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sn-1,2-Diacylglycerols and Phorbol Diesters: Uptake, Metabolism, and Subsequent Assimilation of the Diacylglycerol Metabolites Into Complex Lipids of Cultured Cells

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The cell-permeable diacylglycerol mediators have been shown to mimic partially the effects of 12-O-tetradecanoylphorbol-13-acetate (TPA) on cultured cells. In order to evaluate the metabolic stability of the lipid mediators, several radiolabeled diacylglycerols were synthesized and their uptake and intracellular fate in cultured HL-60 (human promyelocytic leukemia) cells was compared with TPA. In addition to whole cell assessment, the stability of diacyl lipids and TPA was evaluated in a buffer/water system and in the presence of serum and subcellular fractions. The compounds studied include 1,2-dioleoyl-*sn*-glycerol (DiOG), 1-oleoyl-2-acetyl-*sn*-glycerol (OaG), 1-palmitoyl-2-acetyl-*sn*-glycerol (PaG), the ether-linked analog 1-palmityl-2-acetyl-*sn*-glycerol (ePaG), and TPA. TPA was comparatively stable to lipase hydrolysis in all systems examined. First, the data show that within 5 min at pH 7.9, nearly 50% of the PaG (originally > 92% 1,2-isomer) had isomerized, and rapid formation of the 1,3-isomer also occurred with OaG and ePaG. The metabolism of OaG and PaG by serum hydrolases, using a reaction medium containing 10% serum, was chiefly by acetate hydrolysis; however, fatty acid was also liberated. After a 60-min incubation 68% of the [^{14}C]OaG was converted, by serum enzymes, to monooleoylglycerol plus oleic acid. Heat-inactivation of serum reduced the enzymatic formation of fatty acid by 60–70%. ePaG was also metabolized by serum enzymes, but the ether-linked alkylglycerol product was stable. The results of cell-free studies (postmitochondrial supernatant) showed that cellular enzymes were present that could, like serum, convert the diacylglycerols to monoacylglycerols and free fatty acids. Studies using cultured cells showed that radiolabeled OaG, PaG, and ePaG were rapidly taken up by the cells and metabolized. Labeled metabolic products from the diacylglycerols appeared, in a time-dependent manner, in cellular phospholipids and triacylglycerols. The results from experiments employing 1-acyl-2-acetyl-*sn*-[^{3}H]glycerol and [^{3}H]acyl-2-acetyl-*sn*-glycerol indicate that the intracellular mode of mediator metabolism is via complete hydrolysis with subsequent incorporation of ^{3}H-acyl

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groups into complex lipids. Data are also presented which show that a substantial amount of cellular lipid acyl group modification occurs and large amounts of glycerol are produced when cells are cultured with OaG. Collectively, these results demonstrate that the diacylglycerol mediators, when compared with TPA, are not stable and are metabolized by both serum and cellular enzymes. In view of this some of the cellular affects of OaG exposure could be directed by mediator metabolites or be the result of membrane modification.

Key words: 12-O-tetradecanolylphorbol-13-acetate, 1-oleyl-2-acetyl-*sn*-glycerol, HL-60 cells, membrane modification

Phorbol diester tumor promoters have been valuable tools in biochemical and cellular research. 12-O-Tetradecanoylphorbol-13-acetate (TPA) has been used in studies to delineate the mechanism of tumor promotion [1,2], but in addition to the cocarcinogenic action, TPA alone can inhibit or induce cellular differentiation [3]. The human promyelocytic cell line, HL-60, when cultured in the presence of TPA, undergoes terminal differentiation to a cell demonstrating morphological and biochemical markers characteristic of macrophages [4,5]. Because phorbol diesters, which have little physiological significance in mammalian systems, are strong effectors of mammalian cellular response, it has been suspected that they act by mimicking natural cellular agents. Although the mechanism by which TPA enhances carcinogenesis and induces or inhibits cellular differentiation is largely unknown, recent discoveries show that phorbol esters act through a specific receptor, protein kinase C [6,7].

Protein kinase C, which is activated by Ca^{++}, phospholipid, and diacylglycerols[1] [8], is now thought to play a major role in cell signal transduction [9,10]. Among the most intriguing discoveries in this area has been the demonstration that phorbol diesters activate protein kinase C directly [11], and by so doing replace the diacylglycerol requirement of the enzyme. This reaction circumvents the usual path of diacylglycerol production via receptor-mediated hydrolysis of phospholipid by phospholipase C [9]. It now appears that the biological activity of phorbol esters is due, in part, to the ability of these agents to readily enter the cell and imitate the action of diacylglycerols, the lipid second messengers. Such research has spawned the advent and use of new cell-permeable diacylglycerol analogs such as oleoylacetylglycerol (OaG), palmitoylacetylglycerol (PaG), and dioctanoylglycerol (DiC8) [12–15]. Whereas these agents have been used extensively as cellular mediators, very little is known about their stability in biological systems. The metabolism of phorbol diesters has been studied in various cells and tissues [see ref. 16 for brief review]. Although several enzymes that hydrolyze the acetate and/or tetradecanoate moieties of TPA have been described [16], phorbol esters, as far as is known, cannot be utilized as substrates in anabolic reactions, and therefore degradation is the sole means of removal. We, in former studies, have substantiated the idea that TPA parrots diacylglycerols by examining the substrate specificity of a rat serum lipase [17,18]. This enzyme hydrolyzes the myristic acid ester of TPA and the long chain esters of the synthetic mediators [17–19]. These long chain-short chain agents are structurally similar to the diradyl group of TPA, but they are hydrolyzed by serum lipases at 70–

[1]The term diacylglycerol is used collectively for diradyl compounds irrespective of aliphatic chain length and chemical bond (ester, ether).

120 times the rate of TPA [18,19]. Therefore, when compared to the acylacetylglycerol mediators, phorbol diesters are relatively stable compounds.

Diacylglycerols are a new class of bioactive agents. Many recent works have shown that the synthetic analogs are partially active at the cellular level, producing effects similar to TPA, but are unable to fully mimic the phorbol diester response [20–24]. Because the cell-permeable diacylglycerols are used widely in cell biology and biochemistry, we have explored their stability and metabolic fate in HL-60 cells, cells that are target-specific for phorbol diester-induced differentiation, and in serum-containing tissue culture media. Our data show that TPA is not metabolized by cells and is only slowly hydrolyzed by serum lipases. Conversely, the acylacetylglycerol mediators are readily hydrolyzed by serum enzymes and are likewise rapidly metabolized by cells. Therefore, these findings should be considered when evaluating the biological responses of the cell-permeable lipids. When used at high dose, the metabolism of these agents and subsequent assimilation of metabolic products into complex cellular lipids could be influential in governing cell growth and behavior via cellular membrane lipid modification. Therefore, cell response to the lipid agents may not necessarily involve a protein kinase C-mediated mechanism.

MATERIALS AND METHODS

Materials

1-Alkyl-2-acetyl-*sn*-glycerophosphocholine (PAF, platelet-activating factor) and lysopalmitoyl phosphatidylcholine were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). Lysooleoyl phosphatidylcholine, phospholipase C (*Bacillus cereus*), phospholipase A_2 (*Crotalus adamanteus*), and fatty acid-poor bovine serum albumin (BSA) were products of Sigma Chemical Co. (St. Louis, MO). *Rhizopus delemar* lipase was purchased from Miles Laboratories, Inc. (Elkhart, IN). [Alkyl-1′-2′-^{3}H]-PAF (44.0 Ci/mmol), L-1-[oleoyl-1-^{14}C]lysooleoyl phosphatidylcholine (57.0 mCi/mmol), [9,10-^{3}H(N)palmitic acid (27.5 Ci/mmol), [9,10-^{3}H(N)]-triolein (70 Ci/mmol), [20(N)-^{3}H]phorbol-12-myristate-13-acetate (6.5 Ci/mmol), and [2-^{3}H]glycerol (9.0 Ci/mmol) were obtained from New England Nuclear (Boston, MA). [^{14}C]Triolein (carboxyl [^{14}C]; 52 mCi/mmol) was purchased from Research Products International (Mount Prospect, IL). Tissue culture media were purchased from Gibco Laboratories (Grand Island, NY); fetal bovine serum (FBS) was obtained from HyClone (Logan, UT). TPA was purchased from CCR, Inc. (Eden Prairie, MN).

Cell Culture and Manipulations

Suspensions of human Leukemia (HL-60) cells were maintained as previously described [25]. The cells were subcultured every 4–6 days and were given 5 ml of fresh media approximately 24 hr prior to experimental manipulations. For cell-free assays the cells were harvested by centrifugation, washed twice in PBS (phosphate-buffered saline), and sonicated (3.0×10^6 cells/ml PBS). The cell sonicate was centrifuged (Eppendorf microfuge) at 4°C for 10 min, and the supernatant was used as an enzyme source. Protein was determined by the method of Lowry et al [26].

Preparation of Substrates

Radiolabeled 1-palmitoyl-2-acetyl-*sn*-glycerol (PaG) was prepared using LM (mouse fibroblasts) cell monolayers [27]. Approximately 10 μCi of [9,10-

^{3}H(N)]palmitic acid was added (in 10 μl absolute ethanol) to confluent 10-ml cultures (75-cm^2 tissue culture flasks). After a 24-hr incubation period the cells were harvested by scraping with a rubber policeman, washed twice in PBS, and the lipids were extracted by the method of Bligh and Dyer [28]. Total cellular lipids were treated with phospholipase A_2, and the lyso lipids were acetylated using acetic anhydride [18,29]. The reaction products were treated with phospholipase C [18], and the resulting acylacetylglycerols were purified by thin-layer chromatography [18]. As LM cells contain predominately palmitic and stearic acid in the *sn*-1 position of the phospholipids [30], the biosynthetically produced labeled substrate, designated PaG, contains a similar mixture of acyl groups at the *sn*-1 position. 1-Palmitoyl-2-acetyl-[2-^{3}H]-*sn*-glycerol was prepared in a similar manner by in situ labeling of REF52 cells [31] with 100 μCi of [2-^{3}H]glycerol. Synthesis of other diacylglycerols and related preparative procedures have been described previously [12,17,18,29]. 1-[^{3}H]-Palmityl-2-acetyl-*sn*-glycerol (ePaG) was prepared by treating [alkyl-1′2′-^{3}H]PAF with phospholipase C [18]. 1-[Oleoyl-1-^{14}C]-2-acetyl-*sn*-glycerol (OaG) was synthesized by acetylation of L-1-oleoyl-1-^{14}C]lysooleoyl phosphatidylcholine followed by phospholipase C treatment [18]. [^{14}C]Dioleoylglycerol (rac) was prepared by treating the corresponding [^{3}H]trioleoylglycerol with *Rhizopus delemar* lipase [18].

Whole Cell and Cell-Free Metabolic Studies

Details of the cell-free assay conditions are given in the figure legends. Short-term incubations (5–90 min) with HL-60 cells were carried out at 37°C in a Dubnoff metabolic shaking incubator using RPMI-1640 medium buffered with 20 mM HEPES, pH 7.2, and containing 50 μg BSA/ml. In the 24-hr studies, cells were cultured at 37°C in a humidified incubator with an atmosphere of 5% CO_2, 95% air, using chemically defined, serum-free RPMI-1640 medium [32] modified to contain 20 mM HEPES buffer and 100 μg BSA/ml. The radiolabeled compounds were mixed with the corresponding unlabeled lipid to achieve the desired radiospecific activity and added to the cell suspensions in 5–10 μl of absolute ethanol. Reactions were terminated by the addition of lipid extraction solvents [28]. Radiolabeled products and metabolites were resolved by thin-layer chromatography (Silica Gel G) and quantitated by liquid scintillation spectrometry [29]. Boric-acid-impregnated thin-layer plates [33] were used to preserve isomeric integrity of the diradyl lipids. The following solvent systems (all v/v) were employed: I) chloroform/acetone (88/12); II) petroleum ether/diethyl ether/acetic acid (50/50/1); III) chloroform/methanol/acetic acid/water (50/25/8/4); and IV) chloroform/methanol/water (65/25/5). TPA metabolism (whole cells) was assayed as described previously [16–18]. Serum was heat-inactivated by warming at 56°C for 30 min.

Cellular Acyl Group Analysis

Phospholipids and neutral lipids were separated for acyl group analysis by TLC using a hexane/diethyl ether/acetic acid (60/40/1) solvent system. Adsorbent bands were scraped from the plate and sonicated in water/methanol/chloroform (3/7/3) to release the lipids with final extraction by the method of Bligh and Dyer [28]. Gas chromatography (Varian Instruments, model 3300) of fatty acid methyl esters (prepared by reaction with Meth-Prep II; Alltech Associates, Inc., Deerfield, IL) was accomplished using a 6-ft × 1/8-in stainless steel column packed with 5% DEGS-PS (Supelco, Belfonte, PA).

RESULTS

The chemical structures of TPA and the diacylglycerols used in this study are given in Figure 1. In the initial studies we investigated the stability of 1,2-diacylglycerols, in vitro. The incubation of 1,2-ePaG or 1,2-PaG (both >90% 1,2 isomer

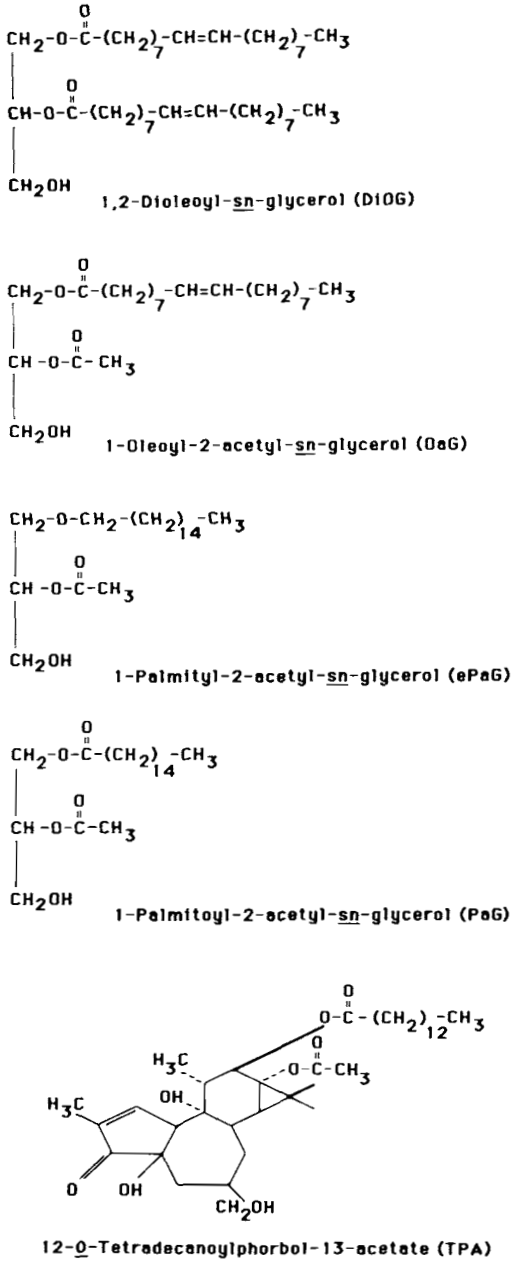

Fig. 1. Structure of diacylglycerols, diacylglycerol analogs, and the phorbol diester, TPA.

prior to the experiment) in a simple water/buffer system at 37°C resulted in a marked increase in the relative abundance of the 1,3-isomers (Fig. 2). Formation of the 1,3-diacylglycerol (via acetyl migration) occurred quickly, and after 1 min >25% of the ePaG and PaG existed as the 1,3-isomers. After 60 min, more than 50% of the acetate originally at the *sn*-2 position had migrated.

As many types of cell culture media contain serum, the effect of serum on the stability of diacylglycerols was also investigated. When PaG and ePaG were incubated in buffer (62 mM Tris-HCl, pH 7.9) containing 10% FBS and 2 nmol of either [^{3}H]PaG or [^{3}H]ePaG, extensive hydrolysis of the compounds to the long chain mono derivative occurred after only 60 min. More than 60% of the ePaG was metabolized to alkylglycerol, and 75% of the PaG was hydrolyzed to monoacylglycerol plus free fatty acid, indicating the presence of an active acetyl hydrolase and lipase in FBS. The liberation of free fatty acid from PaG could be reduced 60–70% by employing heat-inactivated serum, whereas esterase activity (acetate hydrolysis) was not affected. The hydrolysis of lipid mediators by serum enzymes was further assessed using 1-[1-^{14}C]oleoyl-2-acetyl-*sn*-glycerol (OaG). In these experiments high concentrations of OaG were added (50 μM), similar to the amounts used by investigators when comparing the relative reactivity of OaG and phorbol diesters in cell culture [20–24]. As shown by the data in Figure 3, the destruction of OaG by serum components is enzymatic in nature; the decrease in OaG accompanied the increase in the product, monooleoylglycerol, in a time-dependent manner. By 60 min 68% of the OaG had been hydrolyzed. Here, as with PaG, free fatty acid was also produced. The formation of oleic acid from OaG increased in a linear manner and by 60 min fatty acid accounted for 15% of the hydrolysis products. In contrast, TPA is relatively stable in complete serum [16], but enzymatic hydrolysis is expressed upon serum delipidation [17,18].

The acitvity of cellular enzymes on ePaG and PaG was investigated, in vitro, using the postmitochondrial supernatant from HL-60 cells. The data of Figure 4 demonstrate that HL-60 enzymes hydrolyze diacylglycerols. After a 60-min incuba-

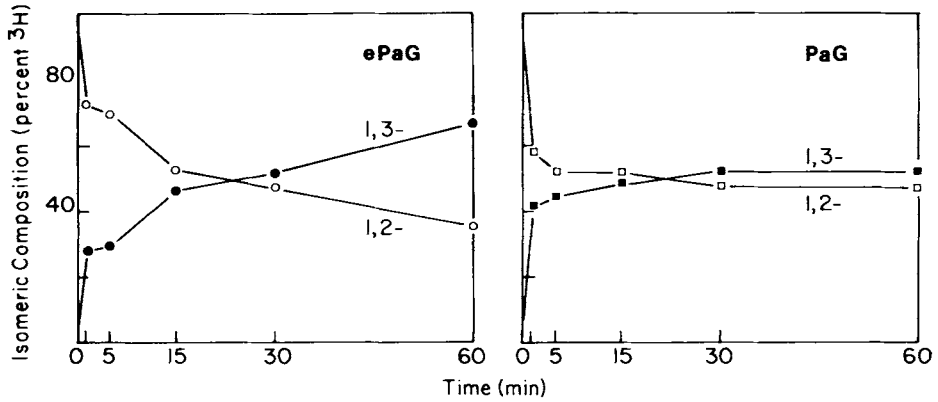

Fig. 2. The influence of incubation time on the isomeric integrity of 1,2-diradyl-*sn*-glycerols. The reactions (0.2 ml), carried out at 37°C, contained 2 nmol [^{3}H]palmitylacetylglycerol (ePaG) or [^{3}H]palmitoylacetylglycerol (PaG), both 100 dpm/pmol (introduced in 5 μl acetone) and 12.5 μmol Tris-HCl buffer, pH 7.9. The reactions were terminated by lipid extraction, and isomers were separated by borate-TLC using solvent system I. The points illustrated represent single values from one experiment; identical results were obtained when the experiment was repeated.

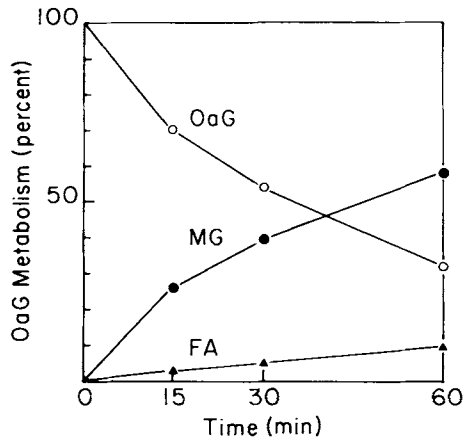

Fig. 3. Hydrolysis of OaG by enzymes of fetal bovine serum. The reactions contained 0.1 ml FBS, 100 μmol Tris-HCl buffer (pH 7.2), 50 nmol [1-^{14}C]OaG (5,000 dpm/nmol) added in 10 μl acetone, and RPMI-1640 medium to a final volume of 1.0 ml. Assays were terminated [28] at the times designated and reaction products were separated by TLC (solvent system II). MG, monooleoylglycerol; FA, fatty acid (oleic). The data points are the averages obtained from duplicates which varied <5%.

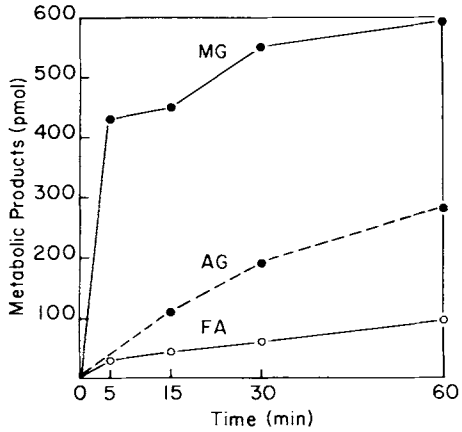

Fig. 4. The metabolism of diacylglycerols by cell-free preparations of HL-60 cells. The enzyme assays (0.2 ml) contained HL-60 cell postmitochondrial supernatant (6.4 μg protein for ePaG; 4.5 μg protein for PaG), 12.5 μmol Tris-HCl buffer (pH 7.9), and 2 nmol [^{3}H]PaG or [^{3}H]ePaG, both 100 dpm/pmol, added in 5 μl absolute ethanol. The reactions were incubated at 37°C for the times shown and terminated by the addition of lipid extraction solvents [28]. The metabolic products of [^{3}H]PaG (MG, monoacylglycerol; FA, fatty acid, solid lines) and [^{3}H]ePaG (AG, alkylglycerol, broken line) were resolved by TLC using solvent system I. Values, which varied <10%, are averages of duplicate and triplicate samples.

tion 14% of the ePaG and approximately 30% of the PaG had been degraded. The cellular enzymes produced approximately six times more monoacylglycerol (from PaG) than fatty acid; however, formation of monoacylglycerol was rapid at early times (5 min) and tapered off thereafter. As expected, the lipase-stable analog ePaG was metabolized solely by acetate hydrolysis, and formation of the alkylglycerol product was nearly linear with respect to time.

Because the neutral lipid mediators are widely employed in tissue culture, we sought to characterize diacylglycerol metabolism further by comparing mediator stability and intracellular fate to that of TPA using cultured HL-60 cells under serum-free conditions. The data of Table I show the uptake and metabolic conversion of several experimental agents, all tested at a concentration of 10 μM. Within 1 hr the long chain-short chain diacylglycerols (PaG, ePaG) and TPA were rapidly internalized (70–90% uptake), whereas only 4% of the dioleoylglycerol was taken up by the cells. Although TPA was rapidly taken up by HL-60 cells, it was metabolized only slightly (2.7%) after 60 min. The diacylglycerols were, on the other hand, quickly metabolized, as shown by the high percentage of conversion to compounds other than the parent. When represented as the disappearance of radiolabel from the parent substrate, 66% of the internalized dioleoylglycerol was metabolized, and ePaG and PaG were metabolized to near completion.

After finding nearly complete cellular metabolism of PaG and ePaG, when tested at a concentration 10 μM, further metabolic studies were designed using higher concentrations (50 μM), in line with the doses of OaG used by other investigators [20,24]. Figure 5 illustrates the uptake (inset) and metabolism of radiolabeled ePaG, PaG, and OaG by HL-60 cells over 90 min. Disappearance of radioactivity from the media was rapid, and by 90 min the cells had taken up 59% of the PaG, 42% of the ePaG, and 72% of the OaG. Whereas the data of Table I do not show the metabolic fate of the lipid mediators, the data of Figure 5 show that OaG and PaG are rapidly metabolized by conversion to complex glycerolipids. Less than 5% of the radiolabel remained as intact OaG, PaG, or ePaG after the 90-min incubation period. The amount of radioactivity in cellular monoacylglycerol and diacylglycerol in OaG and PaG exposed cells remained low as compared to the dynamic appearance of mediator-derived radioactivity in the cellular phosphlipids and neutral lipids. After 90 min, 90% of the intracellular OaG and 94% of the PaG had been converted to triacylglycerols and phospholipids. The metabolism of ePaG was primarily via acetate hydrolysis as shown by the formation of alkylglycerol, which accounted for approximately 80% of the cell-associated radioactivity at 90 min. Therefore, the ether-linked intermediate of ePaG metabolism, alkylglycerol, is relatively stable and increases with time, when compared to the monoacylglycerol produced by cellular metabolism of

TABLE I. Uptake and Metabolic Conversion of TPA and Diacylglycerols by Cultured HL-60 Cells*

Compound	Metabolism[a] (% conversion)	Uptake (% of administered dose)
TPA	2.7	90.3
DiOG	66	4.3
PaG	99	72.5
ePaG	97	82.0

*Cells (3.0×10^6/1.0 ml reaction) were incubated at 37°C for 60 min in Hanks' balanced salt solution, 24 mM Tris-HCl buffer (pH 7.3), 1.4 mM $CaCl_2$, with 10 nmol of the following compounds added in 5 μl ethanol: [^{3}H]TPA (100 dpm/pmol); [1,2(2,3)-^{14}C]DiOG (75 dpm/pmol); [^{3}H]PaG (63 dpm/pmol), [^{3}H]ePaG (100 dpm/pmol). Metabolism was assessed by thin-layer chromatography of the total lipid extract from washed cell pellets. Uptake represents the percent of intracellular radioactivity, the balance being media-associated isotope. Values represent the average from duplicate samples which varied <5%.

[a]Metabolism represents the percent of all cellular radioactivity not found as parent compound.

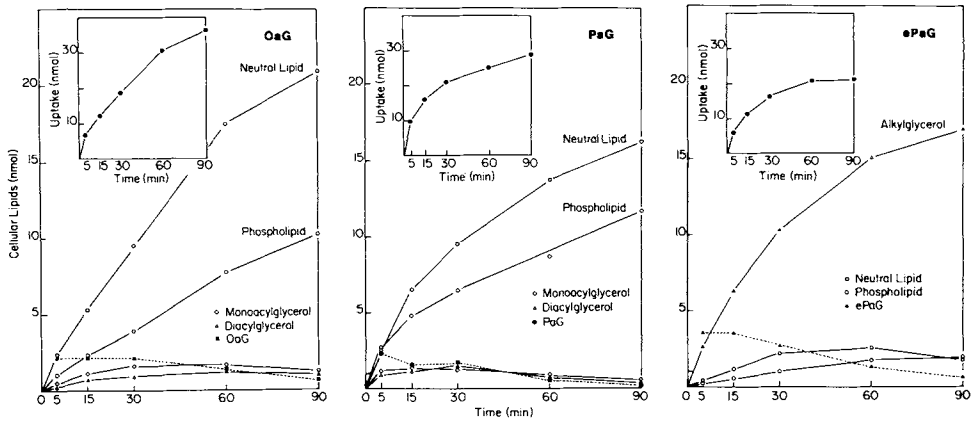

Fig. 5. The effect of time on the uptake and metabolic fate of diacylglycerols by suspensions of HL-60 cells. HL-60 cells (3.0×10^6/1.0 ml reaction) were incubated in serum-free RPMI-1640 medium containing 50 μg BSA and 20 μmol HEPES buffer (pH 7.2), with 50 nmol [^{14}C]OaG, [^{3}H]PaG, or [^{3}H]ePaG (all 6 dpm/pmol) added in 5 μl ethanol. At the times indicated, the cells were harvested by centrifugation and washed two times in cold PBS. Total lipids were extracted [28] from washed cell pellets and aliquots were sampled for radioactivity (**inset**: Uptake). The distribution of isotope in cellular lipids was determined by resolution of total lipids (borate TLC, solvent system I). The neutral lipids formed were triacylglycerols (OaG and PaG) and alkyldiacylglycerols (ePaG). Cholesterol esters were not detected. All data points represent the average values from duplicate samples. Duplicate experiments gave similar results.

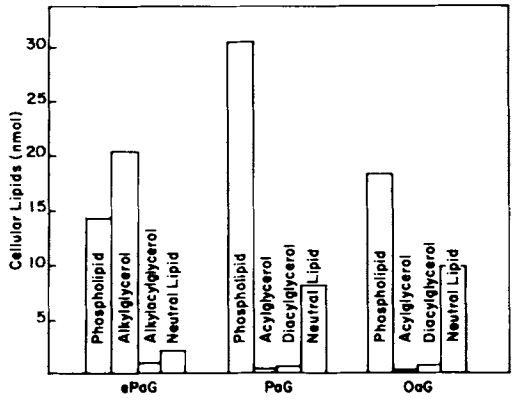

Fig. 6. Long-term metabolism of diacylglycerols by cultured HL-60 cells. The experiment and analyses were carried out as detailed in Figure 5, except that cells were grown in a tissue culture incubator and exposed to the labeled diradylglycerols for a 24-hr period. After the 24-hr incubation, the parent lipids were not detected. Data shown represent average values from duplicate samples.

PaG and OaG. Results from investigations on the long-term metabolism of diradylglycerols by HL-60 cells are shown in Figure 6. After a 24-hr incubation with either radiolabeled OaG or PaG, the cellular phospholipids contained the highest amounts of mediator-derived isotope, 65% and 77%, respectively. However, in cells exposed to ePaG, high amounts of alkylglycerol, which accounted for approximately 53% of the total cellular radioactivity, were present after 24 hr. With all agents examined there was no detectable cell-associated radioactivity present as the parent mediator; therefore, metabolic conversion was complete. In cultures exposed to [^{3}H]TPA,

> 92% of the intracellular radioactivity was present as intact TPA after 24 hr (data not shown).

Because phospholipids are important constituents of cellular membranes, it was of interest to determine the distribution of mediator-derived radioactivity within the different phospholipid classes. After a 24-hr exposure to the various diacylglycerols, the cellular phosphatidylcholine contained approximately 70%, 49%, and 52% of the total phospholipid radioactivity in cells incubated with ePaG, OaG, and PaG, respectively (Table II). Regardless of the compound tested, approximately 27% of the radiolabel was located in the ethanolamine-containing glycerophospholipids. The percent distribution of label within the combined serine- and inositol-containing phospholipids was similar in OaG and PaG-exposed cells (14–15%), whereas radioactivity derived from ePaG in these phospholipids was only slight (1.5%). Cells were also incubated with [^{3}H]palmitic acid in order to compare the metabolism of a free fatty acid to the esterified mediators. As shown in Table II, the distribution of radiolabeled palmitic acid in HL-60 phospholipids was similar to the radioprofile obtained from cells incubated with either [^{3}H]PaG or [^{14}C]OaG.

The route by which the diacylglycerols were metabolized was also investigated. Because the metabolism of [^{3}H]palmitic acid resulted in a radiolabeled lipid distribution pattern similar to that seen with PaG, it was suspected that hydrolysis of the *sn*-1 acyl moiety and subsequent incorporation of the fatty acid into complex lipids was the predominant pathway of intracellular metabolism. Further, the metabolic profile of radiolabeled ePaG (Fig. 5) also suggested that, in HL-60 cells, the monoradyl intermediate is not the preferred substrate for biosynthesis of the complex cellular glycerolipids. Therefore, the metabolism of [^{3}H]glycerol-labeled PaG was examined and compared to the cellular utilization of [9,10-^{3}H]-1-palmitoyl-2-acetyl-*sn*-glycerol that was employed in prior experiments. Table III lists the radiolabeled metabolites recovered after incubating HL-60 cells with PaG (50 μM) for 60 min. The data show that 90% of the radioactivity in the acyl-labeled PaG was converted to phospholipids and neutral lipids, whereas only 11% of glycerol-labeled PaG was found in these lipid classes. The remainder of the radioactivity, 82%, in the cells exposed to the glycerol-labeled compound was shown to be water-soluble metabolites located in the aqueous fraction of the lipid extraction. The water-soluble ^{3}H was found to be glycerol (52%; as determined by thin-layer chromatography; solvent system IV) and an unidentified water-soluble metabolite. These data show that in HL-60 cells, mediator metabolism

TABLE II. The Distribution of Radioactivity From Labeled Diacylglycerols in Phospholipids of HL-60 Cells*

Radiolabeled substrate	Percent of total phospholipid tritium			
	Lyso-PC	PC	PI + PS	PE
ePaG	2.8	69.5	1.5	25.9
OaG	3.0	49.0	15.3	28.6
PaG	3.5	52.4	14.1	26.7
Palmitic acid	5.3	59.7	8.9	22.1

*HL-60 cells (3.0×10^6 cells) were incubated with the diacylglycerols or palmitic acid (50 nmol each) for 24 hr exactly as in Figure 6. Total lipids were extracted from washed cell pellets and the phospholipids were resolved by thin-layer chromatography using solvent system III. The proportion of ether-linked lipids within each class were not quantitated. Lsyo-PC, lysophosphatidylcholine; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine. Values are the average from duplicate samples, which varied < 10%.

TABLE III. The Metabolism of 1-Acyl-2-acetyl-*sn*-[^{3}H]glycerol and 1-[^{3}H]-Acyl-2-acetyl-*sn*-glycerol by HL-60 Cells*

Metabolites	Radiolabeled substrates (nmol formed)	
	[^{3}H]acyl-labeled PaG	[^{3}H]glycerol-labeled PaG
Phospholipids	8.7	1.2
Monoacylglycerol	0.9	0.4
Acylacetylglycerol (substrate)	0.8	0.7
Diacylglycerol	0.8	0.8
Triacylglycerol	13.9	1.7
Glycerol[a]	—	24.4

*HL-60 cells were incubated (as described for Fig. 5) with 50 nmol of either 1-[9,10-^{3}H]-palmitoyl-2-acetyl-*sn*-glycerol (6 dpm/pmol) or 1-acyl-2-acetyl-*sn*-[^{3}H]glycerol (6 dpm/pmol) for 60 min. The lipids were extracted from washed cell pellets, and the metabolites were resolved by TLC. [^{3}H]Glycerol and an unidentified water-soluble metabolite were isolated in the aqueous upper phase of the lipid extraction system. Radioactivity was quantitated by scintillation spectrometry. Values represent the mean from duplicate samples that varied less than 10%.

[a]This value represents the sum of water-soluble ^{3}H metabolites recovered from both the cells and the culture medium.

occurs predominantly via deacylation with subsequent esterification of fatty acid into complex cellullar lipids. Because of this route the cells are also exposed to high concentrations of free glycerol (intra- and extracellular).

Owing to the intracellular deacylation mode of diacylglycerol metabolism, it would be possible, in cells exposed to large or repeated doses of OaG, for a high degree of acyl group membrane modification to occur. Therefore, the fatty acid composition of control and OaG-exposed HL-60 cells was evaluated by gas chromatographic analysis. The results from an experiment in which HL-60 cells were exposed to OaG on a bihourly dose regime [see reference 14 for a similar protocol] are presented in Figure 7. As seen in the chromatographic traces, the phospholipid fatty acid composition of OaG-treated cells was drastically modified, compared to controls. The predominance of 18:1 acyl groups in the treated cells is easily noted as oleic acid comprises 67% of the total phospholipid fatty acids. The doubling in the cellular content of 18:1 in the OaG-treated cells was also accompanied by decreases in the amounts of 16:0 and 16:1, which, overall, produced a general shift (increase) in the ratio of unsaturates to saturates in treated as opposed to control cells. Analysis of cellular total lipids by TLC and GC (data not shown) demonstrated that the OaG-treated cells additionally contained a large amount of triacylglycerols in which the major fatty acid was again 18:1, (74% in OaG-treated compared to 35% in control cells).

DISCUSSION

In previous studies [18] we demonstrated that TPA can be hydrolyzed by serum enzymes; however, the hydrolytic rate is scant when compared to the enzymatic lability of the long chain-short chain diacylglycerols, agents that share close structural similarity to the diradyl group of TPA. Additional studies have shown that TPA, which induces attachment of cultured HL-60 cells, is poorly metabolized in situ [19,34]. As the action of TPA at the cellular level has been compared in many systems

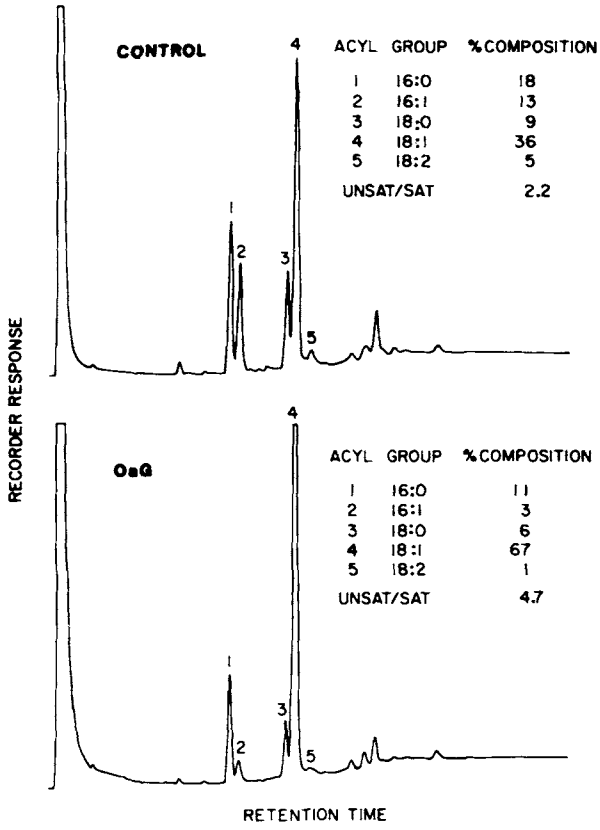

Fig. 7. Phospholipid acyl group composition of HL-60 cells grown in the presence and absence of OaG. HL-60 cells (0.5 $\times 10^6$/ml) were cultured in serum-free medium for 16 hr. Cultures were then supplemented with OaG (delivered in absolute ethanol; 8 μl ethanol for the initial dose, and 4 μl of ethanol thereafter) using an initial dose of 20 μg/ml media followed by 5 bihourly doses of 5 μg/ml on day 1. On day 2, 5 bihourly doses were given, and the cells were again maintained overnight. Control cultures were given ethanol, final concentration, 1%. Cells were harvested by centrifugation, and cell pellets were washed twice with ice-cold PBS. The total cellular lipids were extracted, and the phospholipids were isolated by preparative TLC as outlined in Materials and Methods. Gas-chromatographic analysis of phospholipid acyl groups was carried out as described in Materials and Methods. The experiment was done in triplicate and again repeated on another occasion; identical results were obtained. Cells treated with OaG did not attach to the tissue culture flask, and the only change in morphology (compared to controls) was the appearance of plasma membrane irregularity and intracellular lipid droplets. Viability in treated and control was >90%.

to the effects produced by the cell-permeable mediator OaG [20,21,23,24,35–37], we sought, in the present study, to investigate the metabolic stability of the lipid mediators using HL-60 cells as a culture model. The data show that serum, used by many investigators in tissue culture media, is a rich source of lipolytic enzymes. PAF has been shown to be rapidly metabolized to the lyso derivative by enzymes of cells and serum [38–40], and the serum TPA lipase [16–18], recently determined to be esterase 1 [41], has a wide range of substrate specificity. Our data demonstrate that PaG and OaG, which have both been shown to stimulate protein kinase C activity, in

vitro [12,15], are rapidly metabolized in tissue culture media containing 10% FBS. The favored pathway for mediator metabolism is via acetate hydrolysis, possibly after acetyl migration from the *sn*-2 to the *sn*-3 position of glycerol. The diacylglycerols are also metabolized by hydrolysis of the long chain acyl group; however, the use of heat-inactivated serum in culture media partially reduces the lipase activity. Our results also show that isomeric integrity of the long chain-short chain 1,2-diacylglycerols is not preserved during incubation at 37°C under a controlled pH environment. Collectively, these data demonstrate that when compounds such as 1,2-OaG are employed in cell culture studies, they isomerize and are metabolized by enzymes present in serum. Therefore, the cells are also being exposed to monoacylglycerols, which have been shown, along with diacylglycerols, to possess fusogenic properties [42,43].

The inability of OaG to mimic completely the effects of TPA on cultured HL-60 cells has been a subject of discussion in recent papers [20,23,24]. It is possible that these differential effects are linked to the instability of diradyl lipids vs the metabolic inertness of the phorbol diesters. With the exception of the neutral analog of PAF, alkylacetylglycerol [44], there have been no extensive studies on the metabolism of the neutral lipid mediators. For this reason we synthesized various radiolabeled diacylglycerols and studied their uptake and metabolic fate in HL-60 cells cultured in serum-free medium. When added to cell suspensions, the various compounds were metabolized by the cells within minutes. OaG and PaG displayed similar patterns of metabolism; the majority of mediator-derived radioactivity was distributed in cellular neutral lipids and phospholipids and increased in a time-dependent manner. Although ePaG was metabolized, chiefly to alkylglycerol at early times (5–90 min), this compound, which has been used to induce differentiation in HL-60 cells [23], is also short-lived. By 24 hr, however, radioactivity from ePaG was shuttled into phospholipids, presumably by 1-alkyl-*sn*-glycerol kinase followed by phosphocholine or phosphoethanolamine transferase, which has been shown to occur in cultured LM fibroblasts supplemented with [1-^{14}C]hexadecylglycerol [45]. The data on the metabolism of ePaG (Fig. 5) suggest that fatty acid hydrolysis and reincorporation is the intracellular route by which radioactivity from OaG and PaG enter complex lipids. This is shown by the increase in the amount of lipase-stable alkylglycerol produced from ePaG together with the low degree of phospholipid and neutral lipid labeling (5–90 min). Conversely, in cells incubated with ester-linked OaG and PaG, the labeled monoacylglycerol intermediates remained low, and dynamic increases in labeling of complex glycerolipids occurred. Further, the distribution of radioactivity among the classes of phospholipids was very similar, irrespective of whether labeled fatty acid (palmitic) or labeled acylacetylglycerol were introduced. Phosphorylation of intact acylacetylglycerol has been described in platelets [15]. This is not the major pathway of mediator metabolism in HL-60 cells. In experiments designed to study the metabolic fate of acylacetyl[^{3}H]glycerol, >80% of the radiolabeled products were recovered as glycerol and an unidentified water-soluble metabolite.

We have studied the metabolism of lipids that are often used as second messengers and shown that these compounds are rapidly degraded. TPA, on the other hand, is metabolically stable. The potency of the phorbol diesters may therefore be linked, in part, to this high degree of intracellular stability. We have recently shown that TPA, by virtue of its stability and structural similarity to diacylglycerols, is a potent lipase inhibitor [29]. The failure of OaG to mimic totally the cellular effects elicited

by TPA may be associated with the short intracellular residence time. The work of McNamara et al [23] has demonstrated that OaG treatment does not induce differentiation of HL-60 cells. However, a previously overlooked parameter, that of membrane acyl group modification, may also play a role in cellular response to OaG. The results show that repeated OaG treatment produces dramatic alterations in cellular membrane acyl group composition. The influence of lipid composition on membrane function, cell growth, and behavior has been well documented [27,46,47], and we have recently shown that lipid modification of HL-60 cells also alters phorbol diester binding [34]. While these studies do not demonstrate whether protein kinase C is involved in the OaG-HL-60 response, the data show that vast differences in the metabolic stability of TPA and OaG exist, and that use of OaG as an agonist can result in altered cellular lipid composition. Therefore, in addition to the poor stability of OaG, cells treated with this agent may respond via mechanisms other than protein kinase C. The initiation of such mechanisms could be provoked by cellular acyl group modification.

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