Regulation of Phosphatidylcholine Biosynthesis in Mammalian Cells

III. EFFECTS OF ALTERATIONS IN THE PHOSPHOLIPID COMPOSITIONS OF CHINESE HAMSTER Ovary AND LM CELLS ON THE ACTIVITY AND DISTRIBUTION OF CTP:PHOSPHOCHOLINE CYTIDYLYLTRANSFERASE

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The activity and subcellular distribution of CTP:phosphocholine cytidylyltransferase in LM and Chinese hamster ovary cells in which the phospholipid composition had been altered by supplementary feeding with choline analogues were examined. Decreased levels of cellular phosphatidylcholine with corresponding increased levels of either phosphatidylserine, phosphatidylmonomethylethanolamine, or phosphatidyltrimethylamine resulted in increased CTP:phosphocholine cytidylyltransferase activity in cell homogenates. In addition, a significantly larger fraction of the total cytidylyltransferase activity was membrane-bound in these cells. The activity of the cytidylyltransferase from cytosolic extracts of both cell types was found to be greatly increased when assayed in the presence of either phosphatidylmonomethyl ethanolamine or phosphatidyltrimethylamine. The lysophosphatide forms of these lipids were found to be poor activators of the cytidylyltransferase. These findings suggest that the regulation of phosphatidylcholine biosynthesis is at least partially dependent on information transfer from membranes to CTP:phosphocholine cytidylyltransferase.

Our previous studies on the mechanisms of regulation of phosphatidylcholine biosynthesis have concentrated on a model system in which the CDP-choline pathway was activated in cultured cells by treatment with phospholipase C (1-4). A comparison of the activities of biosynthetic enzymes and levels of metabolic intermediates in phospholipase C-treated and untreated cells led to the conclusion that the rate of phosphatidylcholine production was determined by the activity of CTP:phosphocholine cytidylyltransferase (2-4). A distinctive feature of the cytidylyltransferase is its biomodal distribution in maturing animal cell types (2, 4-8). Membrane-associated cytidylyltransferase is quite active while cytosolic cytidylyltransferase is relatively inactive except when assayed in the presence of certain lipids. A redistribution of the cytidylyltransferase from the cytosolic to the membranous fraction of CHO or embryonic chick muscle cells occurs when the cells are incubated in the presence of phospholipase C (2, 4).

To explain the subcellular redistribution-associated activation of the cytidylyltransferase in phospholipase C-treated cells, we proposed that binding of the cytosolic form of the enzyme to membranes was dependent on membrane phospholipid composition (2, 4). The rapid breakdown of cell surface phosphatidylcholine by phospholipase C suggested that at least a transient decrease in membrane phosphatidylcholine content had occurred. To test our hypothesis further, we began a series of studies to examine the activity and subcellular distribution of CTP:phosphocholine cytidylyltransferase in cultured cells in which the phospholipid composition had been measurably altered by means other than phospholipase C treatment.

MATERIALS AND METHODS

Cell culture—CHO and LM cell lines were maintained as described previously (3). To prepare LM cells with altered lipid compositions, the method of Glaser et al. was used (9). LM cells were placed in choline-free medium and 40 µg/ml of either choline, dimethylthanolamine, monomethylthanolamine, or ethanolamine was added. The cells were placed in a 37°C incubator with a humidified atmosphere for 3 days and then harvested. The culture medium was exchanged for fresh medium every 24 h. (When cultures were maintained for 3 days in the same medium, very low levels of cytidylyltransferase were found.) CHO cells with altered lipid compositions were prepared by maintaining the cells in choline-free F-12 medium containing 10% newborn calf serum that had been exhaustively dialyzed against normal saline, and either choline, dimethylthanolamine, monomethylthanolamine, or ethanolamine were added to a final concentration of 100 µM. The cells were placed in a 37°C incubator with a humidified atmosphere and incubated for 3 days prior to harvest. The culture medium was exchanged for fresh medium every 24 h.

Lipids—Phosphatidylcholine, monomethylthanolamine, and phosphatidyltrimethylamine, prepared by transphosphatidylation of egg yolk phosphatidylcholine, were purchased from Avanti Biochemicals, Inc. The lysophospholipids of these phospholipids were prepared by incubating them with porcine pancreas phospholipase A1 obtained from Boehringer-Mannheim. The lipids were first prepared as 10 mM solutions suspensions in 10 mM Tris-HCl (pH 8.1) containing 3 mM CaCl2 and then incubated at 40°C with 100 units/ml of phospholipase A1. Nine hours later, lipids were extracted from the reaction mixture (10) and the lysophospholipids purified by silicic acid chromatography (11). The preparation of sonicated suspensions of these lipids for use in the cytidylyltransferase assay was described previously (2). The purity of the phospholipids used was determined by thin layer chromatography (22). All of the phospholipids, both before and after sonication, migrated as one spot on the thin layer plates.

Cellular lipid compositions were determined as described by Glaser

1 The abbreviation used is: CHO, Chinese hamster ovary.
et al. (9). Organic phosphate was determined by the procedure of Reuser et al. (13).

Miscellaneous Determinations—Protein was determined by the procedure of Lowry et al. (14) with bovine serum albumin as the standard. CTP:phosphocholine cytidylyltransferase activity was assayed as described (4, 15).

RESULTS

Studies with the CHO Cell Line—The method chosen to alter cellular phospholipid compositions was that described by Glaser et al. (9) in which cells are starved for choline and supplemented with the choline analogues dimethylethanolamine, monomethylethanolamine, or ethanolamine. Under these conditions the levels of cellular phosphatidylcholine decrease while levels of the phosphatidyl-derivatives of the analogues increase. If such production of altered phospholipid compositions in CHO cells is to be used to test the proposed model for regulation of phosphatidylcholine biosynthesis, the different phosphatidyl analogues must be able to activate the cytosolic CHO cytidylyltransferase. The results obtained from assays of the cytosolic cytidylyltransferase in the presence of the added sonicated suspensions of phospholipids are presented in Fig. 1. Both phosphatidyl-dimethyl ethanolamine and phosphatidyl-monomethyl ethanolamine were potent activators of the cytosolic cytidylyltransferase, whereas their corresponding lysophosphatides had little effect on activity. It was previously shown that phosphatidylethanolamine activates the CHO cytidylyltransferase while phosphatidylcholine has little effect (4).

In order to supplement CHO cells with the analogues in a defined medium with choline, initial attempts were made to grow CHO cells in serum-free medium or medium containing 10% dialyzed serum and supplemented with either choline, dimethylethanolamine, monomethyl-

![Fig. 1. Activation of cytosolic CHO cell CTP:phosphocholine cytidylyltransferase by choline analogue-containing phospholipids. Enzyme activity was measured with sonicated suspensions of the following lipids: phosphatidyl-dimethyl ethanolamine (), lysophosphatidyl-dimethyl ethanolamine (), phosphatidyl-monomethyl ethanolamine (), and lysophosphatidyl-monomethyl ethanolamine (). Data points are the averages of duplicate determinations. The concentration of lipid is with respect to phosphorus.](image)

Table I

| Lipid Ethanolamine | Medium supplement | None |
|-------------------|-------------------|------|
| **Choline** | **DME** | **MME** | **Ethanolamine** |
| PC | 53 | 28 | 40 | 49 | 51 |
| PE | 21 | 11 | 7 | 24 | 21 |
| SM | 9 | 9 | 10 | 9 | 10 |
| PS | 5 | 7 | 9 | 6 | 7 |
| PI/PA | 8 | 8 | 8 | 7 | 7 |
| CL | 1 | 1 | 1 | 1 | 1 |
| PDME | 0 | 34 | 0 | 0 | 0 |
| PMME | 0 | 0 | 23 | 0 | 0 |
| Other* | 3 | 2 | 2 | 3 | 3 |

*Other: percentage of total lipid ethanolamine not found in the phospholipid classes identified.

The abbreviations used are: PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin; PS, phosphatidylserine; PI, phosphatidylinositol; PA, phosphatic acid; CL, cardiolipin; PDME, phosphatidyl-dimethyl ethanolamine; PMME, phosphatidyl-monomethyl ethanolamine; DME, dimethyl ethanolamine; MME, mono- methylethanolamine.

ethanolamine, ethanolamine, or no analogue, the growth rate was essentially identical in the different media preparations. The growth rate of cultures in medium with dialyzed serum was slower than that of cultures grown in medium containing complete serum (not shown).

The phospholipid composition of CHO cells grown in choline-free media containing dialyzed serum and different phospholipid head group substrates is presented in Table I. The addition of monomethyl ethanolamine or dimethyl ethanolamine to the medium resulted in a decrease in the levels of phosphatidylcholine and phosphatidylethanolamine and an increase in the levels of either phosphatidyl monomethyl ethanolamine or phosphatidyl dimethyl ethanolamine. Cells grown in the presence of either added choline, ethanolamine, or no supplement had approximately the same phospholipid composition as cells grown in medium containing untreated serum (3). This suggested that the CHO cells could either produce enough phosphatidylcholine through methylation of phosphatidylethanolamine or utilize the choline moiety of sphingomyelin and/or phosphatidylcholine found in the dialyzed serum. CHO cells grown in serum-free medium absolutely require a choline supplement (29) and a CHO variant with a temperature-sensitive cytidylyltransferase cannot survive at the nonpermissive temperature (30, 31); it is doubtful, therefore, that the CHO cells in the present studies can survive simply by increased methylation of phosphatidylethanolamine. It therefore appears likely that these cells can obtain enough choline from the choline-containing phospholipids present in the dialyzed serum. It has been recently demonstrated that phosphatidylcholine can support growth of the strain 58 CHO variant at the nonpermissive temperature (16), indicating that CHO cells can utilize serum phospholipids for membrane biosynthesis.

Because phosphatidylethanolamine, phosphatidyl monomethyl ethanolamine and phosphatidyl dimethyl ethanolamine all activated the CHO cytidylyltransferase, and because the CHO cell phospholipid content could be decreased with respect to phosphatidylcholine and increased with respect to these two lipids, the proposed model predicts a redistribution-associated activation of the cytidylyltransferase in the analogue-supplemented cells. This prediction was proven to be correct (Table II). Cytidylyltransferase activity in homogenates from cells containing either phosphatidyl dimethyl ethanolamine or phosphatidyl monomethyl ethanolamine was...
Activity and distribution of CTP:phosphocholine cytidylyltransferase in CHO cells grown in supplemented media

CHO cells were grown in supplemented media for 3 days as described under "Materials and Methods." The cultures were harvested in 10 mM Tris-HCl (pH 7.5), separated into cytosolic and particulate fractions and assayed as described under "Materials and Methods." DME, dimethylethanolamine; MME, monomethylethanolamine.

| Supplement | Homogenate Specific activity | Cytosolic Specific activity | Particulate Specific activity | Distribution of activity | Percent total activity |
|------------|-------------------------------|-----------------------------|-------------------------------|--------------------------|-----------------------|
| Choline    | 1.79 ± 0.09 | 0.63 ± 0.08 | 0.94 ± 0.03 | 80 | 20 |
| DME        | 2.25 ± 0.06 | 0.62 ± 0.02 | 2.26 ± 0.02 | 52 | 48 |
| MME        | 3.45 ± 0.06 | 0.54 ± 0.02 | 3.62 ± 0.03 | 25 | 75 |

* Assays performed in the absence of exogenous lipids.
* Assays performed in the presence of exogenous lipid.

Total activity = specific activity<sub>cytosolic</sub> × milligrams of protein<sub>cytosolic</sub> + specific activity<sub>particulate</sub> × milligrams of protein<sub>particulate</sub>

Recovery of total homogenate activity after centrifugation was 85%.

Studies with the LM Cell Line—Alteration of the phospholipid composition of LM cells by supplementation with choline analogues provided the opportunity to compare the behavior of the LM cytidylyltransferase with the CHO enzyme. The LM cytidylyltransferase was activated by phosphatidylmonomethylthanolamine and phosphatidylcholine, whereas the lysophosphatidyls of these lipids were poor activators (Fig. 2). The LM cytidylyltransferase was previously shown to be activated by phosphatidylethanolamine but not phosphatidylcholine (4).

When monolayer cultures of LM cells were placed in choline-free medium without added analogue supplements, there was no morphological change over a period of 2 days. On the third day, a large number of cells were rounded, indicating the medium could not support continued growth. If, however, choline analogs or ethanolamine supplements were added to the medium, both the growth and morphological characteristics of the cells were similar to control cultures grown in choline-containing medium. The lipid composition of LM cells grown in medium containing choline or choline analogues (Table III) closely resembled those reported by Glaser et al. (9). There were only very small differences observed in the levels of the minor phospholipid classes found in cultures grown in the different media (data not shown). The lipid compositions of LM cells in which the culture medium was replaced daily were the same as in cells grown for 3 days without medium replacement.

The specific activities of the cytidylyltransferase in LM cell homogenates assayed in the absence of exogenous lipids are presented in Table IV. When assayed under these conditions, the activity is presumed to be representative of the amount of active enzyme present in the cells. The specific activity assayed in the presence of exogenous lipids, presumed to be proportional to the total amount of enzyme in the cell, was increased 25 or 92% as compared to controls. The increased activity was found to be associated with increases of 40 and 175% in the amount of particulate enzyme in phosphatidylcholine, phosphatidylinositol, and phosphatidylcholine-containing cells, respectively. It appears, therefore, that the activity and distribution of CTP:phosphocholine cytidylyltransferase can be governed by the lipid composition of CHO cellular membranes.

**TABLE II**

| Lipid Ethanolamine | DME | MME | Phosphatidylethanolamine | Ethanolamine |
|---------------------|-----|-----|--------------------------|-------------|
| PC                  | 17  | 17  | 25                       | 24          |
| PE                  | 11  | 16  | 45                       | 45          |
| PMME                | 0   | 37  | 0                        | 0           |
| PDME                | 0   | 37  | 0                        | 0           |
| Others*             | 32  | 35  | 32                       | 31          |

*Others: percentage of total lipid phosphate not found in the phospholipid classes identified.

**TABLE III**

| Lipid Ethanolamine | DME | MME | Phosphatidylethanolamine | Ethanolamine |
|---------------------|-----|-----|--------------------------|-------------|
| PC                  | 17  | 17  | 25                       | 24          |
| PE                  | 11  | 16  | 45                       | 45          |
| PMME                | 0   | 37  | 0                        | 0           |
| PDME                | 0   | 37  | 0                        | 0           |
| Others*             | 32  | 35  | 32                       | 31          |

*Others: percentage of total lipid phosphate not found in the phospholipid classes identified.

**TABLE IV**

Activity and distribution of CTP:phosphocholine cytidylyltransferase in LM cells grown in supplemented media

LM cells were grown in supplemented media for 3 days as described under "Materials and Methods." The cultures were harvested in 10 mM Tris-HCl (pH 7.5), separated into cytosolic and particulate fractions, and assayed as described under "Materials and Methods." DME, dimethylethanolamine; MME, monomethylethanolamine.

| Supplement | Specific activity of homogenate | Cytosolic Specific activity | Particulate Specific activity | Distribution of activity | Percent total activity |
|------------|--------------------------------|-----------------------------|-------------------------------|--------------------------|-----------------------|
| Choline    | 0.62 ± 0.04 | 60 | 40 |
| DME        | 1.11 ± 0.11 | 44 | 56 |
| MME        | 1.50 ± 0.19 | 19 | 81 |
| Ethanolamine | 1.47 ± 0.20 | 10 | 90 |
| None       | 1.60 ± 0.05 | 5  | 95 |

* Assays performed in the absence of exogenous lipids. Average ± S.D. of three determinations.
* Assays performed in the presence of exogenous lipid.

*Total activity = specific activity<sub>cytosolic</sub> × milligrams of protein<sub>cytosolic</sub> + specific activity<sub>particulate</sub> × milligrams of protein<sub>particulate</sub>

Recovery of total homogenate activity after centrifugation (105,000 × g) was greater than 85%. 

**Fig. 2.** Activation of cytosolic LM cell CTP:phosphocholine cytidylyltransferase by choline analogue-containing phospholipids. Enzyme activity was measured with sonicated suspensions of the following lipids: phosphatidylcholine (●), linolenylcholine (▲), and linolenylmonomethylthanolamine (□). Data points are the averages of duplicate determinations. The concentration of lipid is with respect to phosphorus.
1.70 ± 0.17 nmol/min/mg of protein for all conditions. The similarity of the results obtained with LM and CHO cells suggests that the regulatory properties of the two enzymes are the same. In cells with a lower phosphatidylcholine content, the activity of the cytidylyltransferase was increased to nearly maximal levels. In addition, the increase in enzyme activity appeared to be associated with more of the enzyme being bound to membranous structures (Table IV).

**DISCUSSION**

By starving cells for choline and making available alternate phospholipid head group substrates, it is possible to replace a large fraction of cellular phosphatidylcholine with analogues of phosphatidylcholine (9). The incorporation of head group analogue into LM cells has been shown to occur to various degrees in plasma membranes, microsomes, and mitochondria (17). In addition to the large changes in the phospholipid composition of the analogue-fed LM cells, small changes in fatty acids, sterols, and ether-linked lipids also occur (17, 18). The question arises as to whether the changes we have observed in cytidylyltransferase activity are the direct result of alterations in the head group composition of membrane phospholipids or are a general property of membrane-bound enzymes placed in artificial phospholipid surroundings.

Several lines of evidence suggest that the activation of the cytidylyltransferase is associated with the alterations in the polar head group composition of the cells. First, the incorporation of analog phospholipid head groups into LM cells has been demonstrated to have no effect on the activities of at least six membrane-bound enzymes: (Na⁺,K⁺)-ATPase, 5’-nucleotidase, NADH-cytochrome c reductase, glucose-6-phosphatase, inosine diphosphatase, and succinate cytochrome c reductase (17). Second, the “characteristic temperatures,” determined by measuring the quantum yield of different fluorescent membrane probes at various temperatures, is not changed in LM cells having an altered phospholipid composition (19). This suggests that the general physical properties of these membranes are maintained despite large alterations in their phospholipid head groups. Third, when the activity of cytosolic or delipidated cytosolic cytidylyltransferase was assayed in the presence or absence of a lipid mixture containing neutral glycerides and sterols, no activation was found (8, 27). Therefore, it is unlikely that changes in the cellular level of neutral glycerides or sterols can be responsible for the increased cytidylyltransferase activity seen in the present study.

In conclusion, the data presented in this paper are consistent with a mechanism of regulation of phosphatidylcholine biosynthesis that is dependent on cellular membrane phospholipid content. We have presented evidence that the replacement of membrane phosphatidylcholine with species of phospholipids known to activate the cytidylyltransferase in vitro results in an increase in the amount of membrane-bound enzyme. Because the membrane-bound form of the enzyme has a much greater activity than the cytosolic form, the shifted subcellular distribution results in an increase in cellular cytidylyltransferase activity. Our early studies employing phospholipase C treatment as a means of activating the cytidylyltransferase demonstrated the same mechanism of activation (2, 4). Presumably, treatment with the phospholipase produces at least a transient decrease in the amount of membrane phosphatidylcholine and in a localized membrane region enriched in phospholipids which are able to activate the cytosolic cytidylyltransferase.

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