Expression of protein kinase C isozymes in guinea pig epidermis: selective inhibition of PKC-β activity by 13-hydroxyoctadecadienoic acid-containing diacylglycerol

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Abstract Prompted by the reversal of skin hyperproliferation to normal by 13-hydroxyoctadecadienoic acid (13-HODE), a 15-lipoxygenase metabolite of linoleic acid, we investigated a possible mechanism for this antiproliferative action. To address this we first demonstrated that 13-HODE is incorporated into epidermal phosphatidyl 4,5-bisphosphate (PtdIns4,5-P_2) and released as 13-HODE-containing diacylglycerol by epidermal phospholipase C. Secondly, we tested the possibility whether this putative 13-HODE-containing DAG (13HODE-DAG) could exert a modulatory effect on epidermal protein kinase C (PKC) activity which previously has been associated with skin hyperproliferation. Unlabeled 13HODE-DAG was generated from 13-HODE-containing phosphatidylcholine after phospholipase C hydrolytic cleavage. The effects of the 13HODE-DAG were determined on: *i)* total epidermal PKC activity; *ii)* dioleinactivated PKC activity; and *iii)* the two identified epidermal PKC-isozymes (PKC-β and PKC-α). Our data revealed over a twofold activation of total basal PKC activity by diolein. In contrast, replacement of diolein (1,2-dioleoylglycerol) with 13HODE-DAG (1-palmitoyl,2-13HODE-glycerol) in the incubation mixture exerted no effect on total basal PKC activity. In an another experiment, 13HODE-DAG inhibited diolein-activated PKC activity in a dose-dependent manner. To determine whether the effects of 13HODE-DAG are selective, we tested its effects on DEAE-Sephacel-purified and Western blot-confirmed PKC isozymes. Our data revealed that 13HODE-DAG selectively inhibited the activity of PKC-β isozyme, while exerting negligible effect on the PKC-α isozyme. This selective inhibitory effect of 13HODE-DAG on a major epidermal PKC isozyme activity suggests that 13HODE-containing DAG seemingly can modulate epidermal PKC activity, which purportedly is associated with epidermal hyperproliferation.—**Cho, Y., and V. A. Ziboh.** Expression of protein kinase C isozymes in guinea pig epidermis: selective inhibition of PKC-β activity by 13-hydroxyoctadecadienoic acid-containing diacylglycerol. *J. Lipid Res.* 1994. **35:** 913–921.

Supplementary key words 13-HODE • 13HODE-PtdIns4,5-P_2 • 13HODE-DAG • PKC • PKC isozymes: (-β, -α) • epidermis • hyperproliferation

Protein kinase C (PKC) belongs to a family of serine/threonine-specific protein kinases and is ubiquitous in most tissues (1). The enzyme seemingly plays an important role in cell surface signal transduction as well as in the control of a variety of physiological/pathophysiological processes such as growth, differentiation, and tumor promotion (2, 3).

A variety of biochemical immunologic and molecular cloning characterizations of the different PKC isozymes have revealed that they can be subdivided into conventional (α, β, γ) and nonconventional (δ, ϵ, ζ, η) classes. The conventional PKCs depend on calcium, phospholipid, and diacylglycerol (DAG) for activation (4–6). In contrast, the nonconventional class lacks a calcium-binding domain, thus their activation appears to be independent of calcium. Although PKC has been implicated in the regulation of many cellular processes such as growth, differentiation, hormone, neurotransmitter release, gene expression, and cellular metabolism (1, 2, 7), the specific roles and control of each of these isozymes on cellular functions are still not fully delineated.

13-Hydroxyoctadecadienoic acid (13-HODE) is a 15-lipoxygenase metabolite of linoleic acid (LA), the most abundant polyunsaturated fatty acid in the skin. We previously reported that this monohydroxy fatty acid does reverse an experimentally induced epidermal hyperproliferation in guinea pig skin (8) after topical application. The mechanism of this reversal is unknown. In an

Abbreviations: PKC, protein kinase C; 13-HODE, 13-hydroxyoctadecadienoic acid; LA, linoleic acid; PtdIns4,5-P_2, phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol; PC, phosphatidylcholine; 13HODE-PtdIns4,5-P_2, 13-HODE-containing PtdIns4,5-P_2; 13HODE-DAG, 13-HODE-containing DAG; PDBu, phorbol-12,13-dibutyrate; TLC, thin-layer chromatography; HPLC, high performance liquid chromatography; PBST, phosphate-buffered saline containing 0.05% Tween 20; PS, phosphatidylserine.

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attempt to delineate the possible mechanism for 13-HODE-induced reversal, we tested the hypothesis that 13-HODE is incorporated into inositol phospholipids (a key phospholipid associated with the signal transduction), and that it is released into diacylglycerol (DAG) as 1-acyl,2-13HODE-glycerol (13HODE-DAG) by epidermal phospholipase C as reported in the preceding report (9). As natural diacylglycerol functions to activate PKC activity, we investigated: *i)* the occurrence of the conventional calcium-, phospholipid-, diacylglycerol-dependent PKC- (α, β) isozymes in the guinea pig epidermis by immunoblotting with isozyme-specific antibodies; and *ii)* we tested whether or not the novel 13HODE-DAG exerts any modulatory effect on the conventional total PKC activity and the PKC-isozymes.

MATERIALS AND METHODS

Materials

1,2-Dioleoylglycerol (diolein) phosphatidylserine (PS), sphingosine, histone (Type III-S), 1-palmitoyl-2-lysophosphatidylcholine (LPC), and DEAE-Sephacel were purchased from Sigma Chemical, Co. (St. Louis, MO). DEAE-cellulose (DE-52) was purchased from Whatman Lab Sales Inc. (Hillboro, OR). [r-^{32}P]ATP and [^{3}H]PDBu (phorbol-12,13-dibutyrate) were purchased from New England Nuclear (Boston, MA). Phospholipase C (*B. cereus*) and glyceride lipase (*C. viscosum*) were purchased from Sigma Chemical, Co. (St. Louis, MO). Monoclonal antibodies specific for each PKC (α, β, δ, ϵ, and ζ) isozyme were purchased from GIBCO BRL (Grand Island, NY). The organic solvents were HPLC grade. All other chemicals were of analytical grade.

Biosynthesis of unlabeled 1-palmitoyl-2-13HODE-glycerol

Preparation of liver microsomal acyltransferase. To determine whether epidermal activity is affected by the novel putative 1-acyl,2-13HODE-glycerol, it was imperative that we synthesize a sufficient amount of unlabeled 13HODE-containing DAG. Specifically, guinea pig liver was removed and homogenized in ice-cold 20 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA and 0.25 M sucrose. The homogenate was first centrifuged at low speed (680 *g*) for 10 min at 4°C to remove debris and nuclear materials. The resulting low-speed supernatant was centrifuged at 105,000 *g* for 90 min to yield a high-speed particulate (microsomal) fraction and a supernatant (cytosolic) fraction. The high-speed supernatant was discarded. The microsomal fraction (which contains acyltransferase) was resuspended at a protein concentration of 1.5 mg/ml in a Tris-HCl (50 mM) buffer (pH 7.4) containing sucrose (0.32 M), CoASH (0.1 mM), dithiothreitol (0.1 mM), ATP (2.5 mM), and $MgCl_2$ (10 mM) for use in the incubations.

Preparation of 1-palmitoyl-2-13HODE-glycerol. To prepare 1-acyl,2-13-HODE-glycerol (13HODE-DAG), commercial 1-palmitoyl,2-lysophosphatidylcholine (LPC) was used as the acceptor lysophospholipid for free 13-HODE. Specifically, excess of 1-palmitoyl,2-lyso-PC (25 μg) and 13-HODE (1.0 μg) were solubilized in 0.1% Triton X-100. To the mixture was added the liver microsomal fraction (containing the acyltransferase) to attain a total volume of 1 ml. The mixture was incubated at 37°C for 20 min and the reaction was terminated by the addition of 2 ml $CHCl_3$–MeOH 2.1 (v/v). The 13HODE-containing phosphatidylcholine (1-palmitoyl,2-13HODE-PC) was extracted by the same solvent mixture and dried under N_2 gas; the residue was redissolved in $CHCl_3$–MEOH 1:1 (v/v) and applied to TLC plates. The silica gel plates were developed in $CHCl_3$–MeOH–HOAc–H_2O 50:37.5:2.0 (v/v/v/v). The putative 1-palmitoyl,2-13HODE-PC was eluted from the silica gel using $CHCl_3$–MeOH 2:1 (v/v) and resuspended in ether for phospholipase C hydrolysis.

Phospholipase C hydrolysis of putative 1-palmitoyl,2-13HODE-PC. To establish that the biosynthesized product eluted from the TLC plate was 1-palmitoyl,2-13HODE-PC, commercial phospholipase C (*B. cereus*) (5 unit/ml) was prepared in Tris-HCl buffer (50 mM), pH 7.4, containing $CaCl_2$, (5 mM). The mixture of 1-palmitoyl,2-13HODE-PC (substrate) and the phospholipase C was incubated at 30°C for 5 h. Incubation was terminated by the addition of 5 ml $CHCl_3$–MeOH 2:1 (v/v) to extract the putative hydrolytic product, (1-palmitoyl,2-13HODE-glycerol). The putative 13-HODE-substituted diacylglycerol was separated by TLC using the solvent system diethyl ether–hexane–acetic acid 30:70:3.5 (v/v/v).

Confirmation of putative 1-palmitoyl,2-13HODE-glycerol (13HODE-DAG). To confirm the formation of 13HODE-substituted-DAG, a portion eluted from the TLC plate containing the putative 13HODE-DAG was hydrolyzed in Tris-HCl buffer (25 μM), pH 8.2, containing $CaCl_2$ (10 μM) and bacterial lipase (*C. viscosum*) (5 unit/ml) at 30°C for 30 min. The release of 13-HODE as the hydrolytic product from 13HODE-DAG was confirmed first by TLC using the solvent system petroleum ether–diethyl ether–methanol–acetic acid 80:20:2.5:1 (v/v/v/v), and second by HPLC at a characteristic wavelength of 238 nm, using an authentic standard of 13-HODE as reported previously (8, 10).

Preparation of total cytoplasmic protein kinase C from guinea pig epidermis

To discern whether the biosynthesis of 13HODE-containing diacylglycerol (13HODE-DAG) also implied that this compound could modulate the epidermal protein kinase C (PKC) activity, we prepared cytoplasmic PKC

enzymes from guinea pig epidermis. Specifically, guinea pig epidermis was homogenized in ice-cold 20 mM Tris-HCl buffer (pH 7.5) containing 2 mM EDTA, 0.5 mM EGTA, 330 mM sucrose, and 2 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was first centrifuged at low speed (680 *g*) to obtain the low-speed supernatant fraction. This fraction was subjected to differential ultracentrifugation at 105,000 *g* for 90 min to obtain the high-speed particulate (membrane) and the soluble (cytoplasmic) fractions. The particulate fraction was discarded. The resulting high-speed 105,000 *g* cytoplasmic fraction that contained epidermal total PKC enzymes was applied onto a column packed with ion exchange DEAE-cellulose (DE-52, Whatman; 0.9 × 2.6 cm) gel. Partial purification of the cytoplasmic total PKC enzymes from other proteins was achieved by linear gradient elution using 15 ml each of buffer A (20 mM Tris-HCl, pH 7.5, 2 mM EDTA, and 2 mM PMSF) followed by buffer A containing 200 mM NaCl as previously described (11). Column elution was at a rate of 1 ml/3 min. Each 1-ml fraction was collected and assayed for total PKC activity. The fractions with PKC activity were pooled and represented the total PKC activity. The protein concentration in each 1-ml fraction was determined by the method of Lowry et al. (12) using bovine serum albumin as a standard.

Assay of total cytosolic protein kinase C activity

The total epidermal protein kinase C activity was assayed as previously described (13). Briefly, the total PKC activity from each 1-ml column effluent was assayed in 0.25 ml of 20 mM Tris-HCl buffer (pH 7.5), containing $MgCl_2$ (10 mM), $CaCl_2$ (500 μM), phosphatidylserine (0.2 mg/ml), 1,2-dioleoylglycerol (diolein) (4.0 μM), histone III-S substrate (50 μg/ml), [γ-^{32}P]ATP (3×10^5 cpm/nmol, 10 μM), and a 100-μl aliquot of the epidermal cytosolic effluent fraction (approximately 50 μg protein). Phosphatidylserine (PS) and diolein were suspended first in 0.3% Triton X-100 prior to addition to the assay mixture. To test the effects of the putative 13HODE-DAG on diolein-activated total cytosolic PKC activity, varying concentrations (2.0, 4.0, 6.0, 8.0, and 10.0 μM) of putative 13HODE-DAG and fixed concentration of PS were suspended in 0.3% Trition X-100 in separate tubes, prior to addition to the assay mixture as described above. Incubations were carried out at 30°C for 10 min, and then terminated by the addition of 2 ml of 25% trichloroacetic acid (TCA). The precipitated labeled proteins were collected with a Millipore HA filter (0.45 μm). Each filter was washed eight times with 3 ml each of 5% TCA and the radioactivity was counted in a Beckman LS3501 liquid scintillation counter using Beckman Ready gel. Control experiments had no cytosolic extract. The nonspecific radioactivity from the control incubations was subtracted from those with total PKC extract prior to estimating enzyme activity. The enzymatic activity was expressed as ^{32}P incorporated (cpm)/mg protein.

Fractionation of total epidermal cytosolic PKC enzyme into its constituent PKC isozymes

In order to delineate the effects of the putative 13HODE-DAG on each cytoplasmic PKC isozyme, the previously partially purified total epidermal cytosolic PKC enzyme was fractionated into constituent PKC isozymes according to the method described by Burns et al. (5) with slight modification. Briefly, the partially purified total cytoplasmic PKC fraction was applied to a second column packed with DEAE-Sephacel (1.5 × 12 cm). The column was pre-equilibrated with buffer B, containing Tris-HCl (50 mM), pH 7.5, mercaptoethanol (50 mM), EGTA (2 mM), and 0.1% Triton X-100. The separation of the cytoplasmic PKC isozymes was achieved by linear gradient elution using 30 ml buffer B and buffer B plus NaCl (200 mM), respectively. The column eluents were collected at a rate of 1 ml/3 min. Each 1-ml eluent was assayed for PKC activity as described previously. Protein concentration in each 1-ml eluent was determined by the method of Lowry et al. (12). Column eluents containing PKC activity were pooled into five separate fractions. Each of the five fractions was subjected to further fractionation by SDS-PAGE (10%) as described under Methods. The proteins were transblotted to nitrocellulose membrane, washed, and incubated with specific monoclonal antibodies against PKC isozymes (α, β, δ, ϵ, and ζ).

Identification of epidermal PKC isozymes by Western blot assay

To ascertain the nature of the specific isozymes in the epidermal cytosolic fraction, approximately 30 μg protein from each of the five pooled fractions with PKC enzyme activity was subjected to further fractionation by sodium dodecyl sulfate, polyacrylamide gel electrophoresis (SDS-PAGE) (10% gel) as described previously (5). The Western blot assay was performed according to the modified procedure by Ohno et al. (14). After separation of the enzyme proteins, the gel was electrophoretically transblotted onto a nitrocellulose membrane and washed with phosphate-buffered saline containing 0.05% Tween 20 (PBST). Each nitrocellulose membrane was soaked in 5% dry non-fat, skim milk in PBS to reduce nonspecific antibody binding. The nitrocellulose membrane was again washed in PBST three times and incubated successively for 30 min with specific monoclonal antibodies against PKC isozymes (α, β, δ, ϵ, ζ). After each incubation, the membrane was washed with PBST and then incubated with the secondary antibody (purified rabbit anti-goat IgG) (Vector Lab, Burlingame, CA) at room temperature for 1 h. The nitrocellulose membrane was again washed with PBST and then stained by incubation with Vectastain ABC reagent (Vector Lab, Burlingame,

CA) for 1 h. This was followed by reaction with the staining substrate (0.5 mg/ml of 3,3-diamino benzidine and 0.01% H_2O_2 dissolved in PBS).

[^{3}H]phorbol dibutyrate (PDBu) binding assay

Guinea pig epidermal homogenate was prepared by low speed (680 *g*) centrifugation after removing the debris as described above. The aliquots of epidermal homogenate containing 25 μg protein were preincubated in 200 μl assay buffer of 20 mM Tris-HCl (pH 7.5) containing $CaCl_2$ (500 μM), $MgCl_2$ (10 mM), phosphatidylserine (0.2 mg/ml), and varying concentrations (2.0, 4.0, 6.0, 8.0, and 10 μM) of 13HODE-DAG at 23°C for 10 min. Phosphatidylserine (PS) and 13HODE-DAG were suspended in 0.3% Triton X-100 prior to addition to the assay buffer. One hundred nM of [^{3}H]PDBu was added and incubated at 23°C for 45 min. The incubations were terminated by the addition of 4 ml cold incubation assay buffer. The samples were filtered on Whatman GF/C filters, washed three times with 3 ml assay buffer to remove free [^{3}H]PDBu, and the washed filters were counted in 10 ml Aquasol II in a Beckman LS3501 counter (15). Specific binding was calculated by subtracting nonspecific binding in the presence of 1000-fold unlabeled PDBu from total binding.

Statistical analysis

Standard statistical methods were used to determine the mean values. For comparison between the observations, Student's *t*-test was used. The probability (*P*) that statistical significance was reached was determined at levels of 0.05 and below.

RESULTS

Concentration-dependent effect of putative 13HODE-DAG on total epidermal cytosolic PKC activity

Diolein (1,2-dioleoylglycerol) was used in our study as a positive control because its stimulatory effect on PKC activity is well established. The partially purified cytosolic extract was delipidized by cold diethyl ether extraction to remove endogenous cellular lipids (particularly diacylglycerols) prior to enzyme assay. The concentration dependent effects of diolein and putative 13HODE-DAG (1-palmitoyl,2-13HODE-glycerol) on DEAE-cellulose purified total cytoplasmic PKC activity is shown in **Fig. 1.** As shown in Fig. 1A, increasing addition of exogenous diolein to the assay mixture containing delipidized epidermal extract stimulated basal total PKC activity (approximately 150%). This stimulatory effect was dose-dependent and maximal at 4.0 μM.

In contrast, as shown in Fig. 1B, increasing addition of exogenous putative 13HODE-DAG to the assay mixture containing delipidized epidermal extract exerted no effect on basal total PKC activity at all concentrations tested. When similar experiments were conducted with non-delipidized epidermal extracts, 13HODE-DAG exerted an inhibitory effect on basal PKC activity, suggesting that

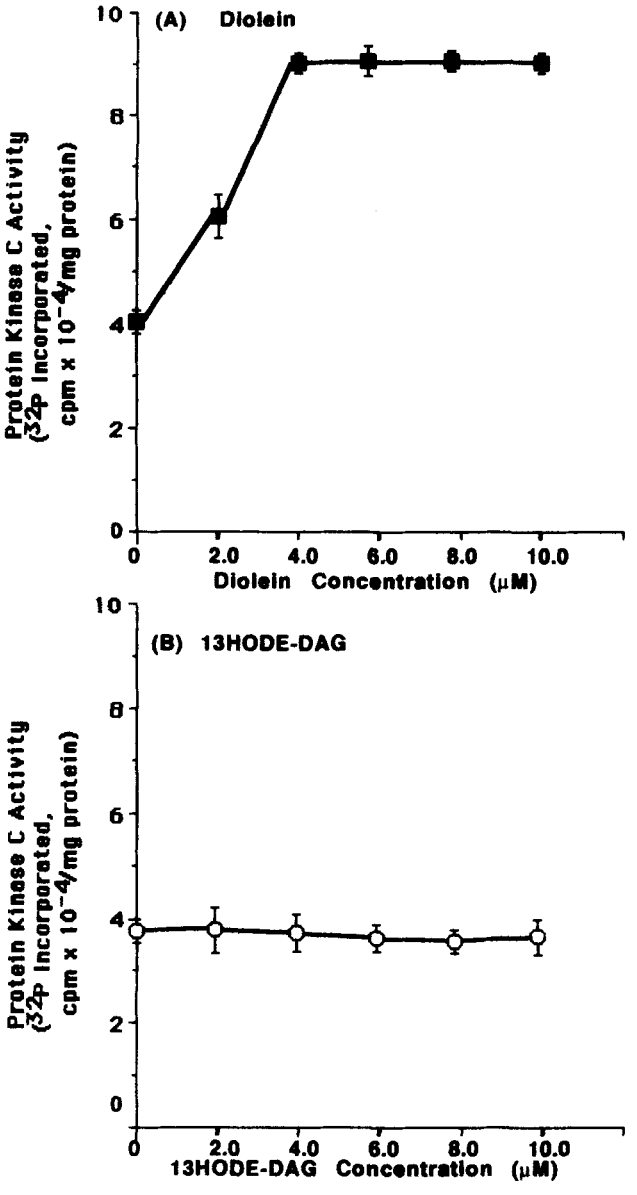

Fig. 1. Concentration-dependent effect of putative 13HODE-DAG on total epidermal cytosolic PKC activity. Epidermal cytosolic PKC enzyme was prepared as described in Methods. The cytosolic extract was delipidized with ice-cold diethyl ether to remove endogenous lipids (particularly diacylglycerols). Total PKC activity was assayed in 20 mM Tris-HCl buffer (pH 7.5) containing 10 mM $MgCl_2$, 0.2 mg/ml histone III-S, 10 μM [γ-^{32}P]ATP, 500 μM $CaCl_2$, 0.2 mg/ml phosphatidylserine, 100 μl (50 μg protein) of cytosolic fraction, and 0.3% Triton X-100 with varying amounts of diolein or 13HODE-DAG. Data are means (± SD) (n = 12) from three separate experiments. Fig. 1A. shows the concentration-dependent effect of diolein whereas Fig. 1B. shows the concentration-dependent effect of putative 13HODE-DAG.

13HODE-DAG seemingly displaced endogenous cellular DAG from the active lipid-enzyme complex. Although putative 13HODE-DAG contains palmitoyl in the *sn*-1 position of the glycerol molecule in contrast to oleoyl in the *sn*-1 position of diolein, it is unlikely that the effects by 13HODE-DAG, as shown in Fig. 1B, are associated with the palmitic acid in the *sn*-1 position. Indeed, it has been reported that 1-palmitoyl,2-linoleoyl-glycerol exerts a similar stimulatory effect on PKC activity as diolein (16) used in these experiments.

Concentration-dependent effects of putative 13HODE-DAG on diolein-activated total cytosolic PKC activity

As diolein is a natural activator of PKC activity, we next tested the effect of putative 13HODE-DAG on diolein-activated PKC. The incubation mixture used to attain maximal diolein-activated PKC activity by the 0.3% Triton X-100 mixed micelle assay contained diolein (4 μM), PS (0.2 mg/ml), $CaCl_2$ (500 μM), and approximately 100 μl (50 μg) of total cytoplasmic PKC fraction and varying amounts of 13HODE-DAG. The concentration-dependent inhibition of diolein-activated total epidermal cytosolic PKC activity by the putative 13HODE-DAG is shown in **Fig. 2.** When maximal diolein is taken to be 100%, putative 13HODE-DAG inhibited diolein-activated PKC activity in a dose-dependent manner. At 4 μM, it inhibited diolein-activated PKC approximately 36.2%, and at 10.0 μM, 13HODE-DAG inhibited diolein-activated PKC activity approximately 50%.

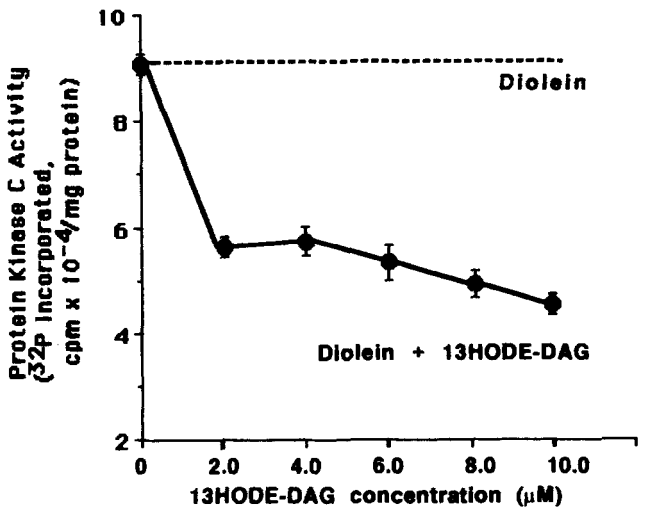

Fig. 2. Concentration-dependent effect of putative 13HODE-DAG on diolein-activated total epidermal cytosolic PKC activity. Epidermal cytosolic PKC enzyme was prepared as described in Methods. Diolein-activated PKC activity was assayed in 20 mM Tris-HCl buffer (pH 7.5) containing 10 mM $MgCl_2$, 50 μg histone III-S, 10 μM [γ-^{32}P]ATP, 500 μM $CaCl_2$, 0.2 mg/ml phosphatidylserine, 4 μM diolein, 100 μl (50 μg of protein) of cytosolic fraction, and 0.3% Triton X-100 with varying amounts of 13HODE-DAG. The data show the concentration-dependent effect of putative 13HODE-DAG on diolein-activated total epidermal cytosolic PKC activity. Data are means (± SD) (n = 12) from three separate experiments.

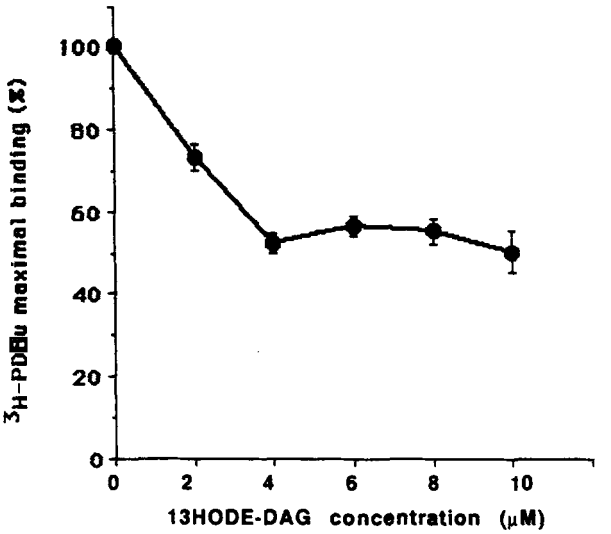

Fig. 3. Inhibition of phorbol dibutyrate binding by 13HODE-DAG. Epidermal homogenate was prepared as described in Methods. Phorbol ester binding was determined in Tris-HCl buffer (20 mM), pH 7.5, containing an aliquot of epidermal homogenate (25 μg protein, 200 μl buffer) $CaCl_2$ (500 μM), $MgCl_2$ (10 mM), phosphatidylserine (0.2 mg/ml), [^{3}H]PDBu (100 nM), and 0.3% Triton X-100, with varying concentrations of 13HODE-DAG at 23°C for 45 min. The data show the concentration-dependent effect of putative 13HODE-DAG on phorbol ester binding. Data are means (± SD) (n = 10) from two separate experiments.

Inhibition of phorbol ester binding by 13HODE-DAG

The results obtained in Fig. 2 prompted us to investigate the mechanism for 13HODE-DAG inhibition of diolein-activated PKC. As phorbol ester is known to exert similar action on PKC as diacylglycerol, we tested the effect of 13HODE-DAG on phorbol ester ([^{3}H]PDBu) binding to epidermal extract as described under Methods. First we demonstrated saturable and displaceable binding of [^{3}H]PDBu to epidermal homogenate (data not shown). As shown in **Fig. 3,** [^{3}H]PDBu binding was inhibited by 13HODE-DAG in a dose-dependent manner. At 2 μM and 10 μM, 13HODE-DAG inhibited phorbol ester binding approximately 26.8% and 50.2%, respectively. The concentration-dependence of 13HODE-DAG inhibition of phorbol ester binding paralleled its ability to inhibit diolein-activated total cytosolic protein kinase C activity (Fig. 2). Thus, 13HODE-DAG inhibition of phorbol ester binding (Fig. 3) is consistent with the view that 13HODE-DAG inhibition may, like sphingosine, interfere with the regulatory domain of protein kinase C.

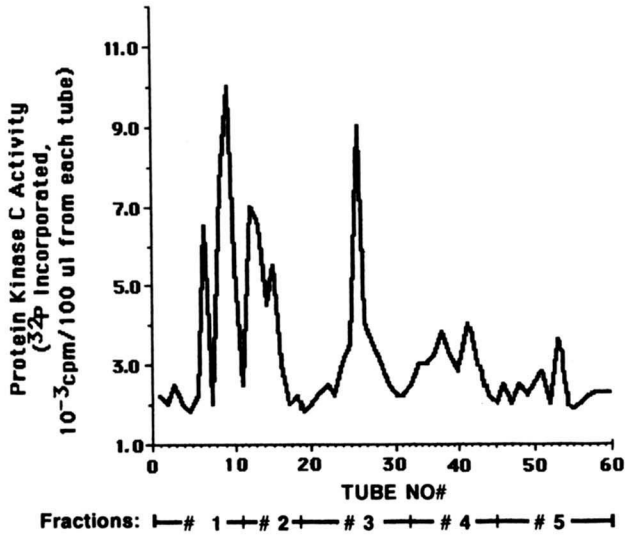

Fig. 4. Partial purification of cytosolic PKC isozyme on DEAE-Sephacel ion exchange chromatography. Purified total epidermal cytosolic PKC was subjected to chromatography on DEAE-cellulose as described in Methods. Five pooled fractions (fraction #1 (tubes 1–10), fraction #2 (tubes 10–20), fraction #3 (tubes 20–30), fraction #4 (tubes 30–45), and fraction #5 (tubes 45–60)) with PKC activity were retained for further separation by SDS-PAGE and identification of PKC isozymes with specific monoclonal antibodies.

Identification of epidermal PKC isozymes by Western blot assay

Partial purification of cytosolic PKC isozymes by ion-exchange chromatography. To determine whether the inhibitory effect of putative 1-palmitoyl,2-13HODE-glycerol (13HODE-DAG) was generalized on all the PKC enzymes or whether it was selective on specific PKC isozymes, we fractionated the partially purified total epidermal cytosolic PKC on a second DEAE-Sephacel column. A typical chromatographic separation of the total cytosolic PKC is shown in **Fig. 4.** Five fractions with PKC activity were pooled: fraction 1 (tubes 1–10); fraction 2 (tubes 10–20); fraction 3 (tubes 20–30); fraction 4 (tubes 30–45); and fraction 5 (tubes 45–60).

To establish the identities of the specific PKC isozymes in the five fractions, an aliquot (30 μg protein) from each fraction was subjected to further fractionation by SDS-PAGE followed by electrophoretic transfer of the proteins onto nitrocellulose membrane (5). The membrane was incubated successively with specific monoclonal antibodies against the following PKC isozymes (α, β, δ, ϵ, ζ) as described in the Methods section. Only fraction 1 stained positively with monoclonal anti-PKC-β. The immunoreactive band corresponded to a molecular mass marker of 80 kDa (**Fig. 5A**). Similarly, fraction 3 stained positively with monoclonal anti-PKC-α (Fig. 5B), with an immunoreactive band corresponding to 80 kDa. Fractions 2, 4, and 5 revealed negligible immunoreactive presence of PKC isozymes either to α, β or to the noncalcium-dependent δ, ϵ, and ζ (data not shown). Thus, normal guinea pig epidermis expresses mainly the two conventional, calcium-dependent PKC-β and PKC-α. These findings are consistent with the reported immunohistological identification of these PKC isozymes in normal human epidermis (17).

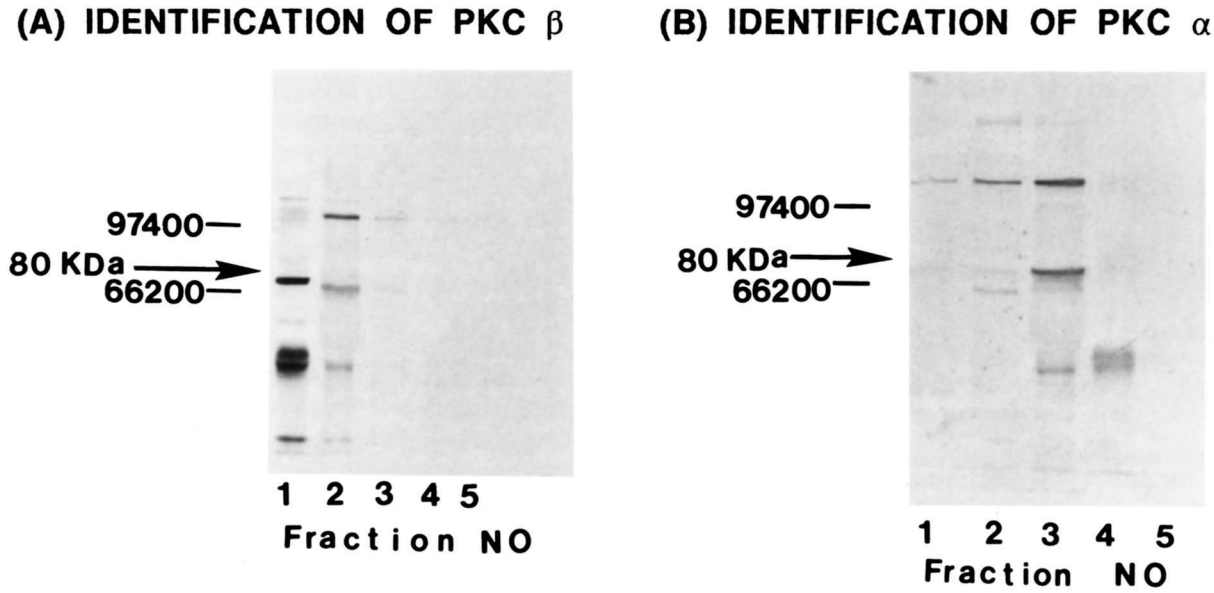

Fig. 5. Identification of PKC isozymes by Western blot assay. To confirm the PKC isozymes in each fraction (Fig. 4) approximately 30 μg protein from each of the five pooled fractions was subjected to separation by SDS-PAGE in 10% gel as described in Methods. Proteins were transblotted to nitrocellulose paper. Western blot assay was performed for the immunostaining of PKC β (FIG. 5A) and α (FIG. 5B) with specific monoclonal PKC isozyme antibodies as described in text.

Effect of putative 13HODE-DAG on epidermal cytosolic PKC isozymes

The inhibition of total cytosolic PKC activity prompted us to determine whether or not the effect of 13HODE-DAG was on all the PKC isozymes or on specific PKC isozymes. We therefore determined the effect of 13HODE-DAG on PKC-β and PKC-α isozymes (the two identified isozymes). Control incubations contained 4 μM diolein, and the test incubation contained 4 μM diolein plus 4 μM 13HODE-DAG. The relative effects of 13HODE-DAG on the epidermal diolein, Ca^{2+}/phospholipid-activated cytosolic PKC isozyme activities are shown in **Fig. 6.** Our data revealed that PKC-β isozyme activity was selectively inhibited approximately 36.1% by 4 μM of the putative 13HODE-DAG, whereas its effects on the PKC-α isozyme at similar concentration were negligible.

Effects of sphingosine on diolein-activated total cytosolic PKC activity

It has been reported that sphingosine (a natural product of lipid metabolism) is a potent and reversible inhibitor of PKC activity in vitro, and of tetradecanoylphorbol-13-acetate (TPA)-induced activation of human platelets, leukocytes, and mouse skin (15, 18–20). Therefore, we tested the dose-dependent effects of sphingosine on diolein-activated total PKC activity. The data in **Fig. 7** revealed that sphingosine at 2 μM inhibited diolein-activated total PKC activity approximately 38.0%. At 10 μM, sphingosine inhibited total cytoplasmic PKC activity approximately 60%. A similar inhibitory effect of 13HODE-DAG at 10 μM was approximately 50% (Fig. 2).

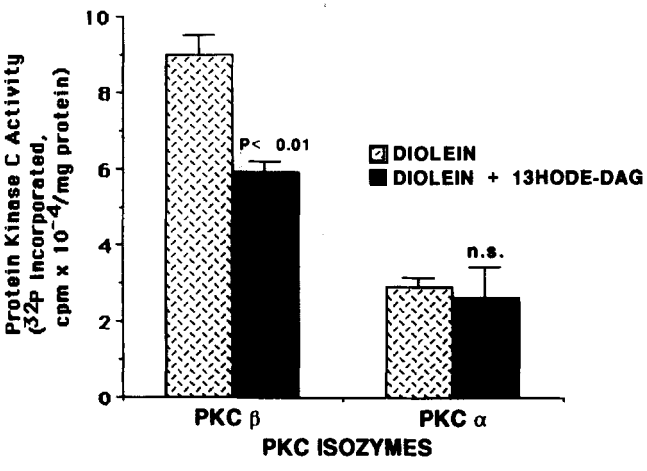

Fig. 6. Effect of putative 13HODE-DAG on diolein-activated epidermal cytosolic PKC isozyme activity. Epidermal cytosolic PKC isozymes were prepared as described in the text. Each PKC activity was assayed in an incubation mixture as described under Fig. 2 containing either 4 μM diolein or 4 μM 13HODE-DAG. After incubation, the reaction was terminated by 25% TCA and the radioactivity in precipitated protein was counted on a scintillation counter. Data are means (± SD) (n = 12) from three separate experiments.

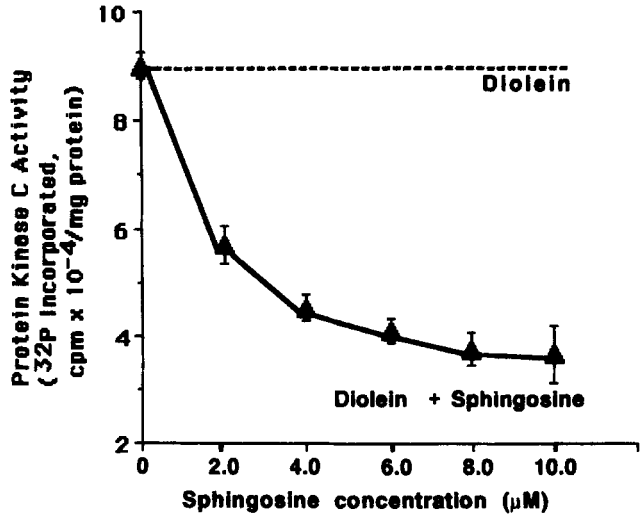

Fig. 7. Effects of sphingosine on diolein-activated total epidermal cytosolic PKC activity. Epidermal cytosolic PKC enzyme was prepared as described in the text. Total PKC activity was assayed in an incubation mixture containing 4 μM diolein and various concentrations of sphingosine. The figure shows the concentration-dependent effect of sphingosine. Data are means (± SD) (n = 12) from three separate experiments.

DISCUSSION

Diaylglycerol (DAG) derived from the hydrolysis of inositol phospholipids or phosphatidylcholine by phospholipase C is the most potent in vivo physiological activator of protein kinase C (PKC). This activation has been associated with the regulation of cellular proliferation (21, 22). It has also been proposed that diacylglycerol may act as phorbol esters to increase cytoplasmic pH (23, 24). Such as increase in pH is a characteristic feature of growth factors and is believed to play an important role in cell growth (25–27).

The specificity of PKC activation by *sn*-1,2-diacylglycerols has been investigated by several investigators (16, 28, 29). Mori et al. (16) demonstrated that only one unsaturated fatty acyl moiety esterified to glycerol seems to play an essential role in PKC activation. Molleyres and Rando (29) also suggested that a certain level of hydrophobicity in DAG, such as long-chain unsaturated fatty acids and saturated medium-chain fatty acids, is required to fulfill the PKC activation. Taken together, it seems certain that at least only one unsaturated fatty acyl moiety is necessary for PKC activation. While the full mechanism(s) of the free 13-HODE remains to be elucidated, it is reasonable to speculate that the generation of a second messenger-substituted-DAG such as 1-acyl,2-13HODE-glycerol raises the possibility that the hydrophilic substitution of hydroxy fatty acid instead of an unsaturated fatty acid to DAG could modulate normal 1,2-diacylglycerol-activated epidermal protein kinase C activity. This, in

turn, can seemingly modulate cellular proliferation/differentiation as well as other transmembrane signalling processes that are associated with PKC activity. Interestingly, our data in Fig. 2 revealed that 13-HODE-substituted DAG (13HODE-DAG) inhibited dolein-activated PKC activity.

Protein kinase C (PKC) is a calcium/phospholipid-dependent kinase that now is recognized to be involved in the regulation of growth, differentiation, and tumor promotion (1–3). The primary structure of PKC has revealed two functional domains, a catalytic domain and a regulatory domain (30). The catalytic domain is homologous to other protein kinases (30). The cysteine-rich regulatory domain distinguishes one protein kinase from other protein kinases and has a binding region for calcium, phospholipids, and diacylglycerol/phorbol esters (30). Several potent and specific inhibitors of PKC have been found and can be classified into two groups depending on whether or not their target is the catalytic or regulatory domain (31). The inhibitors targeted to the catalytic domain, such as staurosporine and calphostin, are not selective for PKC (31). In contrast, the inhibitors targeted to the regulatory domain with calcium, phospholipid, and diacylglycerol/phorbol ester show a high degree of selectivity and sphingosine belongs to this group of inhibitors (31). The parallelism of PKC inhibition by 13HODE-DAG and sphingosine as evidenced in this study (Fig. 2 and Fig. 7) suggests that 13HODE-DAG may, as sphingosine, prevent the formation of an active lipid-enzyme complex by displacement of activator (diolein) from the complex. This suggested mechanism of 13HODE-DAG inhibition of PKC activity was further substantiated with the inhibition of phorbol ester binding as revealed in Fig. 3.

The significance of the monohydroxy fatty acid (13-HODE) in the biology of the normal epidermis is not fully understood. High levels of this monohydroxy fatty acid derived from 18-carbon linoleic acid and others derived from 20-carbon arachidonic acid (15-HETE, 12-HETE) have been reported in lesional scales from psoriatic patients (32). More recently, the topical application of 13-HODE to docosahexaenoic acid (DHA, 22:6n-3)-induced hyperproliferation of guinea pig skin was reported to reverse the lesions towards a normal state (8). This interesting observation suggests that 13-HODE, singly or in complex with other molecules in vivo, can modulate epidermal hyperproliferation. The parallelism of PKC inhibition (Fig. 6) by 13-HODE-DAG and sphingosine (a reported inhibitor of 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-induced skin hyperproliferation) supports the view that reversal of epidermal hyperproliferation by 13-HODE may be, at least in part, due to its release as 13-HODE-substituted-diacylglycerol (13HODE-DAG) and inhibition of DAG-activated protein kinase C activity. Interestingly, altered metabolism of the inositol phospholipids resulting in elevated epidermal PKC activity and DAG content has been reported (33) in hyperproliferative lesion of psoriasis.

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