (Fig. 1B), of which C6-C5-O1-C1 and C7-C4-O2-C2 have a strong preference for the *anti* conformation. A value of 180° was set for these two dihedral angles at the beginning of the calculations and remained substantially unchanged after energy minimization.

A total of 243 conformers can theoretically be generated by rotating the remaining five angles. However, when their energies were minimized, only 209 were found to be stable. The lowest energy conformer (10.9 kcal/mol) has both the esterified residues and the free hydroxyl in a *gauche* conformation with respect to the main chain (O1-C1-C2-O2 = 65° and C1-C2-C3-O3 = −58°, respectively). The cartesian coordinates of this compound are reported in Table 4 and the compound is pictured in Fig. 6B. However, the molecular geometry of this particular conformer is inconsistent with the geometry of the DAG-like structure present in phorbol diesters (Tables 5 and 6). A conformer of 1,2-diacetyl-*sn*-glycerol that closely matches the geometry of this structure exists, and its cartesian coordinates and some relevant geometrical data for this molecule are reported in Tables 4, 5, and 6. As shown in Fig. 6C, it can be superimposed nearly perfectly on the relevant area of 12,13-diacetyl-β-phorbol. It is 3.1 kcal/mol higher in energy than the global minimum and its Boltzmann population is 0.002 at 25°. The difference between O13-C13-C12-O12 in 12,13-diacetyl-β-phorbol and O1-C1-C2-O2 of this conformer in 1,2-diacetyl-*sn*-glycerol is about 24° (Table 6), and the distances and orientations of O3 from O1 and O2 closely match the distances and orientations of O9 from O12 and O13 of 12,13-diacetyl-β-phorbol (Table 5).

1,2-Diacetyl 3-methyl-*sn*-glycerol. The free hydroxyl group at C3 of DAG is essential for the activation of protein kinase C (16, 17, 23), and many DAG analogs modified in this region have been synthesized in order to elucidate the geometric requirements and optimum conformation of this group (16, 17). In order to establish the best orientation of this group with respect to the rest of the molecule, we performed calculations on 1,2-diacetyl 3-methyl-*sn*-glycerol (Fig. 2). The presence of a methyl group at C3 adds an asymmetric carbon to 1,2-diacetyl-*sn*-glycerol and generates two stereoisomers, (2*R*)-acetyl-(3*S*)- and -(3*R*)-hydroxylbutyl acetate (Fig. 2). These compounds have been recently synthesized with six-carbon atom side chains (18). (2*R*)-Hexanoyl-(3*R*)-hydroxylbutyl hexanoate was unable to activate protein kinase C, but its 3*S*-isomer retained some activity, albeit greatly reduced compared with the corresponding nonmethylated DAG (18). Owing to the rigid nature of the phorbol molecule, the hydroxyl group at C9 cannot change position as in DAG, where the hydroxyl group at C3 is



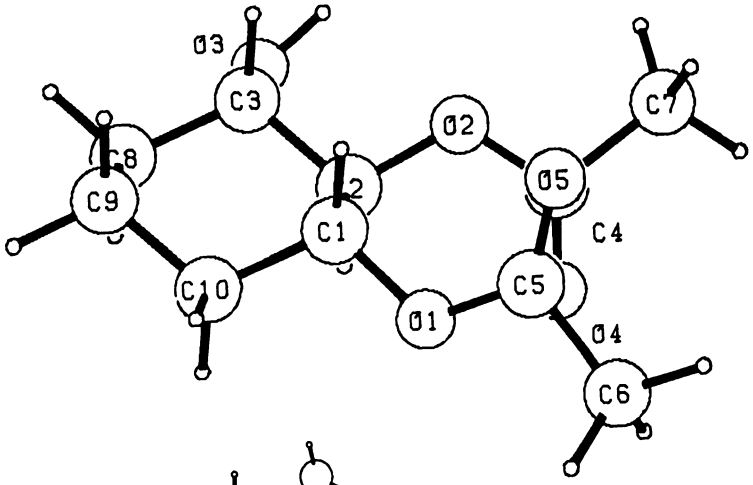


Fig. 3. Structure of 1,2-diacetyl 3-hydroxylcyclohexane and drawing of its lowest energy conformer.

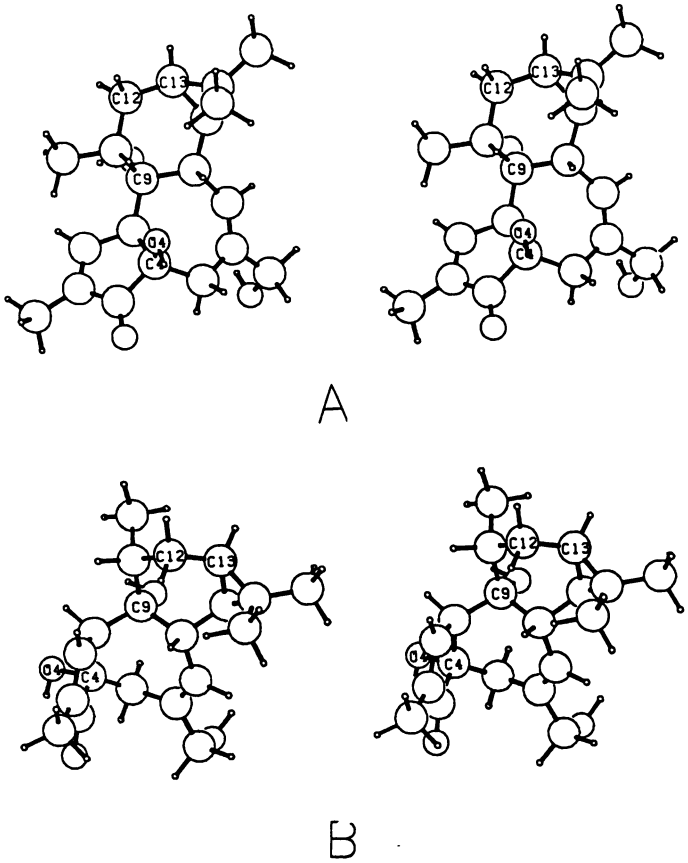


Fig. 4. Stereo projections of 12,13-dideoxy-β- (A) and -α-phorbol (B) conformers of lowest energy.

attached to a carbon atom that can easily rotate around the bond with C2. As a consequence, any modification of the DAG molecule that prevents O3-C3-C2-C1 from assuming a *gauche* conformation with a value around 60° (the geometry of 1,2-diacetyl-*sn*-glycerol that best matches phorbol) (Tables 5 and 6) should hinder its effectiveness as a protein kinase C activator. When we examined the effects of the introduction of the methyl group at C3 of 1,2-diacetyl-*sn*-glycerol, we noticed that the energy preference of the dihedral angle O3-C3-C2-C1 was considerably different in the two isomers. For (2*R*)-acetyl-(3*S*)-hydroxylbutyl acetate, a value of 60° was preferred by 1.0 and 1.7 kcal/mol, compared with 180° and −60°, respectively. For the 3*R*-isomer, a value of −60° was preferred by 1.6 and 5.0 kcal/mol, compared with 180° and 60°, respectively (Fig. 8). Thus, the introduction of the methyl group hinders the hydroxyl group at C3 from assuming the correct position in the 3*R*-isomer, whereas it does not do so in the 3*S*-isomer which retains some residual activity.

1,2-Diacetyl 3-hydroxycyclohexane. According to the results described above, the two esterified residues in DAG must be in a *gauche* conformation with a dihedral angle of about −60° in order for the compound to be similar to the phorbol molecule (Fig. 6, A and C). A series of cyclic analogs of DAG have recently been synthesized by adding one hydroxyl and two acyl residues at different positions to cyclohexane or (*trans*)-bicyclo[4.4.0]decane (17). Only two of these analogs had the acyl residues in the *anti* conformation with a −60° dihedral angle, one derived from cyclohexane and one from (*trans*)-bicyclo[4.4.0]decane (17); however, both were inactive as protein kinase C activators, as were all the other cyclic analogs mentioned in this report (17).

We, therefore, performed calculations on 1,2-diacetyl 3-hydroxycyclohexane in order to measure the distance of the hydroxyl group with respect to the rest of the molecule and we compared it with 12,13-diacetyl-β-phorbol and the conformer of 1,2-diacetyl-*sn*-glycerol with similar geometry (Fig. 6C).

The lowest energy conformer of 1,2-diacetyl 3-hydroxyl-cy-

TABLE 1
Geometry of the ring system of some phorbol derivatives

	Dihedral angle				
	12,13-Diacetyl-β-phorbol	12,13-Dideoxy-		12,13-Dibutyryl-β-phorbol	β-phorbol[a]
		β-phorbol	α-phorbol		
C4-C5-C6-C7	21°	22°	−47°	21°	28°
C5-C6-C7-C8	4°	2°	−1°	4°	−1°
C6-C7-C8-C9	−61°	−65°	−7°	−62°	−77°
C7-C8-C9-C10	34°	43°	59°	35°	56°
C8-C9-C10-C4	49°	41°	−64°	48°	43°
C9-C10-C4-C5	−92°	−89°	−3°	−91°	−87°
C10-C4-C5-C6	31°	33°	64°	31°	26°
C8-C9-C11-C12	64°	11°	2°	62°	73°
C9-C11-C12-C13	−34°	46°	52°	−34°	−42°
C11-C12-C13-C14	6°	−53°	−52°	8°	12°
C12-C13-C14-C8	−9°	2°	−2°	−12°	−7°
C13-C14-C8-C9	40°	55°	56°	42°	34°
C14-C8-C9-C11	−67°	−61°	−54°	−66°	−68°
C1-C2-C3-C4	5°	7°	−1°	5°	8°
C2-C3-C4-C10	−11°	−15°	3°	−11°	−13°
C3-C4-C10-C1	12°	17°	−4°	12°	−12°
C4-C10-C1-C2	−11°	−15°	4°	−11°	−8°
C10-C1-C2-C3	4°	6°	−1°	4°	0°
C11-C12-C13-C15	−68°	−121°	−119°	−65°	−61°
C11-C9-C8-C7	164°	170°	166°	165°	178°
C10-C9-C8-C14	164°	171°	170°	165°	169°
C12-C13-C15-C14	106°	100°	101°	105°	103°
C13-C15-C14-C8	−106°	−98°	−98°	−106°	−107°
C9-C8-C14-C15	109°	123°	123°	109°	104°
C7-C8-C14-C15	−119°	−109°	−104°	−119°	−128°
C1-C10-C9-C8	174°	164°	48°	174°	171°
C1-C10-C9-C11	52°	39°	−75°	52°	53°
C2-C1-C10-C9	−144°	−146°	−139°	−144°	−145°
C3-C4-C5-C6	147°	149°	−63°	147°	147°
C2-C3-C4-C5	−132°	−137°	117°	−132°	−138°
C2-C3-C4-04	107°	102°	−125°	107°	95°
04-C4-C5-C6	−94°	−92°	179°	−94°	−93°

[a] X-ray crystallography (23).

clohexane (16.5 kcal/mol and Boltzmann population of 0.6) is shown in Fig. 3. The dihedral angle between the two esters at O1-C1-C2-O2 is −65° and differs from the corresponding angles in 1,2-diacetyl-*sn*-glycerol and 12,13-diacetyl-β-phorbol by 8° and 32°, respectively (Table 6). In this compound, the region of the molecule that contains the ester bonds is similar to the DAG-like structure of phorbol; however, the distance of the oxygen of the free hydroxyl group from O1 and O2 differs significantly from the corresponding distances in 1,2-diacetyl-*sn*-glycerol by 1.2 and 0.9 Å, respectively, and from the distance between O9 and O12 and O13 of 12,13-diacetyl-β-phorbol by 0.6 and 0.7 Å, respectively (Table 5).

Discussion

The results for 1,2-diacetyl-*sn*-glycerol and 12,13-diacetyl-β-phorbol, which were used as model compounds for DAG and 12,13-diesters of β-phorbol, confirm unequivocally that similarity can exist between DAG and phorbol molecules in the region of the ester bonds, as proposed previously (7). However, the conformer of 1,2-diacetyl-*sn*-glycerol that has a molecular geometry similar to the ester region of β-phorbol is 3.2 kcal/mol above the lowest energy conformer and has the ester residues in a *gauche* conformation at about −60° (Fig. 6). This implies that substantial energy must be put into DAG for it to achieve the correct three-dimensional structure for activation of protein kinase C. This would be consistent with the much lower potency (3–4 orders of magnitude) of DAG compared with phorbol esters.

In the energetically preferred conformation of phorbol esters, the carbonyl dipoles of the ester bonds assume antiparallel stacking, as in 12,13-dibutyryl-β-phorbol (Fig. 7). The reason that this conformer is preferred may be that the calculations were performed on an isolated molecule where there is nothing else for the dipoles to interact with. In the receptor environment, these dipoles could interact with complementary sites that might favor other conformations. Nevertheless, the antiparallel stacking of the dipoles may have some relevance for biological activity. The substitution of the ester with other groups causes loss of activity in DAG and phorbol derivatives (17, 23, 24).

The preferred dihedral angle of 60° for O1-C1-C2-O2 in 1,2-diacetyl-*sn*-glycerol is consistent with NMR studies of phospholipids (28) that contain the DAG moiety but in which the hydroxyl at C3 has been esterified with a phosphate-containing charged group. These studies showed that 60% of the molecules have values of the dihedral angle O1-C1-C2-O2 in the vicinity

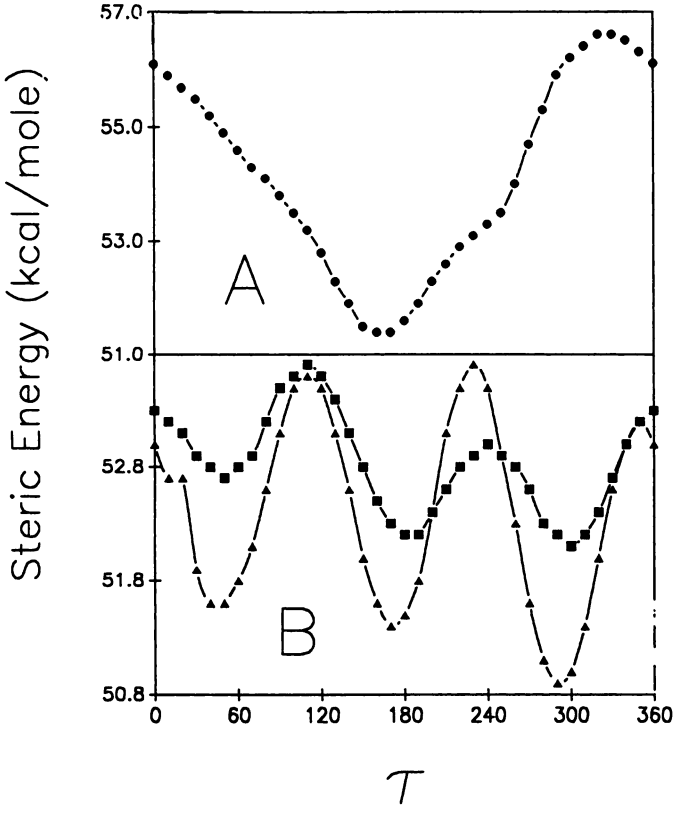


Fig. 5. Rotational barriers of the hydroxyls at C4 (A) and C9 (B) of 12,13-dideoxy-β-phorbol. The six-membered ring is in the chair (▲) or in the boat (■) conformation. A, τ = HO4-O4-C4-C10 (°); B, τ = HO9-O9-C9-C8 (°).

TABLE 2

Some dihedral angles and atomic distances of 12,13-dideoxy-α and -β-phorbols

	Dihedral angle	
	α-Isomer	β-Isomer
H12-C12-C13-H13	−38°	−40°
C18-C11-C12-H12	32°	39°
C18-C11-C9-C10	−88°	−98°
C15-C13-C12-H12	117°	115°
C11-C12-C13-H13	86°	84°
C8-C14-C13-H13	−132°	−133°
	Distance	
	α-Isomer	β-Isomer
	Å	
09-C12	3.33	3.41
09-C13	3.17	3.30
09-H12	4.10	4.24
09-H13	3.35	3.52
09-C18	2.60	2.72

of 60°, that 30% have values in the vicinity of −60°, and that the *anti* conformation is nearly absent, in good agreement with our computed population of rotamers, which have a distribution of 63%, 26%, and 10% for the same dihedral angle. Solvent effects do not appear to affect the conformational equilibrium, because it has been previously shown by NMR studies that the populations of rotamers of the glycerol backbone of phospholipids did not change when the compounds are dissolved either in water or in methanol, both below and above their critical micellar concentrations (29). The shorter acyl chains of our

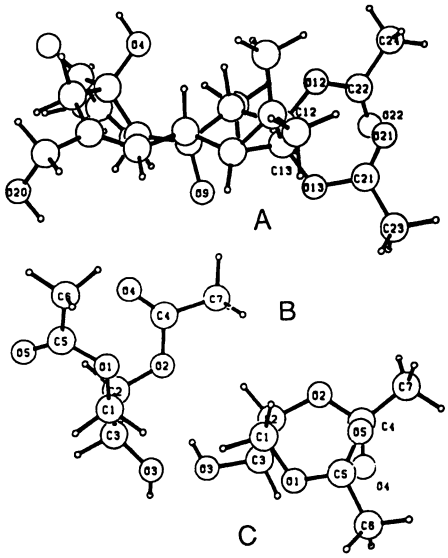


Fig. 6. Comparison of 12,13-diacetyl-β-phorbol (conformer of lowest steric energy) (A) and two conformers of 1,2-diacetyl-*sn*-glycerol. B is the lowest energy conformer; C is the conformer with geometry similar to 1,2-diacetyl-β-phorbol.

model compounds, compared with the DAG and phorbol diesters of higher potency, should also not affect the validity of this result, because the only effect of the chain length is to change the potency of protein kinase C activators (23). Further, NMR analysis has shown that there is no difference in the proportion of rotamers of the glycerol moiety of phospholipids with a *gauche* conformation of O1-C1-C2-O2 (with a value of either 60° or −60°) in diacyl-versus lysocompounds, which lack the fatty acid chain at C2 (28). The fatty acid chains might, therefore, be an anchoring domain immersed in the bilayer, which may position the phorbol and DAG molecules for the binding to the protein. This view is also supported by experiments performed with radioactive phorbol esters containing a photolabile group at the distal end of the esters, in which radioactivity was found to be associated only with lipids (30).

We also investigated the lack of activity of some DAG analogs by determining the position of the free hydroxyl in (2*R*)-acetyl-(3*R*)- and -(3*S*)-hydroxylbutyl acetate and 1,2-diacetyl 3-hydroxycyclohexane, because the free hydroxyl of DAG is essential for the stimulatory activity on protein kinase C (16, 17). The fact that only one of the 3-methyl derivatives of DAG retained some activity (18) suggests that the position of the hydroxyl in space with respect to the two ester bonds has a crucial role in the interaction of the compounds with the enzyme. Thus, our calculations on 1,2-diacetyl 3-methylglycerol showed that the hydroxyl cannot occupy a position in (2*R*)-acetyl-(3*R*)-hydroxybutylacetate (Fig. 2B) (inactive isomer) similar to that proposed for the biologically active conformer of DAG but can do so in the 3*S*-isomer (Fig. 2A), which retains some residual activity. Thus, these experimental results are consistent with what we have identified as the relevant conformation of DAG. We propose, therefore, that the free hydroxyl of DAG corresponds to the hydroxyl at C9 of the phorbol molecule and that its position is crucial. This is further confirmed by the cyclic DAG analog 1,2-diacetyl 3-hydroxylcyclohexane, pictured in Fig. 3, in which the atomic distances between the free hydroxyl and the other relevant atoms are different from the corresponding ones in DAG (see Tables 5 and 6). This

TABLE 3
Computed cartesian coordinates of 12,13-diacetyl-β-phorbol

Atom	x	y	z	Atom	x	y	z
H020	−0.771	0.747	−0.828	020	0.044	0.303	−0.661
LP20	0.216	0.159	−1.218	LP20	−0.106	−0.044	−0.194
C20	0.982	1.262	−0.171	H20	1.120	2.028	−0.968
H20	1.961	0.752	−0.027	C6	0.496	1.868	1.132
C7	−0.056	3.097	1.124	H7	−0.132	3.592	0.143
C8	−0.517	3.935	2.299	H8	0.407	3.969	2.910
C14	−0.842	5.374	1.889	H14	−1.241	5.455	0.880
C15	−0.078	6.541	2.506	C16	1.026	6.338	3.534
H16	1.962	6.009	3.028	H16	0.791	5.606	4.335
H16	1.230	7.305	4.048	C17	0.249	7.655	1.515
H17	−0.526	7.801	0.729	H17	1.200	7.412	0.989
H17	0.388	8.622	2.048	C13	−1.501	6.255	2.927
013	−2.463	7.104	2.318	C12	−2.006	5.635	4.224
H12	−3.118	5.675	4.176	012	−1.534	6.420	5.311
C11	−1.674	4.147	4.473	H11	−0.654	4.107	4.915
C18	−2.598	3.597	5.576	H18	−3.645	3.481	5.218
H18	−2.629	4.275	6.460	H18	−2.239	2.621	5.972
C9	−1.666	3.346	3.148	09	−2.895	3.613	2.454
LP9	−2.898	3.258	1.971	LP9	−2.997	4.201	2.504
HO9	−3.617	3.264	2.948	C10	−1.529	1.820	3.212
H10	−1.803	1.432	2.201	C1	−2.450	1.014	4.080
H1	−3.533	1.212	4.143	C2	−1.845	−0.035	4.650
C19	−2.531	−1.085	5.474	H19	−3.621	−0.881	5.578
H19	−2.091	−1.119	6.497	H19	−2.412	−2.086	5.002
C3	−0.523	−0.028	4.358	03	0.287	−0.859	4.694
C4	−0.158	1.237	3.570	04	0.563	2.079	4.473
LP4	0.209	2.122	4.955	LP4	0.826	2.497	4.135
H04	1.309	1.615	4.817	C5	0.690	0.958	2.331
H5	0.411	−0.064	1.987	H5	1.773	0.918	2.589
LP1	−2.993	6.894	2.500	LP1	−2.275	7.200	1.759
C21	−2.527	8.385	2.782	021	−1.821	8.850	3.636
C23	−3.624	9.144	2.064	H23	−4.610	8.654	2.234
H23	−3.417	9.181	0.970	H23	−3.687	10.190	2.441
LP1	−1.186	6.838	5.062	LP1	−1.387	6.024	5.733
C22	−2.415	7.188	6.007	022	−3.601	7.236	5.816
C24	−1.680	7.989	7.062	H24	−0.931	8.663	6.587
H24	−1.158	7.307	7.773	H24	−2.392	8.618	7.644

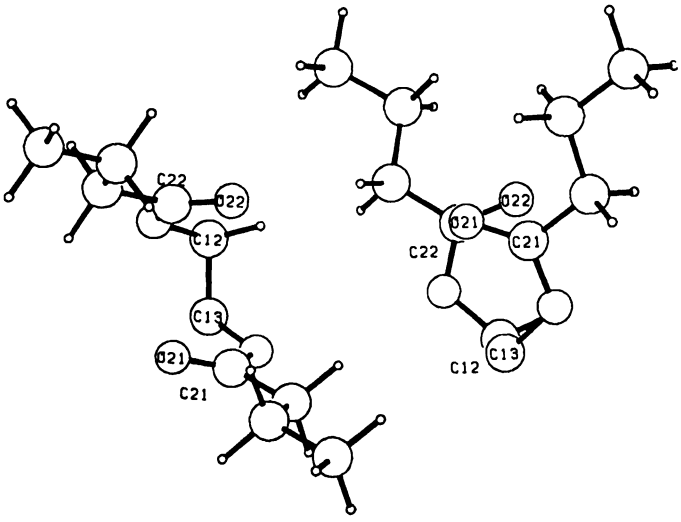


Fig. 7. Two projections of a fragment of the 12,13-dibutyryl-β-phorbol molecule containing the carbonyl groups of the ester bonds, showing the antiparallel stacking of the dipoles at C21-O21 and C22-O22. The phorbol ring system has been omitted for clarity.

compound, synthesized with six carbon atom fatty acid residues, was found to be inactive as a protein kinase C activator (18), as was the similar derivative of (*trans*)-bicyclo[4.4.0] decane (18). Furthermore, addition of one carbon atom to the three-carbon skeleton of DAG decreases its potency, but the compound obtained by the addition of three carbons is completely inactive (16), probably because the hydroxyl in this analog is much farther removed from the ester groups, owing to the longer carbon atom chain.

In view of these results, we suggest that the relative positions of the free hydroxyl and the C1-esterified residue in DAG are critical for its activity and correspond to the free hydroxyl at C9 and the C13 ester group of phorbol, respectively. In addition, the ester bond in position 13 of phorbol seems to be more important than the one at C12, as can be seen from structure-activity studies on phorbol esters as cocarcinogens in mouse skin (1), where 12-deoxy-13-tetradecanoyl-β-phorbol was a potent irritant but the 12-tetradecanoyl-13-deoxy-β-phorbol lacked any activity (1). Similarly, 1-acyl-*sn*-glycerol conserves some residual stimulatory activity on protein kinase C, when compared with the 2-acyl derivative, albeit with greatly reduced potency compared with DAG (16). In addition, the substitution of the ester with an amide group in position 1 causes total loss of activity in DAG, but the substitution in position 2 only decreases potency (16). Therefore, the atomic distances and geometry that we obtained by analyzing 12,13-dibutyryl-β-phorbol and the corresponding 1,2-diacetyl-*sn*-glycerol conformer (Fig. 6, A and C, and Tables 5 and 6) represent a starting point that can be used to design analogs of DAG with higher potency, in which the dihedral angles of the esterified fatty

TABLE 4

Computed cartesian coordinates of 1,2-diacetyl-*sn*-phorbol

Atom	x	y	z	Atom	x	y	z
Lowest energy conformer							
H03	−0.013	0.011	−0.296	03	0.921	−0.024	−0.167
LP3	1.106	−0.277	−0.679	LP3	0.958	−0.218	0.400
C3	1.437	1.308	−0.168	H3	0.958	1.863	0.672
H3	1.139	1.793	−1.126	C2	2.958	1.277	−0.013
H2	3.293	2.341	−0.007	02	3.509	0.599	−1.134
LP2	3.145	0.735	−1.590	LP2	3.670	0.062	−0.926
C4	4.693	1.037	−1.641	04	5.330	1.968	−1.226
C7	5.108	0.170	−2.813	H7	4.364	0.254	−3.639
H7	5.189	−0.896	−2.501	H7	6.099	0.491	−3.206
C1	3.373	0.575	1.283	H1	3.118	−0.509	1.233
H1	2.837	0.994	2.167	01	4.772	0.660	1.492
LP1	5.008	0.637	0.943	LP1	4.884	0.303	1.962
C5	5.261	1.843	1.954	05	4.592	2.798	2.247
C6	6.773	1.786	2.042	H6	7.214	1.530	1.052
H6	7.087	1.022	2.789	H6	7.183	2.771	2.359
Conformer with geometry similar to 12,13-diacetyl-β-phorbol							
H03	−0.861	1.329	−0.197	03	−0.055	0.849	−0.093
LP3	−0.062	0.508	−0.588	LP3	−0.085	0.702	0.488
C3	1.047	1.755	−0.221	H3	0.800	2.463	−1.046
H3	1.925	1.139	−0.518	C2	1.263	2.492	1.100
H2	0.252	2.838	1.430	02	2.009	3.696	0.987
LP2	2.252	3.771	1.529	LP2	1.644	4.067	0.688
C4	3.111	3.781	0.197	04	3.583	2.889	−0.455
C7	3.691	5.180	0.267	H7	3.969	5.433	1.316
H7	2.953	5.926	−0.106	H7	4.608	5.256	−0.360
C1	1.803	1.610	2.231	H1	1.083	0.781	2.426
H1	1.887	2.176	3.188	01	3.037	0.999	1.894
LP1	3.072	1.050	1.301	LP1	3.068	0.505	2.233
C5	4.187	1.622	2.270	05	4.247	2.661	2.870
C6	5.389	0.825	1.805	H6	5.354	0.672	0.702
H6	5.413	−0.168	2.310	H6	6.333	1.363	2.048

TABLE 5

Geometry of DAG and the DAG-like structure of phorbol diesters

	Atomic distance			
	03-01	03-02	03-04	03-05
	Å			
1,2-Diacetyl-*sn*-glycerol				
Conformer with lowest energy	4.26	2.84	4.12	4.40
Conformer with geometry similar to phorbol	3.68	3.68	4.95	5.44
1,2-Diacetyl-3-hydroxycyclohexane	4.85	2.81	4.23	5.65
	Atomic distances			
	09-013	09-012	09-022	09-021
	Å			
12,13-Diacetyl-β-phorbol	3.52	4.23	4.99	5.47
12,13-Dibutyryl-β-phorbol	3.61	4.23	4.99	5.55

TABLE 6

Geometry of DAG and the DAG-like structure of phorbol diesters

	Dihedral angles		
	1,2-Diacetyl-*sn*-glycerol		
	Conformer with lowest energy	Conformer with geometry similar to phorbol	1,2-Diacetyl-3-hydroxycyclohexane
C1-C2-O2-C4	−94°	89°	97°
O1-C1-C2-O2	65°	−73°	−65°
C2-C1-O1-C5	77°	94°	97°
O1-C1-C2-C3[a]	−174°	58°	175°
C1-C2-C3-O3[a]	−58°	70°	−177°
	Dihedral angles		
	12,13-Diacetyl-β-phorbol		12,13-Dibutyryl-β-phorbol
C13-C12-O12-C22	109°		104°
O13-C13-C12-O12	−97°		−93°
C12-C13-O13-C21	83°		85°

[a] There are no dihedral angles equivalent to these in phorbol diesters.

acids as well as the position and distance of the hydroxyl group are optimized.

Our model for the correspondence of DAG with phorbol is not consistent with the previously proposed correspondence of the free hydroxyl of DAG with the hydroxyl at C20 of phorbol (11). Evidence against this proposal is that the geometry of this hydroxyl in β-phorbol is not different from that in the inactive α-isomer, and the atomic distances O20-O21 and O20-O22 are much greater than those between O3-O1 and O3-O2 in 1,2-diacetyl-*sn*-glycerol (25). The proposed correspondence of DAG oxygens O1, O2, and O3 with the hydroxyls at C4, C9, and C20 of phorbol, respectively (14), is also in disagreement with our proposal. Phorbol derivatives lacking the hydroxyl at C4 are still active (1), and the geometry of the ester bonds of phorbol is entirely different from the DAG conformer proposed to be active in this report.

The results of the calculations performed on 12,13-dideoxy-α- and -β-phorbols and the comparison of their geometries showed that the α- and β-isomers differ only in the region of the five-membered rings (Table 1 and Fig. 4). No difference was observed in the six-membered ring in phorbol esters where the fatty acids are attached (C12 and C13) (Tables 1 and 2).

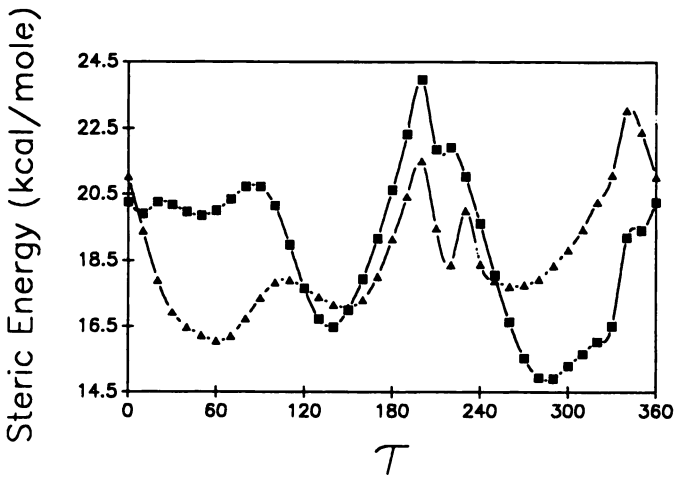


Fig. 8. Rotational barriers of (2*R*)-acetyl-(3*S*)- (▲) and -(3*R*)-hydroxylbutyl acetate (■). τ = C1-C2-C3-O3 (°).

This suggests that the five-membered/seven-membered ring region of β-phorbol may be important for some of its biological activities. This is also supported by the fact that none of the DAG analogs synthesized by Ganong *et al.* (16) was an antagonist and that β-phorbol alcohol is active as a cocarcinogen when administered systemically to mice (1).

In addition, ample evidence exists in support of a different possible mechanism of action of DAG and phorbol. Thus, different effects of the two molecules on protein kinase C have been documented, especially on the subcellular distribution of the enzyme (31, 32) and on its substrate preference (33), as well as on phospholipid metabolism (34, 35) and a series of physiological events involving cell proliferation and differentiation (36–39). DAG, the DAG-like region of the phorbol molecule, and the five-membered/seven-membered ring region of phorbol could act either on separate receptors, which would be consistent with the reported heterogeneity of phorbol receptors (3), or on different isoforms of protein kinase C that exist in different systems (40) that could represent discrete targets for the action of these molecules mediating different physiological events.

Structure-activity relationship studies also support the view that a series of functional groups in the phorbol molecule, distinct from its DAG-like region, may play a crucial role in the cocarcinogenic and inflammatory activities of phorbol esters (1). Thus, it has been shown that the hydroxyl at C20 is essential for the activity as a cocarcinogen and its esterification decreases the potency of phorbol, that the ketone function can be substituted by an ester or reduced to hydroxyl with only some decrease in the activity, and that the C1-C2 double bond is essential (1). Interestingly, the stereochemistry of C4 must be β for the phorbol esters to be active but does not need to carry a hydroxyl group (1). Also, a series of cocarcinogens structurally unrelated to phorbol that do not contain a DAG-like region in their molecule, such as dihydroteleocydin B, aplysiatoxin, and bryostatin, are effective antagonists of phorbol binding to protein kinase C and share some of its biological actions (41, 42). Finally, some high potency inhibitors of protein kinase C that do not compete with DAG, such as staurosporin and K252a, have a portion of their molecule similar to the five-membered/seven-membered ring region of the phorbol molecule (43).

In conclusion, large differences were found in the preferred geometries of α- and β-phorbol in a region of the molecule that is not present in DAG, and the DAG-like structure present in the phorbol molecule is virtually identical in both biologically inactive α- and active β-phorbol. Although the differences in the five-membered/seven-membered ring region of the α- and β-phorbol diesters could prevent the derivatives of the α-isomer from accessing the binding site on protein kinase C due to steric hindrance, the fact that this region is absent in DAG suggests that β-phorbol may contain an additional site essential for the activity. This other site may be responsible for properties that the phorbols do not share with DAG but that they have in common with other cocarcinogens.

Acknowledgments

We gratefully acknowledge the skillful assistance of Ms. Janice Busa in the preparation of the manuscript.

References

1. Hecker, E. Structure-activity relationship in diterpene esters irritant and cocarcinogenic to mouse skin, in *Carcinogenesis. Vol 2. Mechanisms of Tumor Promotion and Cocarcinogenesis* (T. J. Slaga, A. Sivak, and R. K. Boutwell, eds.). Raven Press, New York, 11–48 (1987).
2. Blumberg, P. M., J. A. Dunn, S. Jaken, A. Y. Jeng, K. L. Leach, N. A. Sharkey, and E. Yeh. Specific receptors for phorbol ester tumor promoters and their involvement in biological responses, in *Mechanisms of Tumor Promotion. Vol. 3. Promotion and Cocarcinogenesis In Vitro* (T. J. Slaga, ed.). CRC Press, Boca Raton, FL, 143–184 (1984).
3. Ashendel, C. L. The phorbol ester receptor: a phospholipid-regulated protein kinase. *Biochim. Biophys. Acta* **822**:219–242 (1985).
4. Kikkawa, U., Y. Takai, R. Minakuchi, S. Inohara, and Y. Nishizuka. Calcium-activated, phospholipid-dependent protein kinase from rat brain. *J. Biol. Chem.* **257**:13341–13348 (1982).
5. Kaibuchi, K., Y. Takai, and Y. Nishizuka. Cooperative roles of various membrane phospholipids in the activation of calcium-activated, phospholipid-dependent protein kinase. *J. Biol. Chem.* **256**:7146–7149 (1981).
6. Berridge, M. J. Inositol trisphosphate and diacylglycerol: two interacting second messengers. *Annu. Rev. Biochem.* **56**:159–193 (1987).
7. Nishizuka, Y. The role of protein kinase C in cell surface signal transduction and tumor promotion. *Nature* (*Lond.*) **308**:693–698 (1984).
8. Sharkey, N. A., K. L. Leach, and P. M. Blumberg. Competitive inhibition by diacylglycerol of specific phorbol ester binding. *Proc. Natl. Acad. Sci. USA* **81**:607–610 (1984).
9. Wong, K., and C. Chew. Comparison of 1-oleoyl-2-acetyl-glycerol and phorbol myristate acetate as secretagogues for human neutrophils. *Can. J. Physiol. Pharmacol.* **64**:1149–1152 (1986).
10. Davis, R. J., B. R. Ganong, R. M. Bell, and M. P. Czech. Structural requirements for diacylglycerol to mimic tumor-promoting phorbol diester action on the epidermal growth factor receptor. *J. Biol. Chem.* **260**:5315–5322 (1985).
11. Brasseur, R., V. Cabiaux, P. Huart, M. Castagna, S. Baztar, and J. M. Ruysschaert. Structural analogies between protein kinase C activators. *Biochem. Biophys. Res. Commun.* **127**:969–976 (1985).
12. Sharkey, N. A., and P. M. Blumberg. Kinetic evidence that 1,2-diolein inhibits phorbol ester binding to protein kinase C via a competitive mechanism. *Biochem. Biophys. Res. Commun.* **133**:1051–1056 (1985).
13. Sharkey, N. A., and P. M. Blumberg. Comparison of the activity of phorbol 12-myristate 13-acetate and the diglyceride glycerol 1-myristate 2-acetate. *Carcinogenesis* (*Lond.*) **7**:677–679 (1986).
14. Wender, P. A., K. F. Koehler, N. A. Sharkey, M. L. Dell'Aquila, and P. M. Blumberg. Analysis of the phorbol ester pharmacophore on protein kinase C as a guide to the rational design of new classes of analogs. *Biochemistry* **83**:4214–4218 (1986).
15. Smart, R. C., M. T. Huang, and A. H. Conney. *sn*-1,2-Diacylglycerols mimic the effects of 12-O-tetradecanoyl phorbol-13-acetate *in vivo* by inducing biochemical changes associated with tumor promotion in mouse epidermis. *Carcinogenesis* (*Lond.*) **7**:1865–1870 (1986).
16. Ganong, B. R., C. R. Loomis, Y. A. Hannun, and R. M. Bell. Specificity and mechanism of protein kinase C activation by *sn*-1,2-diacylglycerols. *Proc. Natl. Acad. Sci. USA* **83**:1184–1188 (1986).
17. Molleyres, L. P., and R. R. Rando. Structural studies on the diglyceride-mediated activation of protein kinase C. *J. Biol. Chem.* **263**:14832–14838 (1988).
18. Bonser, R. W., N. T. Thompson, H. F. Hodson, R. M. Beams, and L. G. Garland. Evidence that a second stereochemical center in diacylglycerols defines interaction at the recognition site of protein kinase C. *FEBS Lett.* **234**:341–344 (1988).
19. Laughton, C. A., I. L. Dale, and A. Gescher. Studies on bioactive compounds. 13. Synthesis and lack of growth inhibitory properties of cyclohexane,1,2,4-triol 1,2-diesters which resemble ring C of the phorbol ester molecule. *J. Med. Chem.* **32**:428–433 (1989).

20. Delclos, K. B., D. S. Nagle, and P. M. Blumberg. Specific binding of phorbol ester tumor promoters to mouse skin. *Cell* **19**:1025–1032 (1980).
21. Dunn, J. A., and P. M. Blumberg. Specific binding of [20-^{3}H]12-deoxyphorbol 13-isobutyrate to phorbol ester receptor subclasses in mouse skin particulate preparations. *Cancer. Res.* **43**:4632–4637 (1983).
22. Allinger, N. L., and Y. H. Yuh. *Quantum Chem. Prog. Exc.* **12**:395 (1980).
23. Cabot, M. C., and S. Jaken. Structural and chemical specificity of diradylglycerols for protein kinase C activation. *Biochem. Biophys. Res. Commun.* **125**:163–169 (1984).
24. Heymans, F., C. De Silva, N. Marrec, J. J. Godfroid, and M. Castagna. Alkyl analogs of diacylglycerol as activators of protein kinase C. FEBS Lett. **218**:35–40 (1987).
25. Leli, U., M. Froimowitz, and G. Hauser. Molecular geometries and steric energies of phorbol 10,11-diacetate and 1,2-diacetylglycerol molecules. *Adv. Exp. Med. Biol.* **221**:101–106 (1987).
26. Pettersen, R. C., G. I. Birnbaum, G. Ferguson, K. M. S. Islam, and J. G. Sime. X-ray investigation of several phorbol derivatives: the crystal and molecular structure of phorbol bromofuroate-chloroform solvate at −160°. *J. Chem. Soc.* (*Lond.*) B 890–894 (1968).
27. Froimowitz, M., and P. J. Gans. Comparison of semiempirical classical and semiempirical quantum mechanism calculations on acetylcholine. *J. Am. Chem. Soc.* **94**:8020–8025 (1972).
28. Hauser, H., I. Pascher, and S. Sundell. Preferred conformation and dynamics of the glycerol backbone in phospholipids: an NMR and X-ray simple crystal analysis. *Biochemistry* **27**:9166–9174 (1988).
29. Hauser, H., W. Guyer, I. Pascher, P. Skrobal, and S. Sundell. Polar group conformation of phosphatidylcholine: effects of solvent and aggregation. *Biochemistry* **19**:366–373 (1980).
30. Delclos, K. B., E. Yeh, and P. M. Blumberg. Specific labeling of mouse membrane phospholipids with [20-^{3}H]phorbol 12-*p*-azidobenzoate 13-benzoate, a photolabile phorbol ester. *Proc. Natl. Acad. Sci. USA* **80**:3054–3058 (1983).
31. Ways, D. K., R. C. Dodd, and H. S. Earp. Dissimilar effects of phorbol esters and diacylglycerol derivative on protein kinase activity in the neuroblastoid U937 cells. *Cancer Res.* **47**:3344–3350 (1987).
32. Brockenbrough, J. S., and M. Korc. Effects of 1-oleoyl-2-acetyl glycerol are distinct from those of phorbol ester in rat pancreatic acini. *Life Sci.* **40**:1625–1631 (1987).
33. Kiss, Z., and Y. Luo. Phorbol ester and 1,2-diolein are not fully equivalent activators of protein kinase C in respect to phosphorylation of membrane proteins *in vitro*. *FEBS Lett.* **198**:203–207 (1986).
34. Kolesnick, R. N. 1,2-Diacylglycerols but not phorbol esters stimulate sphingomyelin hydrolysis in GH3 pituitary cells. *J. Biol. Chem.* **262**:16759–16762 (1987).
35. Liscovitch, M., B. Slack, J. K. Blusztajn, and R. J. Wurtman. Differential regulation of phosphatidylcholine biosynthesis by 12-*O*-tetradecanoyl phorbol-13-acetate and diacylglycerol in NG108-15 neuroblastoma × glioma hybrid cells. *J. Biol. Chem.* **262**:17487–17491 (1987).
36. Kamber, M. Phorbol myristate acetate-induced proliferation of an IL-2-dependent T-cell line: action of PMA is independent of IL-2 and cannot be mimicked by diacylglycerols. *Cell. Immunol.* **102**:177–186 (1986).
37. Shinoara, O., M. Knecht, and K. J. Catt. Differential actions of phorbol esters and diacylglycerol on inhibition of granulosa cell maturation. *Biochem. Biophys. Res. Commun.* **133**:468–474 (1985).
38. Yamamoto, S., H. Gotoh, E. Aizu, and R. Kato. Failure of 1-oleoyl-2-acetyl glycerol to mimic cell differentiating action of 12-*O*-tetradecanoyl phorbol-13-acetate in HL-60 cells. *J. Biol. Chem.* **260**:14230–14234 (1985).
39. Stoeck, M., R. Lees, M. Szamel, G. Pantaleo, and H. R. McDonald. Comparison of phorbol-12-myristate-13-acetate and dioctanoyl-*sn*-glycerol in the activation of EL4/6.1 thymoma cells. *J. Cell. Physiol.* **138**:541–547 (1989).
40. Nishizuka, Y. The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature* (*Lond.*) **334**:661–665 (1988).
41. Berkow, R. L., and A. S. Kraft. Bryostatin, a non-phorbol macrocyclic lactone, activates intact human polymorphonuclear leucocytes and binds to the phorbol ester receptor. *Biochem. Biophys. Res. Commun.* **131**:1109–1116 (1985).
42. Fujiki, H., and T. Sugimura. New classes of tumor promoters, teleocydin, aplysiatoxin and palytoxin. *Adv. Cancer Res.* **49**:223–264 (1987).
43. Leli, U., M. Froimowitz, and G. Hauser. Similarities in molecular geometry of K252a, staurosporin and phorbols. *Trans. Am. Soc. Neurochem.* **20**:216 (1989).

Send reprint requests to: Ubaldo Leli, Ralph Lowell Laboratories, Harvard Medical School, McLean Hospital, Belmont, MA 02178.