A Novel Mechanism of Diglyceride Formation

12-O-TETRADECANOYLPHORBOL-13-ACETATE STIMULATES THE CYCLIC BREAKDOWN AND RESYNTHESIS OF PHOSPHATIDYLCHOLINE*

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12-O-Tetradecanoylphorbol-13-acetate (TPA) treatment of Madin-Darby canine kidney cells resulted in an increased incorporation of 32P, and [methyl-3H]choline into choline-containing phosphoglycerides (PC). In pulse-chase experiments, TPA treatment caused an increased release of [methyl-3H]choline from the PC fraction of prelabeled cells. When cells were prelabeled with [3H]arachidonic acid and [14C]palmitic acid, TPA treatment resulted in an increased synthesis of 14C, 3H-diglycerides. Further studies were done to determine the relationship between PC breakdown and diglyceride synthesis. Cells were preincubated with ether-linked 1-O-[3H]hexadecyl-2-lyso-sn-glycero-3-phosphocholine which was acylated to form 1-O-[3H]hexadecyl-2-acyl-sn-glycero-3-phosphocholine. A stimulated release of 3H-labeled diglyceride from prelabeled cells (6) and Paddon and Vance (5) demonstrated that TPA stimulates [3H]choline incorporation into HeLa cell diglycerides. Recent studies have shown that TPA stimulates Ca2+-activated, phospholipid-dependent protein kinase (protein kinase C) which is stimulated by diacylglycerol (12, 13). Thus, it has been suggested that TPA exerts its biological effects by mimicking the actions of diacylglycerol which is generated by phospholipase C degradation of phosphatidylinositol (14). Because of the increasingly appreciated importance of diglycerides as regulatory molecules, we have examined the effects of TPA on diglyceride synthesis from PC.

The tumor promoter TPA1 has been shown to have many effects on cellular lipid metabolism including the stimulation of phospholipid deacylation and prosta glandin synthesis (1-4). TPA also causes an increased incorporation of [3H]choline (5, 6) and 32P (6) into PC, and a stimulated release of choline from prelabeled cells (6). Paddon and Vance (5) demonstrated that TPA stimulates [3H]choline incorporation into HeLa cell phospholipids by stimulating the reaction catalyzed by CTP:choline-phosphate cytidylyltransferase. TPA-stimulated PC synthesis has been observed in a number of other cell types including HL-60 promyelocytic leukemia cells (7) and human neutrophils (8). Incorporation of precursors into PC is stimulated by other, non-phorbol ester, tumor promoters including mezerein (9) and dihydroteicocin B (10). Mufson et al. (6) found that TPA stimulates the release of radiolabeled from PC in cells prelabeled with [3H]choline and suggested that TPA may activate a phospholipase C or phospholipase D. However, the lipid products of these possible pathways (diglyceride or phosphatidic acid) were not identified. Grove and Schimmel (11) demonstrated that TPA causes increased diacylglycerol levels in chick embryo myoblasts and suggested PC may be the source of the diacylglycerol, since the acyl composition of the diacylglycerol is similar to that of the cellular PC.

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The MDCK cell line was chosen for these studies because it is highly sensitive to stimulation by TPA (2) and has been used in many earlier studies of the effects of TPA on lipid metabolism (1-4). Alkylacylglycerides, in addition to diacylglycerides, were investigated as a model system, because recent studies demonstrate that, although the alkylacylglycerides do not stimulate protein kinase C (15), they, like TPA, stimulate HL-60 promyelocytic leukemia cells to differentiate to macrophage-like cells (16).

**EXPERIMENTAL PROCEDURES**

Materials—MDCK cells and cell culture reagents were purchased from Flow Laboratories, Rockville, MD. [methyl-3H]Choline chloride (76 Ci/mmole), [5,6,8,9,11,12,14,15-3H]Arachidonic acid (151 Ci/ mmole), and [1-14C]Palmitic acid (56 mCi/mmol) were obtained from Amersham. 32P (carrier-free) was obtained from ICN, Irvine, CA. All solvents were purchased from Fisher and were either reagent grade or redistilled in the laboratory. Lipid standards for thin layer chromatography, phospholipase C, and other chemicals were obtained from Sigma, Silica Gel 60 plates were from Merck. TPA was obtained from LC Services Corp., Woburn, MA. 1-O-(cis-9')Hexadecenyl-2-lyso-GPC was prepared from the race-mate synthesized chemically as described earlier (17) and tritiated with carrier-free tritium gas using 10% palladium/carbon as the catalyst to yield 1-O-[9,10-3H] Hexadecyl-2-lyso-GPC (56 Ci/mmole), the naturally occurring enantiomer.

Cell Culture—MDCK cells were cultured in Dulbecco’s modification of Eagle’s medium supplemented with 10% (v/v) fetal bovine serum, 100 units of penicillin/ml, 100 µg of streptomycin/ml, 0.22% NaHCO3, and 2 mM L-glutamine. The cells were maintained in 75-cm2 flasks and were subcultured at 3-5-day intervals at a ratio of 1:3. For prelabeling experiments, the cells were plated in 35-mm dishes at 5 x 105 cells/dish and allowed to attach to the plates (2-3 h) before the medium was replaced with fresh medium containing the radiolabeled compounds.

Lipid Extraction and Analysis—The cells were harvested by scraping directly from the cell culture dishes into methanol. The lipids were then extracted by a modification of the method of Bligh and Dyer (15) as described previously (1). After extraction, the lecids were...
dried under a gentle stream of N₂, and resuspended in chloroform. Neutral lipid fractions were separated on layers of Silica Gel 60 developed in a solvent system consisting of hexane/ethyl ether/formic acid (90:6:4, v/v) or a solvent system consisting of the organic phase of ethyl acetate/isooctane/acetic acid/water (55:25:10:5, v/v). Diglycerides were identified by their migration with known standards in both systems (Rᵣ values 0.33 and 0.93, respectively). Phospholipids were separated by TLC on a layer of Silica Gel 60 developed in a solvent system consisting of chloroform/methanol/acetic acid/water (75:48:12:5:4.5, v/v) or (50:25:8:4, v/v). Following chromatography, the lipids were located by fluorography (³H and ¹⁴C) using ENHANCE plates and quantitated by scintillation counting using a Packard liquid scintillation counter.

Water-soluble products of [³H]choline were determined by evaporating the aqueous phase of the Bligh and Dyer lipid extraction mixture under reduced pressure, redissolving the extract in water, and separating the products by TLC in a solvent system consisting of H₂O with 0.9% NaCl/CH₃OH/NH₄OH (50:50:5, v/v) as described by Vance et al. (19).

The content of radiolabeled plasmanagens was determined by two-dimensional TLC as previously described (Daniel et al. 20). The procedure was obtained after incubation of 1-0-[³H]hexadecyl-2-lyso-GPC were resolved on Silica Gel 60 plates developed in chloroform/methanol/acetic acid/water (50:25:8:4, v/v), then the alk-1-enyl groups of the plasmanagens were cleaved by exposure to HCl fumes. The resulting products were next separated in a second dimension using the same solvent system. The radiolabeled products were located by fluorography and identified by their migration in relation to known phospholipid standards run in both dimensions. The radiolabeled products were then scraped from the plate and quantitated by liquid scintillation counting. The products obtained after incubation of 1-0-[³H]hexadecyl-2-lyso-GPC with MDCK cells were first characterized as follows. The PC and PE fractions were isolated by TLC as described and extracted from the silica gel by the method of Bligh and Dyer (18). The phospholipids were then converted to diradyl-glycerols by phospholipase C treatment and converted to benzoate derivatives as described by Blank et al. (20). The reaction mixture was applied to small Silicar columns and the radiolabeled benzoxy diradylglycerols were quantitatively recovered by eluting the columns with >5 column volumes of chloroform. This procedure removed the majority of unreacted benzoic anhydride which interfered with further analysis of the products. The ¹³C-labeled benzoxy diradylglycerols were then separated into three subclasses (1,0-alk-1'-enyl-2-acyl, 1,0-alkyl-2-acyl, and 1,2-diacyl-) by TLC using Silica Gel 60 plates developed in a solvent system consisting of benzene/hexane/diethyl ether (50:45:4, v/v). The radiolabeled products were then located by fluorography and identified by their migration with known standards phosphorylcholine, phospha-tidylglycerol, and PE plasmalogen by Blank et al. (20). The radiolabeled products were scraped from the plates and quantitated by liquid scintillation counting.

Release of Choline from PC upon TPA Stimulation—Cells were preincubated with [³H]choline for 18 h which resulted in >90% of the lipid-associated radiolabel being incorporated into PC with the remainder being in sphingomyelin. Upon stimulation by TPA, there was an increased release of [³H]choline as water-soluble products with a concomitant loss in radioactivity from PC. The water-soluble product contained both [³H]phosphocholine and [³H]choline. In similar experiments in which cells were prelabeled with [³P], the TPA-stimulated [³P]phosphocholine release was similar in time and magnitude to the TPA-stimulated release of [³H]choline products previously observed.

Formation of Diglycerides after TPA Stimulation—The next series of experiments were done to determine the nature of the lipids released upon the TPA-stimulated breakdown of PC. The synthesis of diglycerides would be consistent with phospholipase C hydrolysis, whereas phosphatidic acid formation should result from phospholipase D hydrolysis. In the first series of experiments, MDCK cells were incubated with [³H]arachidonic acid and [¹⁴C]palmitic acid for 18 h. This incubation procedure results in >90% of both labels being incorporated into cellular phospholipids (1). The [³H]arachidonic acid is almost exclusively in the sn-2 position of the phospholipids (95%), whereas [¹⁴C]palmitic acid is predominantly in the sn-1 position (64%); 25% of the [³H]and 40% of the [¹⁴C]are incorporated into the choline-containing phosphoglycerides (1).

We found that TPA stimulation of the prelabeled cells resulted in the synthesis of diglycerides containing both [³H] and [¹⁴C] (Fig. 1). The time course for the release of both [³H] and [¹⁴C] was similar and reached a maximum at 3-4 h. The subsequent decrease in labeled diglyceride probably results from conversion into PC or phosphatidic acid. Although these experiments demonstrate that TPA causes an increased synthesis of diglycerides, they do not prove that the precursor is PC.

In the next studies, we introduced radiolabeled 1-0-[³H]hexadecyl-2-acyl-GPC into the cells by the method of Chilton et al. (23). Cells were incubated for 18 h with 1-0-[³H]hexadecyl-2-lyso-GPC which resulted in >95% of the [³H] label being cell-associated. The labeled products were identified by TLC with known standards employing a solvent system consisting of chloroform/methanol/acetic acid/water (50:25:8:4, v/v) as described under "Experimental Procedures." The distribution of radiolabel in products was as follows: PC, 55.4% ± 1.9; PE, 30.6% ± 0.7; lyso-PC, 3.7% ± 1.3; and neutral lipids, 10.1% ± 1.1 (data are presented as per cent of total labeled products recovered, S. D. = n = 3). The products were then further characterized. The product tentatively identified as PE was shown to co-migrate with authentic PE in a basic solvent system consisting of chloroform/methanol/ammonium hydroxide (65:35:8, v/v). The products were analyzed by two-dimensional TLC and HCl exposure to determine the content of 1-alk-1'-enyl-linked species (see "Experimental Procedures"). We found that 82.4% ± 7.6% (n = 3) of the radiolabel in PE was in an acid-labile species indicating that the PE was predominantly PE-plasmanagen. The major horizon (95%) of the product identified as PC was resistant to acid hydrolysis, indicating that the PC species were predominantly either 1-alkyl-2-acyl-linked or 1,2-diacyl-linked. We further characterized the PE and PC products by treating the...
TPA-induced PC Turnover

![Graphs showing time course of diglyceride formation in MDCK cells prelabeled with [14C]palmitic acid and [3H]arachidonic acid.](image)

Fig. 1. Time course of diglyceride formation in MDCK cells prelabeled with [14C]palmitic acid and [3H]arachidonic acid. MDCK cells were plated under standard conditions; after attachment (2-3 h), the medium was replaced with fresh medium containing [14C]palmitic acid (0.5 uCi/ml) and [3H]arachidonic acid (0.25 uCi/ml) and the cells were incubated for 18 h. The medium was then removed, and the cells were washed and then incubated in the absence or presence of TPA (100 nM) for the times indicated. The cells and medium were then extracted and the radiolabeled products were separated by TLC on layers of Silica Gel 60, developed in a solvent system of hexane/ethyl ether/formic acid (90:60:4, v/v). The radiolabeled diglycerides were identified by comparison with known standards and by fluorography and scintillation counting as described under “Experimental Procedures.” The total amounts of [14C] and [3H] labels recovered in the diglyceride fraction are shown in the left and right panels, respectively. Plus TPA (100 nM), ○-○; control, no TPA, ×-×.

![Graphs showing time course of conversion of 1-O-[3H]alkyl-2-acyl-GPC to 1-O-[3H]alkyl-2-acyl-sn-glycerol by MDCK cells after stimulation by TPA.](image)

Fig. 2. Conversion of 1-O-[3H]alkyl-2-acyl-GPC to 1-O-[3H]alkyl-2-acyl-sn-glycerol by MDCK cells after stimulation by TPA. MDCK cells were plated under standard conditions; after attachment (2-3 h), the medium was removed and replaced with fresh medium containing 1-O-[3H]alkyl-2-lyso-GPC and the cells were incubated for 18 h. The medium was then removed and the cells were washed and treated with fresh medium with or without TPA for the times indicated. The cells and medium were then extracted and the radiolabeled products were separated by TLC on layers of Silica Gel 60, developed in a solvent system consisting of the organic phase of ethyl acetate/isooctane/acetic acid/water (55:25:10:50, v/v). The radiolabeled diglycerides (DG) (left panel), phosphatidic acid (PA) (middle panel), and triglycerides (TG) (right panel) were located by comparison with known standards and by fluorography and scintillation counting as described under “Experimental Procedures.” The results are presented as per cent of total [3H] recovered in the lipid extracts (100% = 1.0 x 10^6 dpm ± 0.06 x 10^4 dpm, n = 22) and are the average of duplicates of one of three separate experiments. Plus TPA (100 nM), ○-○; control, no TPA, ×-×.

Isolated phospholipids with phospholipase C and converting the resulting diglycerides to benzoyl derivatives as described under “Experimental Procedures.” The labeled benzoyl diglycerides were then separated by TLC and their migration was compared to the migration of known standards. This procedure confirmed that the labeled PE was predominantly 1-alk-1'-enyl-2-acyl-sn-glycerol-3-phosphoethanolamine and that the labeled PC was predominantly 1-alkyl-2-acyl-GPC. Both compounds had <3% of the total radiolabel in diacyl species.

The cells thus labeled with 1-O-[3H]hexadecyl-2-lyso-GPC were then stimulated with TPA and the radiolabeled products were analyzed by TLC with two different solvent systems. One solvent system was used to resolve diglycerides, phosphatidic acid, and triglycerides. Using this technique we found that TPA treatment caused an increase in [3H]-diglyceride formation (Fig. 2). There was also an increase in [3H]-labeled phosphatidic acid and triglycerides (Fig. 2); however, the lag in formation of the latter products and the lower amounts produced indicate that they are secondary to formation of diglycerides.

The products were also separated by TLC using a solvent system consisting of chloroform/methanol/acetic acid/water (50:25:8:4, v/v) to resolve the PC and PE fractions. These studies revealed that TPA stimulated a loss of radiolabel from the PC fraction (Fig. 3); there was no decrease in radiolabel in the PE fraction. In fact, there appeared to be a small increase in radiolabel in PE (Fig. 3). This increase was observed in the control cultures and was not significantly different in the TPA-treated cultures. The mechanism of this transfer is unknown; however, a possible mechanism includes base exchange and desaturation of the alkyl chain of the PE to yield plasmalogen PE.

DISCUSSION

The present results indicate that TPA stimulates the degradation of PC to yield phosphocholine and diglycerides. In
phosphate cytidylyltransferase has previously been shown to be a phospholipase that was removed by partial purification. This appeared to be identical with diglycerides.

phospholipase C. However, no such enzyme was known in stimulated by TPA

cyclical degradation and resynthesis of PC analogous to the human neutrophils stimulated with ionophore A23187 (23).
The neutrophils were prelabeled with 1-0-[3H]hexadecyl-2-lyso-GPC as in the present study. In the present studies, the release of phosphocholine and the preponderance of diglyceride, rather than phosphatidic acid, at early time points supports a phospholipase C reaction rather than a pathway mediated by phospholipase D and a phosphohydrolase. However, it is difficult to rule out the possibility that a reversal of the cholinephosphotransferase reaction (27) may be responsible for our observations.

Both 1-acyl-linked and 1-alkyl-linked diglycerides are formed in TPA-treated MDCK cells. The diacylglycerides, in particular 1-oleoyl-2-acyt-sn-glycerol, have been shown to stimulate protein kinase C (14) and mimic the actions of TPA in intact platelets (28) and mouse epidermal cells (29). However, this diacylglycerol does not mimic the effect of TPA in HL-60 cells, although it was reported to stimulate protein kinase C (30). In contrast, the 1-alkyl-linked glyceride, 1-O-hexadecyl-2-acyt-sn-glycerol, does not stimulate protein kinase C (15) but, like TPA, induces differentiation in HL-60 cells (16). Thus, a portion of the effects of TPA may require the generation of endogenous 1-alkyl-linked glycerides and appears to be independent of protein kinase C. The stimulated degradation of PC described herein results in transient increases in 1-alkyl-linked glycerides and these compounds may mediate bioactivities of TPA which do not involve protein kinase C.

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