Phorbol Ester-stimulated Hydrolysis of Phosphatidylcholine and Phosphatidylethanolamine by Phospholipase D in HeLa Cells

EVIDENCE THAT THE BASAL TURNOVER OF PHOSPHOGLYCERIDES DOES NOT INVOLVE PHOSPHOLIPASE D*

(Received for publication, December 3, 1990)

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12-O-Tetradecanoylphorbol-13-acetate (TPA) stimulated the release of [3H]ethanolamine from HeLa cells prelabeled with [3H]ethanolamine within 2 min, and of [3H]choline from cells prelabeled with [3H]choline after a lag of 10–20 min. This result suggests that TPA activates phospholipase D. Propranolol alone or propranolol plus TPA stimulated phosphatic acid (PA) labeling in cells prelabeled with [3H]hexadecanol. In the presence of ethanol, TPA stimulated the accumulation of labeled phosphatidylethanolamine (PEth); no PEth was formed in the absence of TPA. TPA-dependent PEth accumulation was not observed in cells pretreated with TPA to down-regulate protein kinase C, whereas propranolol-induced accumulation of PA was unaffected by TPA pretreatment. Incubation of prelabeled cells with propranolol alone caused a rapid loss of label and phospholipid mass from both phosphatidylinositol and phosphatidylethanolamine, and phosphatidylcholine to together with an accumulation of PA and phosphatidylinositol plus phosphatidylserine. When [3H]hexadecanol prelabeled cells were pulse labeled with [32P] to label nucleotide pools, propranolol induced the accumulation of both [3H]- and [32P]-labeled PA. When cells were prelabeled with lyso-PC double labeled with [3H] and [32P], and incubated with propranolol, only [3H]-labeled PA accumulated, indicating that the pathways involved in the basal turnover of PA resulted in the loss of [32P] from the lipid. These results suggest that the basal turnover of phosphatidylethanolamine and PA involves the sequential actions of phospholipase C, diglyceride kinase, and PA phosphohydrolase.

Agonist-induced hydrolysis of PC is now well established in a number of mammalian cell types (1–8). In most cases phorbol esters also stimulate PC hydrolysis, suggesting a regulatory role of protein kinase C. Although PC hydrolysis by phospholipase C resulting in the direct production of DG probably occurs in some cells (1, 3, 9, 10), recent studies have indicated that hydrolysis by phospholipase D also occurs (8), and in some cells is the major route for PC degradation (7). Phospholipase D produces PA, which can be used for phosphatidylinositol biosynthesis or converted to DG by PA phosphohydrolase. This reaction is of particular interest, both because it provides an alternate source of DG, and because PA itself may have regulatory functions. Recent results have indicated that PE may also be a significant substrate for phospholipase D in a number of mammalian cell lines (11, 12). Phospholipase D catalyzes the hydrolysis of both ester and other forms of phosphoglycerides and both forms of PA and DG accumulate in cells in which the phospholipase is activated (13). The regulatory significance of the accumulation of ether derivatives of these signaling molecules is unknown. In the present paper we have used HeLa cells in which the phospholipids have been labeled by preincubation with [3H]hexadecanol or [3H]palmitic acid. TPA induced the rapid hydrolysis of phospholipid by phospholipase D, whereas the basal turnover of phospholipid appeared to be independent of this enzyme.

EXPERIMENTAL PROCEDURES

Materials—[methyl-3H]Choline (74.5 Ci/mmol), [1-3H]ethanolamine (29.8 Ci/mmol), and [9,10-3H]palmitic acid (64 Ci/mmol) were from Amersham Corp. [32P] (10.8 mCi/mg P) was from Australian Radioisotopes. [3H]Hexadecanol was prepared by reduction of [3H] palmitic acid (14). Precast Silica Gel G thin layer plates (0.25 mm thick) were from Merck and TPA was obtained from P-L Biochemicals. Phospholipase D (cabbage) was obtained from Sigma and used to prepare PEth as described (15). Crotalis durissus terrificus venom phospholipase A, and unlabeled lipids were obtained from Sigma.

Cell Culture and Labeling—HeLa cells were grown in Dulbecco’s minimal essential medium containing 10% fetal calf serum in 35-, 100-, or 150-mm dishes and were used when the cultures were approaching confluency. The 100-mm dishes contained 4–6 × 10⁶ cells. Where appropriate, cultures were labeled with [3H]hexadecanol, [3H] palmitic acid, [3H]choline, or [3H]ethanolamine (1 μCi/ml) for 24 h.

Determination of Water-soluble Phospholipid Metabolites—Cells grown in 35-mm dishes were prelabeled with either [3H]choline or [3H]ethanolamine, washed with phosphate-buffered saline (37°C; 5 ml), and the cells incubated with 2 ml of fresh Dulbecco’s modified Eagle’s medium supplemented with 5 mM choline or 5 mM ethanolamine and either TPA (100 nM) or Me2SO (0.1%). At appropriate times, 1-ml aliquots of medium were collected and metabolites separated by chromatography on AG 1-X8 columns (16). Radioactivity associated with choline, ethanolamine, phosphocholine plus glycerophosphocholine, and phosphoethanolamine plus glycerophosphoethanolamine was quantified by liquid scintillation spectrometry.

Determination of Radioactivity Incorporated into PA and PEth—[3H]Hexadecanol-prelabeled cells (100-mm dishes) were incubated with TPA (100 nM), ethanol (85 mM), propranolol (500 μM), or Me2SO (0.2%) for various times. Propranolol was added 5 min before the other test substances. After incubation, the medium was removed and 2 ml of ice-cold methanol added to each dish. Lipids were extracted as described (17) and PA and PEth separated by thin layer chromatography (15) using internal standards of unlabeled PA and PEth;
compounds were located by iodine staining. In this system, other phospholipids remain close to the origin. PC, PE, and PI plus PS were separated by two-dimensional thin layer chromatography (2) with no internal standards and localized by iodine staining. Radioactivity associated with samples scraped from the thin layer plates was measured using liquid scintillation spectrometry.

To prelabel the cells, cultures (35-mm dishes) were incubated with [3H]hexadecanol for 24 h. This procedure was repeated twice before labeling with TPA. The cells were washed (3 × 2 ml) with phosphate-buffered saline containing 11 mM glucose and finally incubated at 37 °C in complete medium containing 15 mM CaCl₂, 11 mM glucose, 10 mM Hepes, pH 7.4. The cells were washed (3 × 2 ml) with phosphate-buffered saline containing 11 mM glucose and finally incubated at 37 °C in complete medium containing test substances dissolved in MeSO or MeSO alone. The final concentration of MeSO in all incubations was 0.2%. Phospholipids were extracted and separated by two-dimensional chromatography as described above. To determine the incorporation of 32P into soluble organic compounds, the aqueous phases after lipid extraction (17) were mixed with 1 g of activated granular charcoal. After 2 min the suspension was centrifuged (12,000 × g; 1 min) and radioactivity measured by scintillation counting.

Preparation of [3H]Lyso-PC, [3H]Lyso-PE, and [3H,32P]Lyso-PC—HeLa cells (4-7 × 150-mm dishes) were labeled with [3H]palmitic acid (0.6 μCi/ml) or [3H]palmitic acid plus 1.5 μCi/ml of 32P for 24 h. Lipids were extracted, dried, and dispersed by sonication in 20 mM Tris-HCl (pH 8.9) containing 15 mM CaCl₂ and fat-free bovine serum albumin (1 mg/ml). Crotalus phospholipase A₂ (400 μg) was added and the suspension incubated for 4 h at 25 °C. The lipids were extracted and lyso-PC and lyso-PE separated by thin layer chromatography (2). The lipids were extracted, dried, and dispersed in 10 mM Hepes buffer (pH 7.4) containing fat-free bovine serum albumin (1 mg/ml). To prelabel the cells, cultures (35-mm dishes) were incubated with [3H]lyso-PC (0.49 μCi), [3H]lyso-PE (0.07 μCi), or [3H,32P]lyso-PC (1.4 Ci/g of [3H,0.09 μCi of 32P) for 100 min. Details of the incorporation of labeled lyso-PC and lyso-PE into PC and PE are given in the legends to Tables III and V.

Phosphate Determination—P, in PC, PE, PA, and PI plus PS was estimated by a modified malachite green method. Samples were dried and ashed as described (18). Following solubilization of the white precipitate with 1.2 N HCl (0.75 ml), aliquots (210 μl) were mixed with 70 μl of color reagent (19) and 10 N NaOH (25 μl) to develop the color.

Presentation of Data—All results are shown as the mean ± S.E. of at least three separate determinations unless otherwise stated. Statistical analysis was performed using Student’s unpaired t test.

**RESULTS**

The release of water-soluble metabolites from cells prelabeled with [3H]choline or [3H]ethanolamine has frequently been used as an indication of phospholipid hydrolysis by phospholipase D and/or phospholipase C. As shown in Fig. 1, TPA caused a rapid release of [3H]ethanolamine from prelabeled HeLa cells which was detectable within 2 min. Only a small release of phosphoethanolamine plus glycerophosphoethanolamine (less than 1% of ethanolamine) was detected at any time point. TPA stimulation of the release of choline metabolites was also observed, but only after a lag of at least 10 min (Fig. 1). Again release of labeled phosphocholine plus glycerophosphocholine was low (less than 3% of choline release). These data are consistent with the rapid activation of a phospholipase D enzyme with some initial specificity toward PE.

In most of the remaining experiments, phospholipid metabolism was studied in cells in which the phospholipid pools had been labeled by incubation with [3H]hexadecanol for 24 h. Such preincubation resulted in extensive labeling of both PE and PC, and the ratio of label incorporation into ester- and ether-linked derivatives varied from about 15:1 to 50:1 (data not shown). The relatively high incorporation into the ester-linked derivatives is presumably a consequence of the oxidation of [3H]hexadecanol to [3H]palmitic acid (20). In the experiments reported in this paper, total radioactivity associated with phospholipids was determined, and the products were not analyzed for the ether and ester forms.

Stimulation of prelabeled cells with TPA in the presence of ethanol caused a rapid accumulation of PEth (Fig. 2) which could be detected within 2 min. As reported previously (13), PEth accumulation was dependent on the presence of TPA (see Table 1). This result suggests that HeLa cells contain a phospholipase D enzyme which is active only when protein kinase C is stimulated by TPA. Despite this, the addition of propranolol alone to HeLa cells caused a marked accumulation of labeled PA which could be detected after 2 min (see Fig. 3). Propranolol is an inhibitor of PA phosphohydrolase (21) and has been shown to cause the accumulation of PA in cultured mammalian cells (4, 7). In these experiments the cells were preincubated with propranolol for 5 min, so at the 2-min time point the cells had a total exposure time of 7 min to the inhibitor. TPA had little effect on PA radioactivity alone, but caused an enhanced response in the presence of propranolol, which could be detected after 1 min of incubation.
Effect of TPA pretreatment on TPA- and propranolol-stimulated accumulation of labeled PA and PEth

Cells were prelabeled with [3H]hexadecanol for 23 h in the presence or absence of 300 nM TPA and incubated with ethanol (85 mM), TPA (100 nM), and/or propranolol (500 μM) for 20 min. Incorporation into PA and PEth was determined as described under "Experimental Procedures." All incubations contained 0.2% Me2SO. Data are the means ± S.E. of three determinations. Similar data were obtained in two separate experiments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MeSO pretreated PEth dpm/dish</th>
<th>PA dpm/dish</th>
<th>TPA pretreated PEth dpm/dish</th>
<th>PA dpm/dish</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control*</td>
<td>8,874 ± 761</td>
<td>14,181 ± 713</td>
<td>11,43 ± 171</td>
<td>21,828 ± 206</td>
</tr>
<tr>
<td>TPA†</td>
<td>11,542 ± 328</td>
<td>24,568 ± 912</td>
<td>2,808 ± 331</td>
<td>22,519 ± 484</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td>(p &lt; 0.001)</td>
<td>NS†</td>
</tr>
<tr>
<td>Propranolol</td>
<td></td>
<td></td>
<td>(p &lt; 0.01)</td>
<td></td>
</tr>
</tbody>
</table>

* Incubations contained 85 mM ethanol.
† No radioactivity was detected.
† NS, not significant.

When cells were washed to remove propranolol, radioactivity associated with PA decreased rapidly to control values (Fig. 4). Although a maximal accumulation of PA was obtained with 500 μM propranolol, similar results were obtained with 250 or 125 μM propranolol (data not shown). As indicated in Table I, TPA stimulated accumulation of PEth and PA was greatly reduced in cells pretreated with TPA to deplete them of protein kinase C (13). By contrast, propranolol caused an accumulation of PA in both control and TPA-pretreated cells. These data suggest that the transphosphatidylation reaction (PEth formation) is catalyzed by an enzyme which is directly or indirectly activated by protein kinase C, whereas PA accumulation in unstimulated cells involves enzymes which are independent of protein kinase C. It is noteworthy that PA-associated radioactivity in unstimulated cells was substantially higher in the TPA-pretreated cells (Table I), perhaps indicating a role of protein kinase C in regulating the basal rate of PA formation or metabolism. It has previously been reported that incubation of granulosa cells with propranolol increased the basal level of PA (4).

The accumulation of PA radioactivity when HeLa cells were incubated in the presence of propranolol alone is unlikely to be due to phospholipase D activity as PEth accumulation did not occur in the presence of ethanol alone. Despite this, it is clear that incubation with propranolol caused a significant decrease in both PE and PC mass and an increase in PA and PI plus PS mass as determined by total P, measurement (Table II). The decrease in PC mass was maintained for at least 80 min, whereas PE was decreased only transiently (20 min) followed by a significant increase at both 40 and 80 min. The changes in phospholipid mass were mirrored by corresponding changes in radioactive label associated with individual lipids (data not shown). At each time point the total P, associated with PA plus PC plus PE plus PI/PS was not significantly altered in the presence of propranolol. No increases in label associated with phosphatidyglycerol were observed at any time point (data not shown). Taken together, these results indicate that there is a rapid turnover of PC and PE in unstimulated cells which is blocked by propranolol, but which probably does not involve phospholipase D. In separate experiments, PE and PC pools were labeled by incubation of...
cells with [3H]lyso-PC or [3H]lyso-PE (see “Experimental Procedures”). Incubation of these cells with propranolol resulted in the accumulation of labeled PA (Table III), clearly confirming phospholipid as the source of PA radioactivity. One possible explanation of these results is that basal turnover of phospholipid involves the sequential actions of phospholipase C, DG kinase, and PA phosphohydrolase. This was tested in an experiment in which cells prelabeled with [3H]hexadecanol for 24 h were labeled for 30 min with 32P (Table IV). The short incubation period with 32P resulted in the labeling of nucleotides (charcoal-adsorbable radioactivity) but only low labeling of the phospholipid pools (see legend to Table IV). Incubation of the cells with propranolol alone resulted in the rapid accumulation of PA labeled with both 32P and 3H, whereas predominantly 3H-labeled PA accumulated in the absence of TPA. These data are consistent with an involvement of both DG kinase and PA phosphohydrolase in the basal turnover of PC and PE. In a separate experiment, cells were prelabeled with lyso-PC double labeled with 3H and 32P. Incubation of these cells with propranolol resulted in the accumulation of [3H]PA containing essentially no 32P (Table V). Based on the ratio of 3H to 32P in PC after prelabeling (see legend to Table V) about 600 32P cpm in PA would have been expected if production of this compound was the result of phospholipase D activity. In fact in a similar experiment in which prelabeled cells were incubated with TPA plus ethanol, PEth accumulated with a 3H/32P ratio close to that predicted from the ratio present in PC isolated after preincubation (data not shown).

**DISCUSSION**

The present results confirm recent results which demonstrate that TPA stimulates the phospholipase D-mediated hydrolysis of PE (11, 12) and PC (22–29) in a range of cultured mammalian cells. Phospholipase D-mediated breakdown of PC is well established (3, 7, 8) and it is now clear that, in some cells at least, PE is also a potential source of signalling molecules in cells stimulated with phorbol esters or agonists. The results also indicate that there is an extremely rapid basal turnover of PE and PC in HeLa cells which requires the conversion of PA to DG by PA phosphohydrolase. Thus the addition of propranolol alone caused a rapid depletion of both PC and PE mass associated with an increase in PA mass. P, associated with PI plus PS also increased, presumably reflecting diversion of PA towards PI biosynthesis. An earlier report has indicated that incubation of lymphocytes with a PA phosphohydrolase inhibitor led to an increase in PI mass (30), and it has been reported that sphingosine-induced PA accumulation also leads to enhanced PI biosynthesis (31). In the present experiments the total phospholipid mass remained relatively constant, implying that PE and PC hydrolysis products are direct precursors for PA and subsequently PI synthesis. The conversion of PC/PE-derived PA to PI is presumably a consequence of the abnormally high concentration of PA accumulated in the presence of propranolol, and may not represent a physiologically relevant pathway (see Ref. 32 for discussion). Incubation with propranolol also resulted in accumulation of labeled PA in cells prelabeled with [3H]lyso-PC or [3H]lyso-PE. The simplest explanation of all of these results is that DG required for the resynthesis of PE and PC is produced by the sequential actions of phospholipase D and PA phosphohydrolase. However, essentially no labeled PEth accumulated when HeLa cells were incubated in the presence of ethanol alone (Table I; 13), implying that unstimulated cells have only a low basal activity of phospholipase D. While it is possible that propranolol could itself activate phospholipase D as well as inhibiting PA phosphohydrolase, in preliminary experiments we have shown that propranolol does not stimulate the accumulation of PEth (data not shown). Consequently, if propranolol does stimulate phospholipase D, it must be an enzyme which does not catalyze the transphosphatidyl reaction. An alternate explanation which is supported by our data is that the basal turnover of phospholipid is initiated by phospholipase C, but that resynthesis requires the conversion of DG to PA by DG kinase. PA phosphohydrolase would then generate DG in an appropriate membrane compartment for phospholipid resynthesis. This hypothesis is supported by the results of two experiments. First, when cells were prelabeled with [3H]hexadecanol and pulse labeled with 32P to label nucleotide pools, PA accumulated in the presence of propranolol contained both 3H and 32P, whereas PA accumulated in the presence of TPA contained predominantly 3H label. This result, together with the observation that propranolol results in loss of total PE and PC is consistent with a role of DG kinase in the basal turnover of these lipids. Second, PA accumulated in the presence of propranolol in cells prelabeled with 3H, [32P]lyso-PC contained only 3H label. It therefore seems certain that phospholipase

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### Table II

**Effect of propranolol on PA, PE, PC, and PI/PS mass in HeLa cells**

<table>
<thead>
<tr>
<th>Phospholipid and treated</th>
<th>Phospholipid</th>
<th>20 min</th>
<th>40 min</th>
<th>80 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC (MeSO)</td>
<td>356 ± 7</td>
<td>344 ± 18</td>
<td>357 ± 5</td>
<td></td>
</tr>
<tr>
<td>PC (propranolol)</td>
<td>336 ± 6</td>
<td>300 ± 9</td>
<td>302 ± 5</td>
<td></td>
</tr>
<tr>
<td>(p &lt; 0.01)</td>
<td>(p &lt; 0.05)</td>
<td>(p &lt; 0.001)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PE (MeSO)</td>
<td>142 ± 3</td>
<td>128 ± 3</td>
<td>136 ± 6</td>
<td></td>
</tr>
<tr>
<td>PE (propranolol)</td>
<td>128 ± 4</td>
<td>150 ± 4</td>
<td>156 ± 5</td>
<td></td>
</tr>
<tr>
<td>(p &lt; 0.01)</td>
<td>(p &lt; 0.001)</td>
<td>(p &lt; 0.05)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA (MeSO)</td>
<td>6.5 ± 2</td>
<td>7.7 ± 4</td>
<td>7.1 ± 8</td>
<td></td>
</tr>
<tr>
<td>PA (propranolol)</td>
<td>12.3 ± 1.3</td>
<td>10.3 ± 0.3</td>
<td>9.5 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>(p &lt; 0.001)</td>
<td>(p &lt; 0.001)</td>
<td>NS*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PI/PS (MeSO)</td>
<td>48 ± 3</td>
<td>47 ± 2</td>
<td>45 ± 4</td>
<td></td>
</tr>
<tr>
<td>PI/PS (propranolol)</td>
<td>84 ± 2</td>
<td>80 ± 5</td>
<td>89 ± 2</td>
<td></td>
</tr>
<tr>
<td>(p &lt; 0.001)</td>
<td>(p &lt; 0.001)</td>
<td>(p &lt; 0.001)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* NS, not significant.

### Table III

**Effect of propranolol on labeling of PA in cells prelabeled with [3H]lyso-PC or [3H]lyso-PE**

HeLa cells were prelabeled with [3H]lyso-PC or [3H]lyso-PE for 100 min as described under “Experimental Procedures.” Propranolol (500 μM) or MeSO (0.1%) was added, and the cells were incubated for 25 min. Radioactivity associated with PA was determined as described under “Experimental Procedures.” After preincubation with [3H]lyso-PC or [3H]lyso-PE, a total of 18,320 ± 3,352 and 32,44 ± 192 dpm were incorporated into PC and PE, respectively. Data are the mean ± S.E. of three determinations. Similar data were obtained in two separate experiments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PA radioactivity</th>
<th>PC labeled</th>
<th>PE labeled</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dpm/dish</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>400 ± 13</td>
<td>132 ± 6</td>
<td></td>
</tr>
<tr>
<td>Propranol</td>
<td>3677 ± 42</td>
<td>635 ± 20</td>
<td>(p &lt; 0.001)</td>
</tr>
</tbody>
</table>
Hydrolysis of Phosphoglycerides by Phospholipase D

### TABLE IV

**Effect of TPA and propranolol on PA labeling in cells preincubated with \(^{3}H\)hexadecanol and \(^{32}P\)**

HeLa cells in 35-mm dishes were prelabeled with \(^{3}H\)hexadecanol for 24 h followed by labeling with \(^{32}P\) for 30 min as described under "Experimental Procedures." The cells were incubated with propranolol (500 μm) or TPA (100 nm) and incorporation of label into PA was determined. All incubations contained 0.2% MeSO. Data are the mean ± S.E. of three or four (40-min time point) determinations. After preincubation with charcoal-adsorbable fraction from the aqueous phase remaining after lipid extraction contained 85,208 ± 3,832 \(^{32}P\) cpm, and the total PC plus PE pool 118,947 ± 18,083 \(^{3}H\) dpm and 759 ± 119 \(^{32}P\) cpm. Similar data were obtained in two separate experiments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>5 min</th>
<th>10 min</th>
<th>20 min</th>
<th>40 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(^{3}H) (dpm/dish)</td>
<td>5,270 ± 342</td>
<td>4,775 ± 178</td>
<td>3694 ± 55</td>
<td>7,316 ± 225</td>
</tr>
<tr>
<td>(^{32}P) (dpm/dish)</td>
<td>254 ± 19</td>
<td>204 ± 20</td>
<td>149 ± 9</td>
<td>134 ± 7</td>
</tr>
<tr>
<td>Propranolol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(^{3}H) (dpm/dish)</td>
<td>7,505 ± 321a</td>
<td>8,558 ± 286b</td>
<td>10,389 ± 447b</td>
<td>12,350 ± 606b</td>
</tr>
<tr>
<td>(^{32}P) (dpm/dish)</td>
<td>816 ± 14a</td>
<td>1,449 ± 39b</td>
<td>1,643 ± 39b</td>
<td>1,497 ± 99b</td>
</tr>
<tr>
<td>TPA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(^{3}H) (dpm/dish)</td>
<td>6,379 ± 411c</td>
<td>7,253 ± 262c</td>
<td>6,647 ± 98c</td>
<td>8,271 ± 404</td>
</tr>
<tr>
<td>(^{32}P) (dpm/dish)</td>
<td>277 ± 10d</td>
<td>207 ± 21d</td>
<td>210 ± 18c</td>
<td>159 ± 8c</td>
</tr>
</tbody>
</table>

*Significantly different from control, p < 0.01.

**Significantly different from control, p < 0.001.

aSignificantly different from control, p < 0.05.

NS, not significantly different from control.

### TABLE V

**Effect of propranolol on labeling of PA in cells preincubated with lyso-PC double labeled with \(^{3}H\) and \(^{32}P\)**

HeLa cells were prelabeled with \(^{3}H,^{32}P\)lyso-PC for 100 min as described under "Experimental Procedures." Propranolol (500 μm) or MeSO (0.1%) was added, and the cells were incubated for 25 min. Radioactivity associated with PA was determined as described under "Experimental Procedures." After preincubation with \(^{3}H,^{32}P\)lyso-PC, a total of 115,984 ± 2,633 \(^{3}H\) dpm and 4,791 ± 145 \(^{32}P\) cpm were incorporated into PC. Data are the mean ± S.E. of three determinations. Similar data were obtained in two separate experiments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PA radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(^{3}H)</td>
</tr>
<tr>
<td>Control</td>
<td>892 ± 57</td>
</tr>
<tr>
<td>Propranolol</td>
<td>14,668 ± 539</td>
</tr>
</tbody>
</table>

D activity is not responsible for the accumulated PA in unstimulated cells.

The contribution of de novo PA biosynthesis to the present data is difficult to assess. The mass determinations suggest that all of the changes can be accounted for by the turnover of existing phospholipid. However, in both of the experiments described above, it is possible that labeled PA accumulated in the presence of propranolol is a consequence of de novo synthesis. This could obviously account for the incorporation of \(^{32}P\) from the nucleotide pool. Similarly, in the experiments in which cells were prelabeled with lyso-PC or lyso-PE, the \(^{3}H\) label is associated with the 1-position. Consequently, \(^{3}H\) PA could arise by phospholipase A2 activity, followed by incorporation of labeled palmitic acid via the de novo pathway. In separate experiments we have shown that HeLa cells incorporate \(^{3}H\)palmitic acid rapidly into PA and that this incorporation is strongly inhibited by the inclusion of excess unlabeled palmitic acid in the incubation. However, the addition of unlabeled palmitic acid did not reduce the propranolol-induced accumulation of labeled PA in cells prelabeled with \(^{3}H\)lyso-PC (data not shown). Finally, we have shown that the addition of propranolol to \(^{3}H\)hexadecanol-prelabeled cells results in the loss of both ether and ester forms of PC and PE and the accumulation of PA with an ether lipid composition, which is similar to that of the phospholipid pool.2

Accumulation of the stable ether derivative of PA argues strongly that a major portion of the labeled PA is derived from phospholipid hydrolysis and not de novo synthesis.

Possible functions for PA generated by phospholipase D remain speculative. Apart from providing an alternate source of DG, the recent reports that lyso-PA is strongly mitogenic are intriguing (33, 34). It has not yet been established whether lyso-PA interacts with a surface receptor and whether internally produced lyso-PA is involved in metabolic regulation. However, it will be of interest to determine whether stimuli which activate phospholipase D also result in the accumulation of lyso-PA.

### REFERENCES

Hydrolysis of Phosphoglycerides by Phospholipase D