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Identification of a Protein Kinase C (PKC) Activator, Daphnoretin, That Suppresses Hepatitis B Virus Gene Expression in Human Hepatoma Cells

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ABSTRACT. We examined the antiviral activity of a crude extract prepared from a Chinese medicinal herb *Wikstroemia indica* C.A. Mey. One active component, daphnoretin (7-hydroxyl-6-methoxy-3,7′-dicoumaryl-ether), was identified, which showed strong suppressive effects on the expression of the hepatitis B surface antigen (HBsAg) in human hepatoma Hep3B cells. To examine the signaling pathway of daphnoretin on the Hep3B cells, we pretreated Hep3B cells with 12-*O*-tetradecanoylphorbol-13-*O*-acetate (200 nM) for 24 hr to down-regulate intracellular protein kinase C (PKC) levels and found that the PKC-down-regulated Hep3B cells did not respond at all to daphnoretin. Furthermore, daphnoretin induced translocation of PKC from the cytosol to the membrane and down-regulated intracellular PKC levels in the Hep3B cells, indicating that it may directly activate PKC. This hypothesis was supported by the observation that daphnoretin directly competed with [^{3}H]phorbol dibutyrate for the binding of PKC in the whole cell and activated purified PKC activity *in vitro*. Our results demonstrated that daphnoretin, with a structure distinct from phorbol ester, is a PKC activator and has suppressive effects on HBsAg gene expression in human hepatoma cells. BIOCHEM PHARMACOL 52;7:1025–1032, 1996.

KEY WORDS. natural product; daphnoretin; protein kinase C activator; human hepatoma cells; hepatitis B virus surface antigen; gene expression

Regulation of HBsAg¶ gene expression in Hep3B cells has been studied intensively. Cultured human hepatoma Hep3B cells contain one or two copies of HBV genomes in their chromosomes [1] and actively secrete HBsAg into the medium [2]. Gene expression of HBsAg is up-regulated by all-*trans* retinoic acid [3] and glucocorticoid [4], but is suppressed by insulin [5] and the phorbol ester TPA [6]. Therefore, expression of the HBsAg gene in Hep3B cells provides an excellent way for screening biological active substances from natural resources.

In our continual search for anti-hepatitis B viral agents from Chinese medicinal herbs, we have found that daphnoretin, which was isolated from the *Wikstroemia indica* C.A. Mey [7], can suppress HBsAg production by Hep3B cells. A similar response was also observed in the tumor promoter TPA-treated Hep3B cells [6]. Daphnoretin is a PKC activator in rabbit platelets [8]. We then investigated whether daphnoretin acted similarly to TPA through the PKC pathway in regulating gene expression of HBsAg on Hep3B cells.

PKC was originally characterized as a calcium- and phospholipid-dependent protein kinase and has been implicated in the control of various biological functions including cell growth and differentiation [9–13]. The enzyme is activated by DAG under physiological conditions [14–16] and by phorbol esters [17, 18] that apparently substitute for the DAG in the activation process [19]. In addition to the phorbol esters, a number of structurally distinct natural products have been identified which bind and activate PKC both *in vivo* and *in vitro*. They include alkaloid teleocidins [20–22], polyacetate aplysiatoxins [23], bryostatins [24–25], debromo-aplysiatoxins [26–28], and mezereins [29]. Although these compounds are structurally distinct from the phorbol esters, they all contain a three-dimensional array of functional groups homologous to the postulated phorbol ester pharmacophore on PKC [30–32]. In this report, we present evidence showing that daphnoretin, with a structure distinct from phrbol ester, is a novel PKC activator and acts through the PKC pathway to regulate gene expression of HBsAg in Hep3B cells.

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¶*Abbreviations:* HBsAg, hepatitis B surface antigen; TPA, 12-*O*-tetradecanoylphorbol-13-*O*-acetate; DAG, (*S*)-1-oleoyl-2-acetylglycerol; EIA, enzyme immunoassay; DMEM, Dulbecco's modified Eagle's medium; and PBt_2, phorbol 12,13-dibutyrate.

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MATERIALS AND METHODS

Materials

Daphnoretin (see Fig. 8) was isolated from the plant, *Wikstroemia indica* C.A. Mey as described [7]. Fetal bovine serum was purchased from Hyclone (Logan, UT, U.S.A.). DMEM balanced salt solutions were obtained from Gibco/BRL (Gaithersburg, MD, U.S.A.). Purified PKC, composed of a mixture of Ca^{2+}-dependent isozymes (α, β, γ), was obtained from UBI (Houston, TX, U.S.A.). [α-^{32}P]dCTP (3000 Ci/mmol), [γ-^{32}P]ATP (3000 Ci/mmol), [^{3}H]PBt_2 (25 Ci/mmol), and monoclonal anti-PKC antibody which recognizes the α + β form were obtained from the Amersham Corp. (Buckinghamshire, U.K.). Daphnoretin was acetylated with Ac_2O and pyridine to give 7-acetyl-daphnoretin, which was purified on silica gel chromatography using 15% CH_2Cl_2/*n*-hexane as eluent. TPA and other chemicals were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Cell Culture

Stock cultures of human hepatoma Hep3B cells were maintained in DMEM supplemented with 10% fetal bovine serum and antibiotics (100 U/mL each of penicillin and streptomycin) in a humidified atmosphere containing 5% CO_2 and 95% air at 37°. The cultures were passaged by trypsinization every 4 days. Cells were plated either in 24-well plates at a density of 1.0×10^5 cells/well or in 100 mm culture dishes at a density of 1.5×10^6 cells/dish in DMEM containing 10% fetal bovine serum.

Determination of HBsAg Production

Cells cultured in DMEM with 10% fetal bovine serum for 24 hr were changed to serum-free DMEM with or without drugs and incubated for 48 hr. The HBsAg in culture medium was measured by EIA kits. The viability of cells was determined using a hemocytometer and trypan blue dye exclusion.

Northern Blotting Analysis

Total cellular RNA was isolated by centrifugation through cesium chloride according to the method of Schwab *et al.* [33]. The RNA (20 μg) was denatured in 6.5% formaldehyde and fractionated by electrophoresis in a 1% agarose gel. The RNA was transferred to a nitrocellulose filter. The filter was prehybridized for 6 hr at 42° in a solution containing 35% formamide, 5× Denhardt's reagent (0.1% Ficoll, 0.1% BSA, 0.1% polyvinylpyrrolidone), 5× SSPE (0.75 M NaCl, 50 mM NaH_2PO_4, 5 mM EDTA, 5% SDS, and 500 μg/mL denatured salmon sperm DNA, pH 7.4). A specific HBsAg cDNA was labeled with [α-^{32}P]dCTP using random primers. A denatured ^{32}P-labeled cDNA probe (10^9 dpm/mg) was added directly to the prehybridization buffer, and hybridization was carried out for 36 hr at 42°. The membrane filter was washed twice in 0.2× SSC (1× = 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0), 0.2% SDS at 42° for 15 min and once in 0.1× SSC, 0.1% SDS at 65° for 15 min. Autoradiography was performed at −70° with an intensifying screen (Quanta IV, Du Pont, U.S.A.) for 12 hr on Kodak SAFET X-ray film.

Preparation of Cell Lysate

The cultured cells were washed once with cold phosphate-buffered saline (pH 7.0) and lysed in 1 mL of Buffer A [20 mM Tris-HCl (pH 7.5), 10 mM EDTA, 10 mM EGTA, 0.3% (v/v) β-mercaptoethanol, 50 mg/mL phenylmethylsulfonyl fluoride, 10 mg/mL leupeptin] plus 1% Triton X-100. The cell lysate was cleared by centrifugation at 30,000 *g* for 10 min. For subcellular fractionation, cells were scraped into 1 mL of Buffer A and homogenized (30 strokes in a Dounce homogenizer). The homogenate was then centrifuged at 100,000 *g* for 30 min. The pellet was extracted further by Buffer A containing 1% Triton X-100. The detergent-solubilized membrane fraction of proteins was collected by centrifugation at 100,000 *g* for 30 min.

Immunoblot Analysis of PKC

The proteins were separated by electrophoresis in a 7.5% polyacrylamide gel in the presence of SDS followed by electrotransfer to a nitrocellulose membrane. The filters were blocked by 3% skim milk in TBS buffer (20 mM Tris–HCl, pH 7.4, 150 mM sodium chloride) at 25° for 1 hr. Membranes were then incubated with anti-PKC monoclonal antibody (1 μg/mL) in TBS buffer for 2 hr. The blot was incubated subsequently with a horseradish peroxidase-linked anti-Ig antibody. The second antibody was determined with an ECL Western Enhanced ChemiLuminescence detection system (Amersham).

Assay of [^{3}H]PBt_2 Binding

[^{3}H]PBt_2 binding to the cells was analyzed as described [34]. Briefly, the Hep3B cells were washed twice with binding medium [DMEM, 1 mg/mL BSA, 20 mM HEPES (pH 7.0)] followed by incubation at 37° for 30 min in binding medium containing 5 nM [^{3}H]PBt_2 plus various concentrations of TPA or daphnoretin. After washing three times with ice-cold PBS, cells were lysed by 1 N NaOH, and the cell-bound radioactivity was measured by scintillation counting. Nonspecific binding of [^{3}H]PBt_2 in Hep3B cells was determined in the presence of 40 μM unlabeled PBt_2.

PKC Activity Assay

PKC activity was assayed by the method described previously [35]. Enzyme activity was assayed in 50 μL of a reaction buffer consisting of 50 mM Tris–HCl (pH 7.5), 15 mM magnesium acetate, 1 mM calcium acetate, 2.5 mM dithiothreitol, 50 μM [γ-^{32}P]ATP (10^5 cpm/nmol), 25 mg/mL phosphatidylserine, 0.5 mg/mL histone III-S, 2 ng of puri-

fied PKC, and various concentrations of TPA or daphnoretin at 37°C for 10 min. The radioactivity of ^{32}P-labeled histone III-S was measured by trichloroacetic acid precipitation and scintillation counting.

Determination of Protein

Protein concentration was determined according to the method of Bradford [36], using BSA as standard.

Computer Modeling Calculation

Distances were measured between the centers of mass of the oxygens attached to the indicated carbons (see Fig. 8) of TPA, diacylglycerol, and daphnoretin. The conformation of each of these compounds gave an rms (root mean square) gradient of 0.1 kcal/mol when calculated using Hyperchem Auto Desk MM^+ (molecular mechanics, geometry optimization program).

RESULTS

Suppression of HBsAg Production by Daphnoretin

Hep3B cells were plated into a 24-well plate and allowed to attach overnight. The medium was then changed to serum-free DMEM, and various concentrations of daphnoretin were added. After 48 hr of incubation, HBsAg in the culture medium was determined. The results showed that daphnoretin drastically suppressed HBsAg production with an IC_{50} of about 0.1 μM (Fig. 1). The suppression activity of daphnoretin was not due to its cytotoxicity, since the treated cells were still viable and continued to proliferate during the 48-hr incubation period. Northern blot analysis revealed a dramatic decrease of HBsAg mRNA in the Hep3B cells during drug treatment, suggesting that the suppression of HBsAg gene expression in Hep3B cells by daphnoretin was mainly on the mRNA level (Fig. 2).

Involvement of PKC in the Suppression of HBsAg Production by Daphnoretin

We have shown previously that TPA suppresses HBsAg gene expression in Hep3B cells [6]. It was, therefore, of interest to determine if the suppression activity of daphnoretin on HBsAg gene expression is mediated through a pathway similar to that of TPA on Hep3B cells. We pretreated Hep3B cells in serum-free DMEM with TPA (200 nM) for 24 hr to down-regulate the intracellular PKC level. The PKC-depleted cells were then treated in the presence of daphnoretin (1 μM) or TPA (200 nM) for 48 hr, and the production of HBsAg in the medium was determined by EIA. As shown in Fig. 3, the suppressive activity of TPA and daphnoretin on HBsAg production was abolished completely in the PKC down-regulated Hep3B cells.

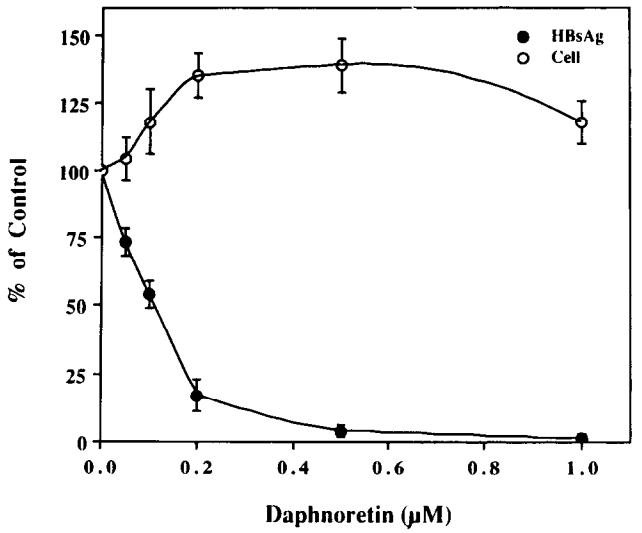


FIG. 1. Concentration-dependent effect of daphnoretin on HBsAg production in Hep3B human hepatoma cells. Hep3B cells were plated at a density of 1.0×10^5 cells/cm^3 in DMEM with 10% fetal bovine serum and allowed to attach overnight. The cells were then washed three times with phosphate-buffered saline (pH 7.0) and treated with various concentrations of daphnoretin in serum-free DMEM for another 48 hr. The cell number in each well was counted with a hemocytometer, and the amount of HBsAg in the culture medium was determined by EIA. Control cells produced 15 ng of HBsAg/10^6 cells/48 hr. Data are expressed as means ± SD (N = 3).

Effect of Daphnoretin on PKC Translocation and Down-regulation in Hep3B Cells

Immunoblot analysis using anti-PKC antibody as a probe was performed to examine whether daphnoretin may cause the translocation and down-regulation of PKC in Hep3B cells. When the Hep3B cells were treated with daphnoretin for 4 hr, the membrane-bound PKC was increased by ~20-fold with a concomitant decrease in cytosolic PKC (Fig. 4A). Furthermore, when cells were treated with either TPA (200 nM) or daphnoretin (800 nM) for 24 hr, the total extractable cellular PKC was diminished to a level barely detectable in both cases (Fig. 4B). These results suggest that daphnoretin, like TPA, can induce the translocation of PKC from cytosol to membrane and consequently down-regulate the intracellular PKC level in Hep3B cells.

Effect of Daphnoretin on Phorbol Ester Binding to the Cells and on Purified PKC In Vitro

To see whether daphnoretin interacts with PKC at the same site as TPA intracellularly, we tested the ability of daphnoretin to compete with the binding of ^3H-labeled PBt_2 to PKC on Hep3B cells. TPA and daphnoretin both competed for the binding of [^3H]PBt_2 at an IC_{50} = 20 nM and 10 μM, respectively (Fig. 5). Furthermore, we examined whether daphnoretin could directly activate purified

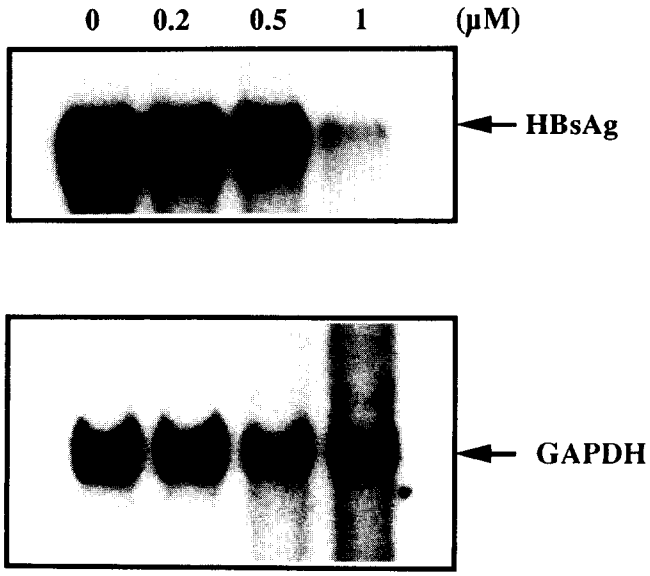


FIG. 2. Effect of daphnoretin on the steady-state mRNA level of HBsAg in Hep3B cells. Hep3B cells were seeded on a 100-mm culture dish and treated with 0, 0.2, 0.5, and 1.0 μM daphnoretin in serum-free DMEM for 48 hr. Total RNA was extracted and analyzed by northern hybridization with HBsAg DNA as described in Materials and Methods. The constitutively expressed glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an internal marker.

PKC *in vitro*. As shown in Fig. 6, the PKC activity was stimulated in a concentration-dependent manner by daphnoretin and TPA with a half-maximal concentration of 600 and 5 nM, respectively.

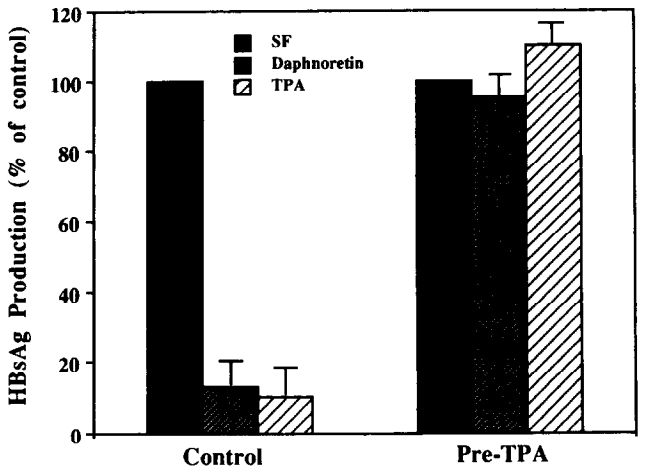


FIG. 3. Effect of TPA pretreatment on HBsAg production by daphnoretin in Hep3B cells. Hep3B cells were plated and treated with 200 nM TPA in serum-free (SF) DMEM for 24 hr. Hep3B cells also were plated and treated without TPA in serum-free DMEM for 24 hr to serve as the control. The cells were then washed three times with phosphate-buffered saline (pH 7.0) and treated with TPA (200 nM) or daphnoretin (1 μM) in serum-free DMEM for another 48 hr. The cell number in each well was determined with a hemocytometer, and the amount of HBsAg present in the culture medium was determined by EIA. Control cells produced 15 ng of HBsAg/10^6 cells/48 hr. Values are the means ± SD of three experiments, each conducted in triplicate.

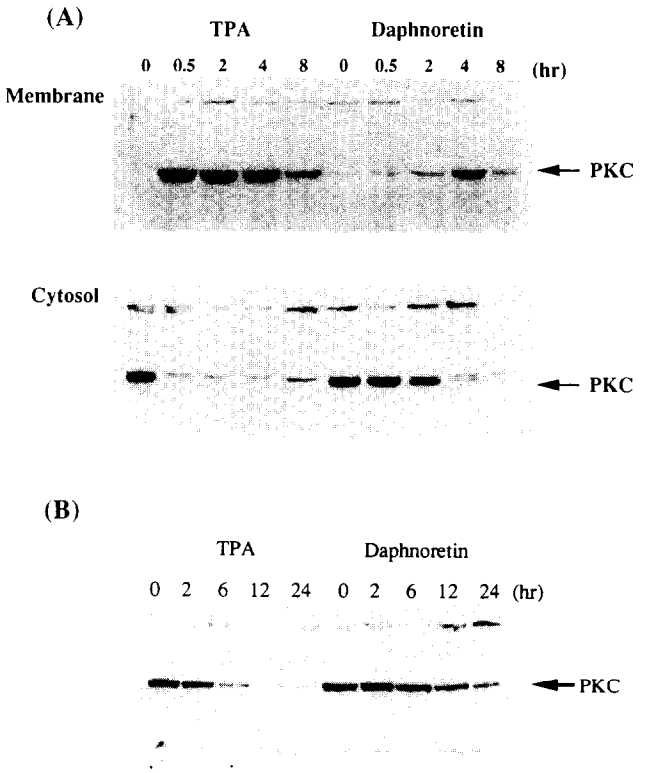


FIG. 4. Effects of daphnoretin and TPA treatment on cytosolic and membrane PKC. Hep3B cells deprived of serum for 24 hr were incubated with either daphnoretin (1 μM) or TPA (200 nM) for the duration as indicated. Cytosolic and membrane proteins from the homogenized cells were obtained as described in Materials and Methods. Membrane and cytosol proteins (A) or the unfractionated total proteins (B) were separated by electrophoresis in a 7.5% SDS-polyacrylamide gel. Proteins were transferred to a nitrocellulose filter, and western blotting was performed with anti-PKC monoclonal antibody as described in Materials and Methods.

Effect of Acetylated Daphnoretin on the Suppression of HBsAg Production and Activation of PKC in Hep3B Cells

To test whether daphnoretin specifically binds to the regulatory domain of PKC and causes the activation of PKC toward substrate, we examined the effect of the C_7 hydroxyl group of daphnoretin on suppression of HBsAg production and probed the nature of the PKC effector binding site. 7-Acetyl-daphnoretin was derived from daphnoretin by minor modification. When the Hep3B cells were treated with 7-acetyl-daphnoretin (5 μM) for 48 hr, both the suppression of HBsAg production and the down-regulation of the intracellular PKC level were abolished (Fig. 7).

DISCUSSION

We have demonstrated that the production of HBsAg in human hepatoma Hep3B cells is very sensitive to various agents [37–39] and can be developed as an effective assay system for screening biologically active substances in Chinese herb medicine. We have identified a compound, daph-

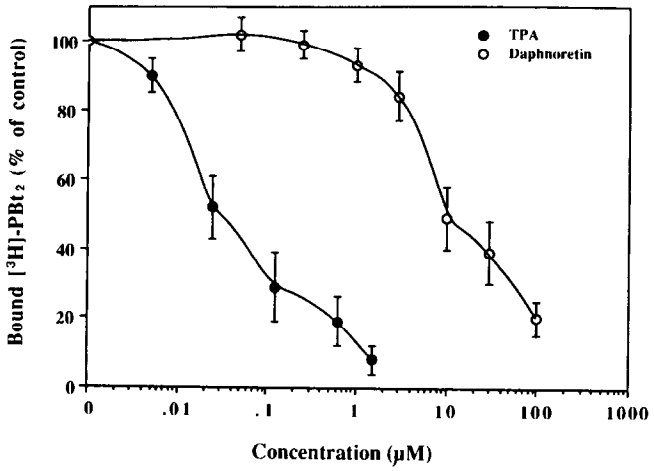


FIG. 5. Effect of daphnoretin on the binding of [^{3}H]PBt$_2$ to PKC in Hep3B cells. Hep3B cells were seeded and incubated at 25° with 5 nM [^{3}H]PBt$_2$ in the presence of various concentrations of TPA or daphnoretin for 25 min. Cells were then washed three times with cold phosphate-buffered saline (pH 7.0) and lysed in 2 N NaOH solution. The bound radioactivity of [^{3}H]PBt$_2$ was measured with a liquid scintillation counter. Nonspecific binding of [^{3}H]PBt$_2$. Control binding was 5200 ± 700 dpm/10^6 cells, and nonspecific binding was 720 ± 100 dpm/10^6 cells. Values are the means ± SD of three experiments, each conducted in duplicate.

noretin, isolated from *Wikstroemia indica* C.A. Mey. This compound severely suppresses the production of HBsAg on Hep3B cells. A similar observation was reported previously in Hep3B cells treated with the phorbol ester TPA [6].

In the present study, we clearly demonstrated that daph-

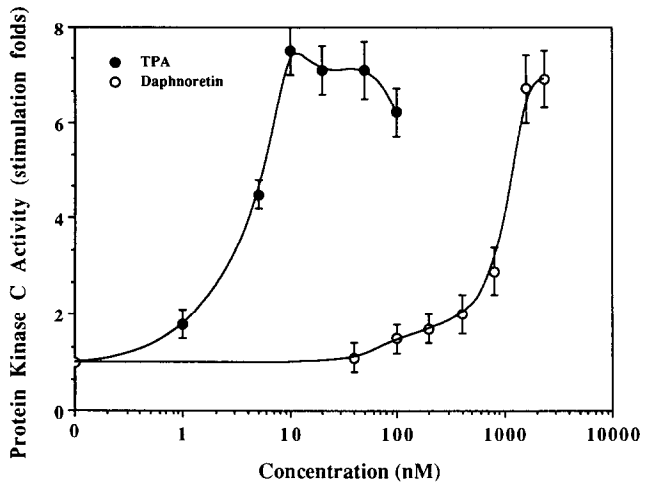


FIG. 6. Effects of daphnoretin and TPA on PKC activity in a cell-free system. PKC activity was assayed at various concentrations of daphnoretin or TPA in a reaction mixture (50 μL) containing 50 mM Tris–HCl (pH 7.5), 15 mM magnesium acetate, 1 mM calcium acetate, 2.5 mM dithiothreitol, 50 μM [γ-^{32}P]ATP, 25 mg/mL phosphatidylserine, 0.5 mg/mL histone III-S, 2 ng of purified PKC before incubating at 37° for 10 min. The incorporation of [^{32}P]phosphate in histone III-S was measured by a liquid scintillation counter. Control enzyme incorporated 2.3 ± 0.7 × 10^9 cpm/mg protein/min. Values are the means ± SD of three experiments, each conducted in duplicate.

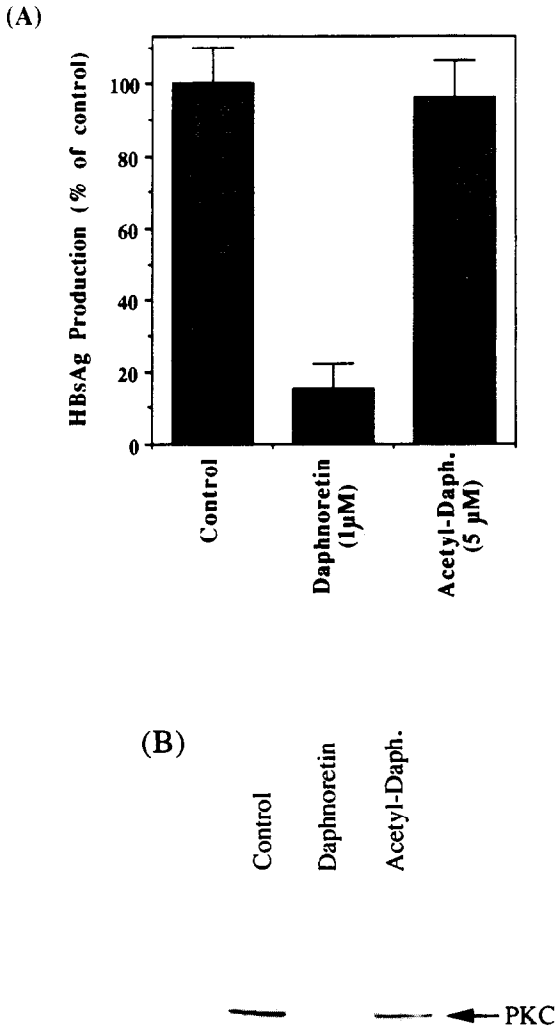


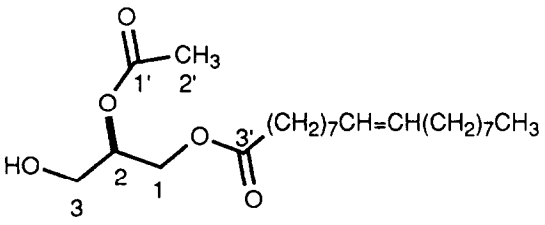
FIG. 7. Effects of daphnoretin and its acetylated analog on HBsAg production and PKC down-regulation in Hep3B cells. Hep3B cells were treated with daphnoretin (1 μM), acetyl-daphnoretin (5 μM) or control solvent in serum-free DMEM for 48 hr. (A) The amounts of HBsAg in cultured medium were determined by EIA. Control cells produced 15 ng of HBsAg/10^6 cells/48 hr. Values are the means ± SD of three experiments, each conducted in triplicate. (B) The intracellular protein levels of PKC were analyzed by SDS–gel electrophoresis followed by immunoblotting with anti-PKC antibody.

noretin mimics the biological activities of TPA in Hep3B cells. Daphnoretin was able to activate the purified PKC *in vitro* and to compete for the [^{3}H]PBt$_2$ binding to the phorbol ester's receptor *in vivo*. In addition, daphnoretin induced translocation of intracellular PKC from cytosol to plasma membrane and led to down-regulation of intracellular PKC levels. This evidence suggests that daphnoretin,

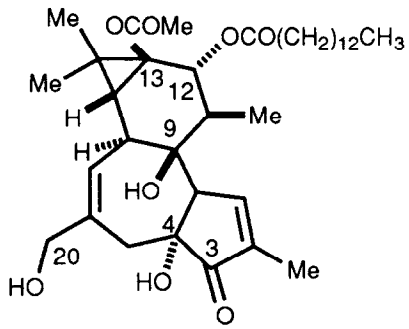
though structurally different from TPA (Fig. 8), is a PKC activator.

PKC can be activated when DAG binds to its regulatory domain [13]. This regulatory domain can also be bound by a group of structurally diverse PKC activators [16–19, 26–28]. The structure–activity relationships of DAG have been illustrated on the basis of (i) substitution of an ether or a halogen for the 3-hydroxyl group abolishes activity [31, 32]; (ii) elimination of one or more of the carbonyl groups of the ester leads to the disappearance of activity [21]; and (iii) extension of the length of the DAG backbone produces decreasingly active molecules [20]. These results indicate that the hydrophilic hydroxyl, the ester moiety, and the distances between the hydrophilic moieties are important in the interactions of DAG with PKC.

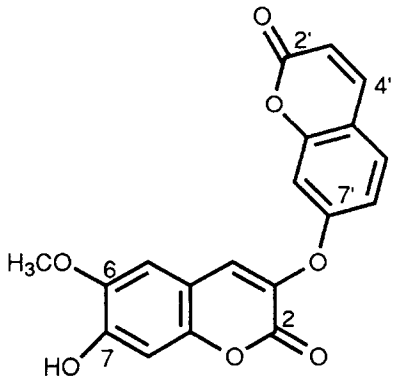
Daphnoretin has a chemical structure distinct from TPA or DAG. How it interacts with PKC is not clear yet. Analyzing the structure of daphnoretin using DAG as a template suggests that the relevant hydrophilic moieties of C_7 and C_2 oxygen atoms of daphnoretin (Fig. 8) may correspond to C_3 and $C_{3'}$ oxygen atoms of DAG, respectively [14]. Further evidence is needed, however, to substantiate



(S)-1-Oleoyl-2-acetylglycerol
(DAG)



12-*O*-Tetradecanoylphorbol-13-*O*-acetate
(TPA)



7-Hydroxyl-6-methoxy-3,7'-dicoumarylether
(Daphnoretin)

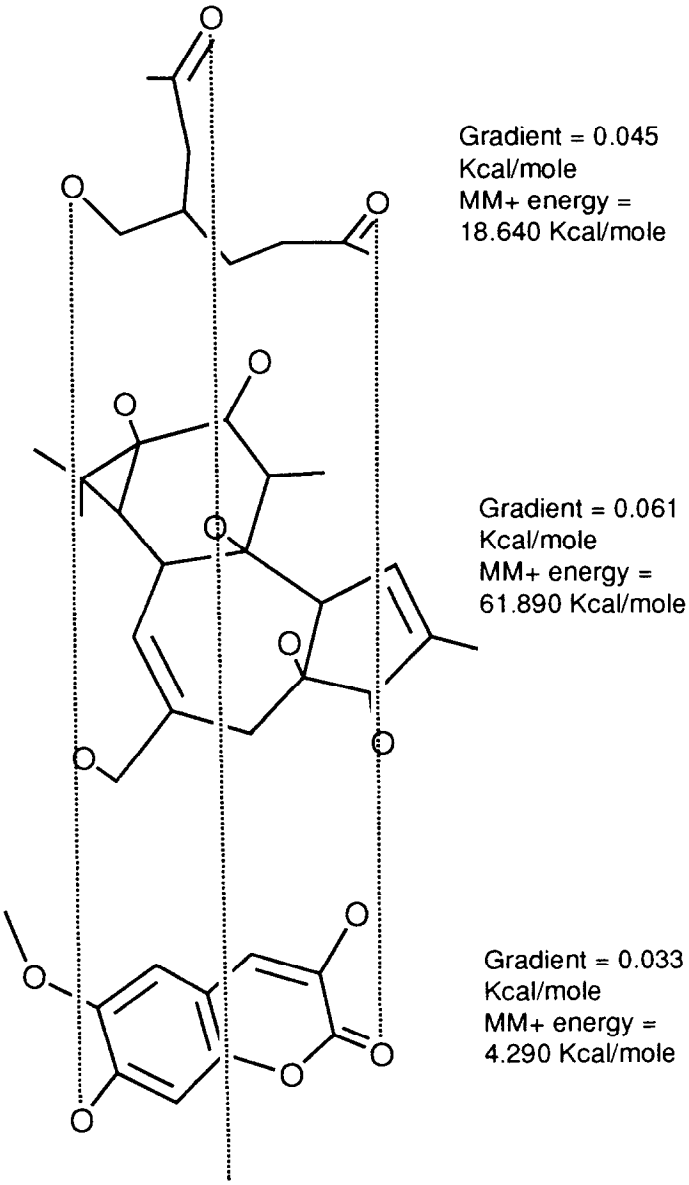


FIG. 8. Chemical structures of DAG, TPA, and daphnoretin.

TABLE 1. Interatomic distances between corresponding oxygen atoms in TPA, DAG, and daphnoretin

Compound	Distances* (Å)					
TPA	(C_3–C_{20})	6.4	(C_9–C_{20})	4.1	(C_3–C_9)	5.6
DAG	($C_{3'}$–C_3)	6.9	($C_{1'}$–C_3)	4.3	($C_{1'}$–$C_{3'}$)	5.8
Daphnoretin	(C_2–C_7)	6.4	($C_{2'}$–C_7)	10.3	(C_2–$C_{2'}$)	9.2

* Distances were measured between the centers of mass of the oxygens attached to the indicated carbons (Fig. 8) of TPA, DAG and daphnoretin. The conformation of each of these compounds gave an rms (root mean square) gradient of 0.1 kcal/mol when calculated using Hyperchem Auto Desk MM+ (molecular mechanics, geometry optimization program).

whether the $C_{2'}$ oxygen atom of daphnoretin may correspond to the $C_{1'}$ oxygen atom of DAG. The C_7 hydroxyl group is thought to be a hydrogen bond donor, and the C_2 carbonyl oxygen is by necessity, a hydrogen bond acceptor [22, 23]. The hydrophobic side chain of the $C_{2'}$ carbon atom of daphnoretin may help the PKC activity. The interatomic distance between the C_3 hydroxyl oxygen and the $C_{3'}$ carbonyl oxygen of DAG is identical to the distance between the C_7 hydroxyl oxygen and the C_2 carbonyl oxygen in daphnoretin (Table 1). This shows that the atoms are involved in hydrogen bonding to the same receptor sites. Daphnoretin may be metabolized *in vivo* and activated. In addition, substitution of a C_7 hydroxyl group with an acetyl group in daphnoretin abolished its PKC activation activity (Fig. 7). The C_7 hydrophilic moieties of daphnoretin could be important for the pharmacophore for PKC activation.

We have provided further evidence which supports the hypothesis that daphnoretin may act through the PKC-dependent pathway to suppress the expression of the HBsAg gene on Hep3B cells. Down-regulation of the intracellular PKC level by chronic treatment of TPA completely abolishes the suppressive activity of daphnoretin. The concentration of daphnoretin at the half-maximal activation of PKC *in vitro* is close to that required for the half-maximal suppression of HBsAg production. 7-Acetyldaphnoretin suppressed neither the HBsAg production nor the down-regulation of intracellular PKC on Hep3B cells. How the activated PKC suppresses the expression of the HBsAg gene is not clear yet. The promoter region of the HBsAg gene contains two short sequences, GCAGTCAG and AGAGTCAG [40], which are homologous to the consensus AP1 site (TGAGTCAG) [41]. Whether these sequences are responsible for PKC-mediated suppression of HBsAg production is currently under study.

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