ABSTRACT

The tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) caused a marked stimulation of inorganic \[^{32}P\]orthophosphate incorporation into HeLa-cell phosphatidylcholine (PC), phosphatidylethanolamine (PE), and lysophosphatidylethanolamine. The increased incorporation of inorganic \[^{32}P\]orthophosphate into PE and lysophosphatidylethanolamine in the presence of TPA was not associated with an increase in PE synthesis as detected by the incorporation of \[^{3}H\]serine or \[^{3}H\]ethanolamine. The PC-specific exchange protein from beef liver was used to insert PC labeled with \[^{3}H\]choline, inorganic \[^{32}P\]orthophosphate, or \[^{14}C\]arachidonic acid plus \[^{3}H\]palmitic acid into the outer monolayer of intact HeLa cell membranes. Radioactivity from the latter two compounds was rapidly incorporated into PE and lysophosphatidylethanolamine; the incorporation was stimulated by TPA. It was concluded that TPA stimulated the formation of PE by base exchange between ethanolamine and PC.

INTRODUCTION

Recent studies have demonstrated that the phorbol ester tumor promoters cause rapid changes in PC turnover in cultured mammalian cells (7, 8, 18). Both increased incorporation of \[^{3}H\]choline into PC (8, 18) and increased release of radioactivity into the medium from cells prelabeled with \[^{3}H\]choline have been reported (8, 18). The current evidence suggests that the primary reaction activated by promoters is a PC-specific phospholipase C resulting in the production of DG and phosphocholine (7, 8, 18). Grove and Schimmel (7) have provided evidence that a portion of the DG is converted into PA, which, in turn, is a precursor for PI biosynthesis.

Perturbation of PC turnover by promoters occurs rapidly (8, 18) and is a prime candidate for the initial event triggered by promoters. For HeLa cells, at least, it has been established that the enhanced turnover is closely coupled to receptor occupancy, and removal of the promoter by washing causes a rapid return to control rates (9).

In the present paper, 2 approaches have been used to explore further the effects of tumor promoters on HeLa cell phospholipid metabolism: (a) overall patterns of \[^{32}P\] incorporation into individual phospholipids have been analyzed; (b) a technique has been developed to insert PC molecules, prelabeled in a defined way, into intact HeLa cell membranes; the effect of TPA on the redistribution of the labeled PC has then been determined. Insertion of the PC was accomplished using a PC-specific exchange protein from beef liver, which catalyzes the exchange of PC between liposomes and a variety of membrane preparations (4, 12, 15).

MATERIALS AND METHODS

Materials. TPA was obtained from P-L Biochemicals, Inc., Milwaukee, Wis. Phospholipase C (Clostridium perfringens), phospholipase A2, and A23187 were gifts from Eli Lilly and Co., Indianapolis, Ind. MEM and fetal calf serum were obtained from Commonwealth Serum Laboratories, Melbourne, Australia. Supplemented MEM contained a 50% increase in essential amino acids, glutamine, and vitamins, a 100% increase in nonessential amino acids, and 1 mM sodium pyruvate.

Phosphatidylcholine (specific activity, 77 Ci/mmol), \[^{3}H\]palmitic acid (specific activity, 15.2 Ci/mmol), myo-\[^{2}H\]inositol (specific activity, 3.9 Ci/mmol), \[^{3}H\]ethan-1-ol-2-amine (specific activity, 8.8 Ci/mmol), \[^{3}H\]serine (specific activity, 28 Ci/mmol), \[^{14}C\]arachidonic acid (specific activity, 58.4 Ci/mmol), and \[^{32}P\] were obtained from the Radiochemical Centre, Amersham, England.

HeLa cells were maintained in 35- or 60-mm plastic dishes (Lux Scientific Corp., Newbury Park, Calif.) in MEM supplemented with 10% fetal calf serum and antibiotics (penicillin, 100 IU/ml; streptomycin, 100 μg/ml; complete medium). Incubations were carried out at 37° in a humidified atmosphere of 5% CO\(_2\) in air, and cultures were used at cell densities of 1.5 to 2 x 10\(^6\) cells/35-mm dish or 3.8 to 4.3 x 10\(^6\) cells/60-mm dish.

Polyethyleneimine cellulose plates (glass backed) and Silica Gel 60 plates (aluminum backed) were from E. Merck, Darmstadt, Germany. Autoradiographs were carried out using Kodak X-Omat RP film.

PC-specific exchange protein was purified from beef liver essentially as described (12) and used after the carboxymethylcellulose step. The enzyme was stored at −20° in 50% glycerol. Exchange protein activity was assayed by measuring radioactivity transferred from \[^{3}H\]PC-labeled liposomes to unlabeled liposomes.

Methods. The following procedure was used to measure the incorporation of precursors into phospholipids. Complete medium was removed from culture dishes, and the cells were washed twice with MEM. MEM (3 ml), together with either \[^{3}H\]choline (0.3 Ci/ml), \[^{3}H\]ethanolamine (1 μCi/ml), \[^{3}H\]serine (1 μCi/ml), \[^{3}H\]inositol (1 μCi/ml), or \[^{32}P\] (20 μCi/ml), was added, and incubations were carried out for the appropriate time. TPA was added as a solution in DMSO; control incubations contained DMSO alone (final solvent concentration, 0.1%). The medium was aspirated, and the cells were washed with ice-cold PBS prior to extraction as described by Wertz and Mueller (21). The extracted phospholipids were dried in air, dissolved in 100 μl of chloroform:methanol (2:1, v/v), and stored on ice under a N\(_2\) atmosphere. Phospholipids were resolved by 2-dimensional chromatography on silica gel plates. Control experiments established that the phospholipid patterns obtained from air-dried samples were identical to those obtained when samples were dried in a stream of nitrogen. A solvent system of chloroform:methanol:ammonia (65:25:5, v/v) was used in the first direction, after which the plates were air dried for 10 min and oven dried (50–60°) for 30 min. The plates were developed in the second direction with a solvent system of chloroform:acetone:methanol:acetic acid:H\(_2\)O (30:25:5:10:11)
containing phospholipid were scraped into vials, and radioactivity was measured using a Triton:xylene scintillation fluid (1).

The various phospholipids were identified using authentic standards and by determination of labeling patterns after incubation with [3H]-choline, [3H]inositol, [3H]serine, or [3H]ethanolamine. The identity of lyso-derivatives was confirmed by incubation of 3H- or 32P-labeled PC, PI, or PE (isolated from chromatograms) with phospholipase A2 and redeveloping the 2-dimensional chromatograms with appropriate unlabeled standards. The 2-dimensional system described above did not completely resolve PI and PS. A separate solvent (17) was used to separate these 2 phospholipids.

Incorporation of 32P, into HeLa-cell ATP was measured as follows. Cells were incubated in MEM containing 32P, (10 µCi/ml). At appropriate times, the medium was removed, the cells were washed with ice-cold PBS (3 x 2 ml), and nucleotides were extracted with cold 5% trichloroacetic acid. After removal of the trichloroacetic acid by extraction with water-saturated ether, an aliquot of the acid-soluble fraction was fractionated by chromatography on polyethyleneimine plates (14). Radioactivity associated with ATP was determined by scintillation counting.

 Autoradiography or by staining with iodine vapor. Areas of silica gel showing the separation of the individual phospholipids is shown in Fig. 1. The criteria used for identification of the separated compounds are summarized in “Materials and Methods.” Not all the radioactive compounds were identified on these chromatograms, particularly the more polar derivatives with low mobilities in both solvents. This low-mobility group includes both phosphatidylinositol-4-phosphate and phosphatidylinositol-4,5-bisphosphate. In these experiments, 32P, was added at Time 0, and it was necessary to determine whether TPA caused any change in the incorporation of label into ATP which could lead to artifactual changes in phospholipid labeling. A continuous increase in 32P, labeling of ATP was observed over 120 min, and 10-7 M TPA. After 2-dimensional chromatography, phospholipids were visualized by radioautography; the direction of the second development is indicated with an arrow. LPA, LPE, and LPC, the lyso-derivatives of PA, PE, and PC, respectively. PG, phosphatidylglycerol. Left, 2-hr exposure to DMSO; right, 2-hr exposure to 10-7 M TPA.

RESULTS

We have shown previously that TPA causes a rapid stimulation of the release of radioactive choline and phosphocholine from HeLa cells prelabeled with [3H]choline (8). These products are derived from the PC pool and are a consequence of phospholipase C action (8). Release is followed by a TPA-enhanced rate of PC synthesis, as detected by the incorporation of [3H]choline into phospholipid.

A series of experiments was initially carried out to determine the effect of TPA on the overall patterns of 32P, incorporation into individual HeLa phospholipids. An example of an autoradiograph showing the separation of the individual phospholipids is shown in Fig. 1. The criteria used for identification of the separated compounds are summarized in “Materials and Methods.” Not all of the radioactive compounds were identified on these chromatograms, particularly the more polar derivatives with low mobilities in both solvents. This low-mobility group includes both phosphatidylinositol-4-phosphate and phosphatidylinositol-4,5-bisphosphate. In these experiments, 32P, was added at Time 0, and it was necessary to determine whether TPA caused any change in the incorporation of label into ATP which could lead to artifactual changes in phospholipid labeling. A continuous increase in 32P, labeling of ATP was observed over 120 min, and 10-7 M TPA. After 2-dimensional chromatography, phospholipids were visualized by radioautography; the direction of the second development is indicated with an arrow. LPA, LPE, and LPC, the lyso-derivatives of PA, PE, and PC, respectively. PG, phosphatidylglycerol. Left, 2-hr exposure to DMSO; right, 2-hr exposure to 10-7 M TPA.

The results from a typical 32P, incorporation experiment are shown in Table 1; similar data have been obtained in 10 separate experiments. 32P, incorporation into PC was rapidly stimulated by TPA. It is likely that there was some increase in the labeling of PI and PA, particularly at the later time points, but the stimulation was not marked. This is also indicated by the observation that TPA caused a delayed and relatively minor stimulation of [3H]inositol incorporation into phospholipid (Chart 2).

A surprising result was that TPA caused a rapid and marked increase in the 32P, labeling of PE and lyso-PE (Table 1). However, in contrast to PC (8), this was not accompanied by enhanced phospholipid synthesis, as detected by the incorporation of [3H]ethanolamine (Table 2). Another mechanism for the 32P, labeling of PE and lyso-PE is the decarboxylation of [32P]PS. PS
incubation for the appropriate time in the presence of DMSO or 10^-7 M TPA. G. R. Guy and A. W. Murray explained by gross changes in PE synthesis de novo or from PS. 32P, labeling of PE and lyso-PE induced by TPA cannot be concluded from Tables 2 and 3 is that the dramatic increase in absence and presence of TPA is higher with PS (2.3 to 3.6) than with PE (1.1 to 1.5). Thus, in the presence of TPA, a higher proportion of the labeled PS pool is converted into PE. The incorporation of 32P, but not of [3H]ethanolamine into PE would be a base exchange reaction between PC and ethanolamine (13, 19). In the presence of TPA, 32P, incorporation into PC is stimulated, and this would lead to increased label in PE if base exchange occurred. Consequently, in an attempt to determine whether such exchange reactions occurred, experiments were carried out in which prelabeled PC was inserted into the outer monolayer of HeLa cell membranes. It was hoped that this approach would enable a distinction to be made between phospholipid labeling by base exchange and by de novo biosynthetic pathways.

Preliminary experiments established that beef liver exchange protein catalyzed the efficient transfer of [3H]choline-labeled PC into HeLa cell membranes. In a typical experiment, in which vesicles containing approximately 2.5 x 10^5 cpm in PC were incubated with HeLa cells as described in "Materials and Methods," about 6.5 x 10^4 cpm were inserted into the membranes after a 2-hr incubation. Less than 1% of this incorporation was observed in the absence of added exchange protein. Approximately 95% of the radioactivity was associated with PC, 4% with lyso-PC, and 1% with sphingomyelin (data not shown). As expected, the majority of the label was initially in the outer monolayer of the membrane, as indicated by its susceptibility to exogenous phospholipase attack. Thus, incubation of prelabeled cells with exogenous phospholipase C (1 unit/ml) resulted in the loss of 85% of the radioactivity associated with PC over a 3-hr period. In addition, the incorporated PC was susceptible to increased breakdown in the presence of TPA, as detected by the release of radioactivity into the medium (Chart 3). In other

Table 1

Effect of TPA on the incorporation of 32P, into HeLa cell phospholipids

<table>
<thead>
<tr>
<th>Incubation time (min)</th>
<th>Treatment</th>
<th>Radioactivity (cpm/dish)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PC</td>
<td>Lyso-PC</td>
</tr>
<tr>
<td>30</td>
<td>DMSO</td>
<td>188</td>
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<tr>
<td></td>
<td>TPA</td>
<td>794</td>
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<tr>
<td>60</td>
<td>DMSO</td>
<td>726</td>
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<td></td>
<td>TPA</td>
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</tr>
<tr>
<td>90</td>
<td>DMSO</td>
<td>1,062</td>
</tr>
<tr>
<td></td>
<td>TPA</td>
<td>4,502</td>
</tr>
<tr>
<td>120</td>
<td>DMSO</td>
<td>1,492</td>
</tr>
<tr>
<td></td>
<td>TPA</td>
<td>9,462</td>
</tr>
</tbody>
</table>

Table 2

Effect of TPA on the incorporation of [3H]inositol into HeLa cell phospholipids

<table>
<thead>
<tr>
<th>Incubation time (min)</th>
<th>Treatment</th>
<th>Radioactivity (cpm/dish x 10^5)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>PE</td>
<td>Lyso-PE</td>
</tr>
<tr>
<td>30</td>
<td>DMSO</td>
<td>385</td>
</tr>
<tr>
<td></td>
<td>TPA</td>
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<tr>
<td>60</td>
<td>DMSO</td>
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<td></td>
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<td>DMSO</td>
<td>633</td>
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<tr>
<td></td>
<td>TPA</td>
<td>520</td>
</tr>
</tbody>
</table>

Table 3

Effect of TPA on incorporation of [3H]serine into HeLa cell phospholipids

<table>
<thead>
<tr>
<th>Incubation time (min)</th>
<th>Treatment</th>
<th>Radioactivity (cpm/dish)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PS</td>
<td>PE</td>
</tr>
<tr>
<td>30</td>
<td>DMSO</td>
<td>3,914</td>
</tr>
<tr>
<td></td>
<td>TPA</td>
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<td>60</td>
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<td>TPA</td>
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<tr>
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<td>3,272</td>
</tr>
</tbody>
</table>

is not synthesized de novo in animal cells (13, 19) and is probably synthesized by base exchange enzymes. As shown in Table 3, [3H]serine is incorporated rapidly into PS and PE, with lower incorporation into other phospholipids. TPA caused a marked decrease in [3H]serine incorporation into PE and a smaller decrease in lyso-PE labeling (Table 3). This decrease was not due to TPA inhibition of serine uptake into the acid-soluble pool (data not shown). It is noteworthy that the ratio of incorporation in the absence and presence of TPA is higher with PS (2.3 to 3.6) than with PE (1.1 to 1.5). Thus, in the presence of TPA, a higher proportion of the labeled PS pool is converted into PE. The conclusion from Tables 2 and 3 is that the dramatic increase in [32P] labeling of PE and lyso-PE induced by TPA cannot be explained by gross changes in PE synthesis de novo or from PS decarboxylation.

We have shown previously (8) that enhanced turnover of PC in HeLa cells is also induced by incubating cells with exogenous C. perfringens phospholipase C, or with the Ca++ ionophore A23187. As shown in Table 4, these agents also stimulated the incorporation of 32P, into PC, PI, lyso-PI, and PA; these results were reproducible in 3 separate experiments. Stimulation of lyso-PI labeling by A23187 was particularly marked and presumably reflects a Ca++ stimulation of a phospholipase A2 (3). However, it is noteworthy that neither the phospholipase C nor the Ca++ ionophore stimulated the 32P, labeling of PE or lyso-PE.

One possible mechanism for a selective TPA stimulation of the

Chart 2. Effect of TPA on the incorporation of [3H]inositol into HeLa-cell phospholipids. Each point is the mean of triplicate determinations. Bars, S.E.
The possibility that HeLa cells catalyze a rapid base exchange reaction between ethanolamine and PC, which is stimulated by TPA, is supported by the data in Table 6. In these experiments, PC labeled with both [3H]palmitic acid and [14C]arachidonic acid was inserted into HeLa cell membranes. There was a rapid incorporation of both 14C and 3H label into PE and of 3H label into lyso-PE; both incorporations were stimulated by TPA. This result suggests that direct transfer of both fatty acid moieties from PC to PE occurs via a base exchange enzyme. There is evidence also for an enrichment of PI and possibly other phospholipids with arachidonic acid (14C label), which is unaffected by TPA. This could either result from the reincorporation of [14C]-arachidonic acid released by phospholipase A2 action or from a "shuttle" involving the transfer of arachidonic acid from PC to lyso phosphatidyls catalyzed by lyso phosphatide acyltransferase (11, 16, 20).

Accumulation of 14C and 3H label also occurred in PS, and this is presumably a consequence of serine exchange with either PE or PC. This reaction was slightly inhibited by TPA. In animal cells, PS is believed to be synthesized by base exchange between serine and other phospholipids rather than by de novo biosynthesis (13, 19). In the experiments in which 32P incorporation was measured, only low levels of radioactivity were associated with PS, even at the longer incubation times (data not shown). Consequently, it does seem that the PC pools labeled via de novo synthesis and exchange with exogenous PC are not identical. Although both are perturbed by TPA, the pool labeled with exogenous PC seems relatively more available for a subsequent exchange reaction with serine than that labeled with 32P.

**DISCUSSION**

The present results confirm that a major and early response of HeLa cells to the phorbol ester tumor promoters is an enhanced rate of PC turnover. Some delayed perturbation of PI metabolism is also induced, but this may largely be an indirect consequence of the close interrelationship between the various phospholipid pools. Evidence for such an interrelationship has been reported from several laboratories (5, 10). For example, although adrenocorticotropin hormone is thought primarily to activate the PI cycle, it also causes a marked stimulation of PC and PE metabolism (5). Similarly, platelet-derived growth factor enhances PI turnover in 3T3 cells but also increased choline incorporation into PC (10). However, it should be stressed that the analysis of PI metabolism in HeLa cells is not yet complete, as the fractionation methods used in the present study did not separate the polyphosphoinositides phosphatidylinositol-4-phosphate and phosphatidylinositol-4,5-bisphosphate. A rapid effect of promoters on the hydrolysis of these derivatives has, therefore, not been ruled out.
An unexpected finding was the marked stimulation by TPA of 
\[^{32}P\] incorporation into both PE and lyso-PE. This stimulation was 
not accompanied by increased incorporation of \[^{3H}\]ethanolamine or \[^{3H}\]serine. These 2 precursors feed into the pathways which 
are not metabolically distinct PC pools. Presumably, such labeling will be dependent only 
on accessibility to the exchange protein, and may, therefore, "hide" the possible existence of metabolically distinct PC pools.

It is clear that TPA causes major changes in phospholipid metabolism in culture cells. In addition to base exchange, TPA stimulates the turnover of PC initiated by phospholipase C (7, 8, 15) and the breakdown of PC by phospholipase A2 (2, 6). These changes might be expected to modify the activities of intrinsic membrane proteins and to alter the interactions of membranes with cytoplasmic proteins.

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Effects of a Tumor Promoter on Phospholipid Metabolism in HeLa Cells

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