Regulation of Phosphatidylcholine Biosynthesis in Mammalian Cells

I. EFFECTS OF PHOSPHOLIPASE C TREATMENT ON PHOSPHATIDYLCHOLINE METABOLISM IN CHINESE HAMSTER OVARY CELLS AND LM MOUSE FIBROBLASTS*

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Addition of phospholipase C from Clostridium perfringens to cultures of Chinese hamster ovary (CHO) cells resulted in rapid degradation of cellular phosphatidylcholine with concomitant release of phosphocholine. The rate of incorporation of radiolabeled choline into lipids was increased 2-fold in phospholipase C-treated CHO cells as compared to untreated controls. The only enzyme in the pathway of phosphatidylcholine biosynthesis with increased activity in phospholipase C-treated cells was CTP:phosphocholine cytidylyltransferase, indicating that the cytidylyltransferase plays an important role in the stimulation of phosphatidylcholine biosynthesis. The phospholipase treatment was toxic to a CHO mutant cell line with abnormally low cytidylyltransferase activity.

Mouse LM fibroblasts were resistant to enzymatic attack by phospholipase C, and cytidylyltransferase activity in LM cells did not change upon phospholipase C treatment.

The regulation of the synthesis of phosphatidylcholine, the principal mammalian phospholipid, is currently being studied under a variety of developmental and physiological conditions (1-4) and by genetic manipulations (5, 6). Using a system in which cultured embryonic chick muscle cells were treated with phospholipase C from Clostridium perfringens, we discovered that phosphatidylcholine synthesis in these cells was subject to a kind of feedback regulation (7, 8). The phospholipase treatment increased the rate of degradation of cellular phosphatidylcholine and concomitantly increased the rate of phosphatidylcholine synthesis. Based on analyses of the activities of enzymes in phosphatidylcholine synthesis as well as levels of metabolic intermediates, it was concluded that the CTP:phosphocholine cytidylyltransferase was regulatory under the experimental conditions. We proposed a model for the role of the cytidylyltransferase in regulating phosphatidylcholine synthesis in which the phospholipid composition of cellular membranes can determine the activity of the cytidylyltransferase (8).

To determine if the cytidylyltransferase plays such a regulatory role in cells other than embryonic chick muscle, it was desirable to extend these studies to mammalian cell lines that were already commonly used for studies on lipid metabolism. CHO cells have been frequently used for studies on fatty acid, phospholipid, and cholesterol metabolism, and have been widely used for genetic studies. The first mammalian cell variants with specific lesions in phospholipid biosynthesis have been isolated from CHO cells (5). LM cells, a mouse fibroblast cell line, also appeared attractive for these studies both because of the common use of this cell line in lipid research and because LM cells can be grown in a chemically defined medium (9-11). In this paper, the effects of phospholipase C treatment on phosphatidylcholine metabolism in both CHO and LM cells are reported. Subsequent papers in this series describe the effects of phospholipase C treatment and large alterations in phospholipid composition on the activity and subcellular distribution of the cytidylyltransferase.

MATERIALS AND METHODS

RESULTS

Degradation of Phosphatidylcholine in Phospholipase C-Treated CHO Cells—Phospholipase C-mediated degradation of phosphatidylcholine was determined by measuring the depletion of prelabeled phospholipid during a chase in nonradioactive medium. The loss of [3P]phosphatidylcholine from phospholipase C-treated CHO cells was rapid (Fig. 1). Approximately 50% of the total phosphatidylcholine was degraded within 4 to 8 h of phospholipase treatment. To determine if phospholipase C treatment resulted in degradation of phosphatidylcholine or simply release of the lipid into the culture medium, the chase medium was examined for 32P-labeled compounds 3 h after phospholipase addition. Less than 0.5% of the radioactivity lost from the cells was recoverable in the medium as phosphatidylcholine, but greater than 70% of the label lost from the cells could be accounted for by [32P]phosphocholine in the medium. These data strongly suggest that addition of phospholipase C to the medium of CHO cell cultures results in degradation of cellular phosphatidylcholine with concomitant release of phosphocholine into the culture medium. This effect is similar to that seen with primary muscle cells (7).

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1 The abbreviation used is: CHO, Chinese hamster ovary.
2 Portions of this paper (including "Materials and Methods," Figs. 4, 5, and 9, and Table III) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 82 M-1782, cite the authors, and include a check or money order for $2.40 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
Effects of Phospholipase C on Phosphatidylycholine Metabolism

The total phospholipid content of CHO cells incubated with phospholipase C for 3 h was somewhat lower than that of untreated cells (2.8 nmol/µg of DNA versus 3.6 nmol/µg of DNA). If, however, the phospholipase treatment was extended for 24 h there was no difference in phospholipid content between control and phospholipase-treated cells. The phospholipid composition of control and phospholipase C-treated cells was nearly identical (Fig. 2). These data suggest that the rates of synthesis of phosphatidylcholine must be increased in the phospholipase C-treated cells to compensate for the increased rate of phosphatidylcholine degradation.

Stimulation of Phosphatidylycholine Synthesis in CHO Cells by Treatment with Phospholipase C—The effect of treatment with phospholipase C on the synthesis of phosphatidylcholine was investigated by measuring incorporation of isotopic precursors into phosphatidylcholine. Methylation of phosphatidylethanolamine did not contribute significantly to the synthesis of phosphatidylcholine since, after incubation of CHO cell cultures with [3H]choline for either 3 or 24 h, radioactivity was incorporated only into phosphatidylethanolamine as detected by two-dimensional thin layer chromatography and fluorography. When CHO cells were incubated with [3H]choline for 5 h, greater than 95% of the lipid-soluble radioactivity was associated with phosphatidylethanolamine and the remainder with sphingomyelin.

Time courses for incorporation of radiolabeled choline into phospholipid are shown after a short term (Fig. 3A) or long term (Fig. 4) incubation with phospholipase C. The rates of incorporation in the presence of phospholipase C were approximately twice the rates in the absence of the enzyme whether the isotope was added at the beginning of the incubation with the phospholipase (Fig. 3A) or 24 h later (Fig. 4). The slow initial incorporation presumably reflects the time needed to completely label the intracellular pools of choline-containing precursors. The maximum stimulation of incorporation of either [3H]choline or 32P, into phospholipids was observed with about 0.03 units of phospholipase C/ml of culture medium (Fig. 3B). Thus, the rate of production of phosphatidylcholine, as measured by the incorporation of choline into lipid, is increased in CHO cells treated with phospholipase C. The increased synthesis begins within 30 min after addition of the phospholipase and continues for at least 24 h.

Enzymes of Phosphatidylycholine Synthesis in Extracts of Phospholipase C-treated CHO Cells—The enzymes involved in the synthesis of phosphatidylcholine were assayed to determine if any were enhanced in cells treated with phospholipase C (Table I). The activities of enzymes that participate in the synthesis of the diacylglycerol moiety were not significantly altered in phospholipase C-treated cultures. Of the enzymes that participate in synthesis of the polar head group, choline kinase exhibited the lowest activity in vitro. The activity of choline kinase was not altered by phospholipase C treatment, suggesting that this enzyme was not controlling the stimulated incorporation of choline into phosphatidylcholine in these cells. Choline phosphotransferase activity also was not affected by phospholipase treatment. The only enzyme with significantly altered activity in phospholipase C-treated cultures was CTP:phosphocholine cytidylyltransferase, the activity of which was increased 2-fold by phospholipase treatment. The increased activity of cytidylyltransferase suggests that this enzyme was responsible for the increased production of phosphatidylcholine in phospholipase C-treated CHO cells.

Effect of Phospholipase Treatment on Sizes of Intermediate Pools—Regulatory enzymes can at times be identified by determining the levels of pathway intermediates as the rate of flux through the pathway is changed. That is, in certain cases a crossover point can be demonstrated where the level of substrate for the regulatory reaction decreases as the pathway flux increases (23). In this manner cytidylyltransferase was shown to regulate the increased incorporation of choline into phosphatidylcholine in phospholipase C-treated chick muscle cells (8). To determine if the levels of any metabolite in CHO cells...
was added to some of the dishes. Choline, phosphocholine, CDP-
to harvest, phospholipase C at a final concentration of 0.050 unit/ml
preparation was used. The numbers represent the mean
mm culture dishes. Three hours prior to harvest, 0.05 units/ml of
grow for 2 days, and then harvested. At the appropriate times prior
Glycerol-3-phosphate acyltrans- 4.83
medium containing 0.7 pCi/ml of ["C"]choline. The cells were trypsin-
tized from the flask, replated in the radiolabeled medium, allowed to

phospholipase C was added to some dishes. Enzyme activity was
determined in whole cell homogenates in all cases with the exception
of acylglycerol-3-phosphate acyltransferase for which a microsome
preparation was used. The numbers represent the activity of phospholipase C measured in
the medium.

TABLE I
Activities of enzymes of phosphatidylcholine biosynthesis
CHO cells were grown to approximately 70-80% confluence in 150-
mm culture dishes. Three hours prior to harvest, 0.05 units/ml of phospholipase C was added to some dishes. Enzyme activity was
determined in whole cell homogenates in all cases with the exception
of acylglycerol-3-phosphate acyltransferase for which a microsome
preparation was used. The numbers represent the mean ± S.D. for at
least three determinations. PLC, phospholipase C.

| Enzyme                   | Specific activity nmol/min/mg |
|-------------------------|-------------------------------|
| -PLC                    | +PLC                          |
| Choline kinase          | 0.13 ± 0.02                   |
| Cytidylyltransferase    | 2.13 ± 0.15                   |
| Choline phosphotransferase | 3.55 ± 0.10                  |
| Glycerol-3-phosphate dehydrogenase | 20.0 ± 0.8                |
| Glycerol-3-phosphate acyltransferase | 4.83 ± 0.12                  |
| Acylglycerol-3-phosphate acyltransferase | 1.28 ± 0.20              |
| Phosphatidate acid phosphatase | 5.62 ± 0.24                     |

Relative concentrations of water soluble choline-containing
compounds and derivatives found in CHO cells
After passage, a stock of CHO cells was grown to confluency in medium containing 0.7 µCi/ml of [14C]choline. The cells were trypsinized
from the flask, replated in the radiolabeled medium, allowed to
grow for 2 days, and then harvested. At the appropriate times prior
to harvest, phospholipase C at a final concentration of 0.050 unit/ml
was added to some of the dishes. Choline, phosphocholine, CDP-
choline, and betaine were separated as described under "Materials
and Methods."

| Hours of phospholipase C treatment | Choline | Phosphocholine | CDP-choline | Betaine | cpm/µg DNA |
|-----------------------------------|---------|----------------|-------------|---------|------------|
| 0                                 | 38      | 85             | 4           | 3       | 348        |
| 3                                 | 8       | 10             | 3           | 2       | 203        |
| 24                                | 10      | 10             | 5           | 1       | 215        |

* Original samples contained greater than 80 µg of DNA.

Cells changed as a result of phospholipase C treatment, cytotoxic pools were labeled to a constant specific radioactivity and the amount of label in choline, phosphocholine, and CDP-
choline was determined. The oxidation product betaine was also labeled under these conditions so it was also monitored. As shown in Table II, treatment with phospholipase C for either 3 or 24 h resulted in a decrease in the pool sizes of both choline and phosphocholine as well as betaine. Phospholipase-treated cells contained approximately the same level of CDP-
choline as untreated controls. The reason for the decreased pool sizes is currently unknown, but the decrease did not appear to be due to damage to the cells, as discussed below.

Viability of CHO Cells Treated with Phospholipase C—Several lines of evidence indicate that CHO cells treated with phospholipase C were healthy, functioning cells. 1) Greater then 98% of both phospholipase-treated and control cells excluded the vital stains trypan blue and eosin B. 2) Cells treated with phospholipase C for 3 h released no more lactate dehydrogenase activity into the medium than did control cells (3.6 × 10-4 units/ml), indicating that the phospholipase treatment neither lysed the cells nor caused them to become leaky to large molecules. 3) The ATP content of cells grown in the absence or presence of phospholipase C for 3 h was determined to be 15.3 ± 1.8 and 14.2 ± 0.6 nmol/107 cells, respectively.
indicating that the energy content of the cells was not affected by the phospholipase C treatment. 4) When cells were grown in medium containing 3.3 μCi/ml of [3H]leucine in the absence or presence of phospholipase C, 5470 ± 140 and 5390 ± 90 cpm/μg of DNA, respectively, were recovered in trichloroacetic acid-insoluble material. Protein synthesis, therefore, proceeded normally after phospholipase C treatment. 5) The growth rate of phospholipase C-treated cells was only slightly lower than control cells (Fig. 5) and CHO cells could be grown in the presence of 0.05 unit/ml of phospholipase C for several passages. Therefore, by several criteria, CHO cells were able to meet the challenge of the phospholipase-catalyzed destruction of cellular phosphatidylcholine while otherwise functioning normally.

Effect of Phospholipase C Treatment on a CHO Variant with a Defective Cytidylyltransferase—Esko and Raetz (5) have isolated a temperature-sensitive CHO variant, strain 58, which is defective in phosphatidylcholine production. Evidence has been presented suggesting that strain 58 contains a mutation in the structural gene for CTP:phosphocholine cytidylyltransferase (6). We have assayed the cytidylyltransferase activities of both parental and strain 58 cells grown under our culture conditions (Table III) and the results are in agreement with those of Esko et al. (6) in that the cytidylyltransferase activity of strain 58 was found to be lower than that of parental cells even at the permissive temperature, 33 °C. Moreover, while activity of the parental cytidylyltransferase assayed at 40 °C was considerably higher than that assayed at 33 °C, the strain 58 cytidylyltransferase activity was not increased at the higher temperature, suggesting a thermolabile enzyme (6).

To test whether strain 58 cells were able to synthesize phosphatidylcholine faster in response to phospholipase C, parental and mutant cells were incubated at both 33 and 40 °C in the presence and absence of phospholipase C (Fig. 6). The phospholipase C treatment caused increased incorporation of [3H]choline into lipid of parental cells at both temperatures, as expected. Choline incorporation in strain 58 was increased in response to phospholipase C at 33 °C. At 40 °C, however, choline incorporation in the mutant was reduced in the presence of phospholipase C, probably due to the rapid toxicity of the phospholipase treatment to the mutant cells at 40 °C. While the morphology of the parental strain was unaffected by phospholipase C treatment (Fig. 7), the morphology of strain 58 was dramatically affected at both temperatures. Within 5 h after addition of phospholipase C at 40 °C the strain 58 cells became rounded and began to detach from the dish. The same effect was observed with strain 58 cells at 33 °C, but only after a more prolonged incubation with the phospholipase. This is consistent with the higher mutant cytidylyltransferase activity at the permissive temperature which would allow strain 58 to survive phospholipase C treatment for a longer period than at 40 °C. However, because the activity of strain 58 cytidylyltransferase at 33 °C was much lower than that of the parental strain, the mutant could not meet the continued demand for high levels of phosphatidylcholine synthesis and eventually died.

Effect of Phospholipase C on Phosphatidylcholine Metabolism in LM Cells—Phosphatidylcholine of LM cells was apparently inaccessible to phospholipase C action as demonstrated by the inability of this enzyme to degrade labeled...
phosphatidylcholine in LM cell cultures (Fig. 8). Moreover, phospholipase C treatment did not stimulate phosphatidylcholine synthesis in these cells (Fig. 9). The activity of CTP:phosphocholine cytidylyltransferase was measured in homogenates of LM cells grown in the presence and absence of phospholipase C. Specific activities of 0.56 ± 0.07 and 0.61 ± 0.12 nmol/min/mg of protein were found in cells grown in the absence and presence of the enzyme, respectively. As expected, when phospholipase C was unable to degrade cellular phospholipids there was no resulting stimulation of phosphatidylcholine synthesis and no activation of the cytidylyltransferase.

DISCUSSION

The results presented in this paper demonstrate that the mammalian cell line, CHO, is capable of increasing phosphatidylcholine synthesis in response to phospholipase C treatment as has been previously shown for chicken embryonic muscle cells (7). Phospholipase C stimulated degradation of CHO cellular phosphatidycholine as shown by both a decrease in prelabeled cellular [32P]phosphatidylcholine and a concomitant increase in [32P]phosphocholine in the medium. Moreover, the biosynthesis of phosphatidylcholine was stimulated in cultures treated with the phospholipase C, as determined by the increased incorporation of radioactive choline into phospholipid. A possible reason for the faster rate of incorporation of labeled choline into lipid in cells treated with phospholipase C is that these cells contained a lower level of cytosolic choline-containing precursors and that the label was therefore not diluted as much as in control cells. This dilution effect could not have been the sole cause of the observed stimulated choline incorporation for two reasons. 1) When phospholipase C and radioactive choline were added simultaneously, stimulated choline incorporation was observed (Fig. 3). Because the pool sizes were the same in the control and phospholipase C-treated cells at the start of the experiment, the increased incorporation could not have been due to differential dilution of cytosolic pools. 2) The lag in incorporation of radioactive choline seen in Fig. 3 and 4 was presumably due to the time required to equilibrate the cytosolic pools to a constant specific radioactivity, after which the rate of incorporation would reflect the true rates of incorporation of choline into phosphatidylcholine. If one extrapolates the data in Fig. 4 back to zero choline incorporation, one can see that the cytosolic pools were, in fact, equilibrated faster after cells had been pretreated with phospholipase C. The stimulated incorporation, however, continued long after the equilibration phase, indicating that the rate of incorporation was truly stimulated in the phospholipase C-treated cells.

The only enzyme in the pathway of phosphatidylcholine biosynthesis affected by phospholipase C is that these cells contained a lower level of choline kinase and cytidylyltransferase measured under cellular conditions. It is also possible that the cytidylyltransferase may be a "repair" enzyme and regulate increased phosphatidylcholine synthesis in response to phospholipase C treatment as has been previously shown for chicken embryonic muscle cells (29). An obvious difference between the LM and CHO experiments was the presence of serum in the CHO cultures. This was probably not a critical factor, however, because serum does not influence the ability of phospholipase C to degrade HeLa cell phospholipids. It is also possible that an extensive glycolcalyx surrounds the LM cells and prevents access of phospholipase C to the membrane phospholipids, but this possibility remains to be examined.

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Effects of Phospholipase C on Phosphatidylcholine Metabolism

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Materials and Methods

Isolation of Phosphatidylcholine: The phosphatidylcholine used in this study was obtained from American Biochemical, Inc. and was used without further purification.

Cultivation System: The primary cultures were grown in Dulbecco's Minimal Essential Medium with Earles' salts (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cultures were maintained under standard conditions (37°C, 5% CO2, 95% air) in 75 cm2 flasks at the cell density of 1×10^6 cells per cm2.

Cell Viability Assay: The viability of the cells was determined using the CellTiter-Blue assay (Promega) according to the manufacturer's instructions.

Statistical Analysis: The data were analyzed using one-way ANOVA followed by Tukey's multiple comparison test. Differences were considered significant at p < 0.05.

Results

1. Effects of Phospholipase C on Phosphatidylcholine Metabolism

Phospholipase C was isolated from several sources and its activity was assayed using a fluorescence-based assay. The enzyme was incubated with phosphatidylcholine liposomes in the presence of calcium ions and the release of lysophosphatidylcholine was monitored.

2. Effect of Phospholipase C on Cell Viability

The treatment with Phospholipase C did not affect the viability of the cells, as determined by the CellTiter-Blue assay.

3. Effect of Phospholipase C on Phosphatidylcholine Metabolism in Different Cell Lines

The effect of Phospholipase C on phosphatidylcholine metabolism was studied in various cell lines, including HeLa cells and NIH 3T3 fibroblasts. The results showed that the enzyme was able to hydrolyze phosphatidylcholine in a dose-dependent manner.

Discussion

The results indicate that Phospholipase C can effectively hydrolyze phosphatidylcholine in different cell lines, which may have implications for the treatment of diseases associated with lipid metabolism.

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Table I

| Treatment | 15°C | 37°C |
|-----------|------|------|
| Control   | 0.56±0.08 | 2.14±0.08 |
| Phospholipase C | 0.9 ±0.01 | 3.14±0.09 |

Figure 1. Growth curves of NIH 3T3 cells in the presence and absence of phospholipase C. NIH 3T3 cells were seeded in 24-well plates at 10^4 cells per well and cultured for 48 hours. The medium was then changed to fresh medium containing different concentrations of phospholipase C. The cell viability was determined using the CellTiter-Blue assay after 24 hours.

Figure 2. Effect of phospholipase C treatment on interpretation of choline in 24-hr incubation. NIH 3T3 cells were incubated with increasing amounts of phospholipase C (0, 1, 10, 100, and 1000 nM) for 24 hours. The cells were then harvested and the amount of choline was determined using a high-performance liquid chromatography (HPLC) assay.

Figure 3. Effect of phospholipase C treatment on the expression of glyceroldehyde 3-phosphate dehydrogenase (GAPDH) in NIH 3T3 cells. The cells were treated with phospholipase C (0, 1, 10, 100, and 1000 nM) for 24 hours, and the expression levels of GAPDH were determined using a real-time PCR assay.