Stimulation of Cholinephosphotransferase Activity by Phosphatidylcholine Transfer Protein

REGULATION OF MEMBRANE PHOSPHOLIPID SYNTHESIS BY A CYTOSOLIC PROTEIN*

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The effect of rat liver phosphatidylcholine transfer protein on the incorporation of CDP-choline and dioleoylglycerol into phosphatidylcholine catalyzed by rat liver microsomal CDP-choline:1,2-diacyl-sn-glycerol cholinephosphotransferase was studied. In the presence of phosphatidylcholine transfer protein, the incorporation of CDP-choline into phosphatidylcholine was markedly stimulated. Phosphatidylcholine transfer protein, isolated from either rat or bovine liver, was capable of this stimulatory effect; in contrast, phosphatidylinositol transfer protein from rat liver had no effect on phosphatidylcholine synthesis. Kinetic analysis showed that microsomal phosphatidylcholine synthesis increased 2.4-fold after 1 min and reached a maximum of approximately 10-fold within 10 min in the presence of phosphatidylcholine transfer protein; in the absence of this protein phosphatidylcholine synthesis stopped after 2-4 min. These results suggest that phosphatidylcholine transfer protein permits phosphatidylcholine synthesis to proceed further. With the addition of phospholipid vesicles, as an acceptor membrane in the reaction mixture, there was a significant amount of protein-mediated transfer of synthesized phosphatidylcholine to the vesicles. Measurable transfer of synthesized phosphatidylcholine to vesicles could only be detected after a lag of 2-4 min. The stimulation of cholinephosphotransferase could be nearly abolished by increasing the amount of added phospholipid vesicles; concurrently, a greater transfer to the vesicles was observed. These results describe a new property of phosphatidylcholine transfer protein which may be of physiological significance in the regulation of phosphatidylcholine synthesis in mammalian tissues.

The majority of enzymes utilized in the biosynthesis of phospholipid in mammalian tissues is located on the endoplasmic reticulum (Vance and Vance, 1988). In rat liver PtdCho is mainly synthesized by the *de novo* CDP-choline pathway, which contributes about 70-80% of PtdCho formation (Sundler and Akesson, 1975; Vance and Choy, 1979). The final step of this pathway involves the reaction between CDP-choline and 1,2-diacyl-sn-glycerol to yield PtdCho and CMP (Weiss et al., 1958), a step catalyzed by CDP-choline:1,2-diacyl-sn-glycerol cholinephosphotransferase (EC 2.7.8.2). While there is considerable evidence for regulation of PtdCho synthesis at the level of CDP-choline, several recent reports suggest additional control at the terminal step. Lin et al. (1986) have suggested that the activity of cholinephosphotransferase may be limited by the availability of both CDP-choline and diacylglycerol in HeLa cells permeabilized with digitonin. Cholinephosphotransferase activity was stimulated by analogues of cAMP, such as chlorophenylthio-cAMP, and CTP:phosphocholine cytidylyltransferase activity was reduced in cultured rat hepatocytes (Pelech et al., 1981). Moreover, insulin increased cellular diacylglycerol content (Farese et al., 1997) and activated protein kinase C, increased the labeling of phosphocholine but not PtdCho (Bocchino et al., 1987). An increase in the level of endogenous diacylglycerol led to a corresponding increase in PtdCho synthesis, supporting the idea that the availability of this substrate, under certain conditions, may be rate limiting for the synthesis of PtdCho in rat lung microsomes (Rustow and Kunze, 1987).

Protein-catalyzed transport of phospholipids between membranes was first described in rat liver preparations by Wirtz and Zilversmit (1968). Intracellular phospholipid transfer proteins differ not only in physical and chemical properties but more dramatically in their catalytic activities (Helmkamp, 1986). Rat tissues contain the following three well-defined phospholipid transfer proteins: (a) PtdIns transfer protein, which transports both PtdCho and PtdIns (Helmkamp, 1985), (b) nonspecific lipid transfer protein, which facilitates the movement of most phospholipids and other amphiphilic lipid such as cholesterol and glycolipids (Bloj and Zilversmit, 1977), and (c) PtdCho transfer protein. Rat PtdCho transfer protein is a cytosolic protein that has been purified to homogeneity (Teerlink et al., 1983), has a molecular weight of 28,000 (Poorthuis et al., 1980), and is localized mainly in liver (Lutton and Zilversmit, 1976) and small intestinal mucosa (Yamada et al., 1978). Like other transfer proteins, it can mediate the transport of lipid between isolated cellular membranes, including endoplasmic reticulum, intact mitochondria, synaptosomes, and myelin (Wirtz, 1982); it also functions with a variety of artificial lipid membranes. PtdCho transfer protein is highly specific for PtdCho and does not transfer phospholipids of other polar head group classes (Poorthuis et al., 1980; Teerlink et al., 1981). Catalytically, it operates as a carrier of...
monomeric PtdCho molecules in the aqueous phase between membranes or vesicles.

An important event in membrane biosynthesis is the movement of phospholipids from their sites of synthesis on the endoplasmic reticulum to the plasma membrane. While the ability of transport proteins to effect intermembrane phospholipid transport in vitro has been clearly demonstrated, any direct evidence of a physiological function is lacking. Recognizing the substrate specificity of PtdCho transfer protein and its ability to interact with endoplasmic reticulum, we tested the hypothesis that this protein may play a role in the de novo synthesis of PtdCho. Our results provide evidence that PtdCho transfer protein stimulates PtdCho biosynthesis through increased cholinephosphotransferase activity; simultaneously, it is capable of transporting the newly synthesized PtdCho to small unilamellar vesicles.

**EXPERIMENTAL PROCEDURES**

**Materials**—PtdCho was purified from crude egg PtdCho (Sigma) by dry column elution chromatography on silica gel G using chloroform/methanol/water (65:25:4, v/v) as a developing system (Weil and Silburt, 1982). Crude pig liver PtdIns (Srclary Research Laboratories, London, Ontario) was similarly purified, using the solvent system chloroform/methanol/acetic acid/water (50:25:7.5, v/v). Phospholipids were used to determine phospholipid concentration by dry column elution chromatography on Silica Gel-G using chloroform/methanol, 7 N ammonium hydroxide (330:90:0:14, v/v) (Abramson and Blecher, 1964). PtdCho zones were scraped into scintillation vials for radioactivity measurement.

**Vesicle Preparation and Characterization**—Solvent injection vesicles were prepared by the method of Batzri and Korn (1973) from 30 mM phospholipid solutions in ethanol/dimethyl sulfoxide (3:1, v/v). In all cases acceptor vesicles contained PtdCho/PtdIns (95:5, mol%). These vesicles were similar in size, as measured by chromatography on Sepharose 4B, and in participation in assays as the small, sonicated, single blayer vesicles previously used in this laboratory (Helmkamp, 1980). Each assay was performed in triplicate and reported as a mean ± S.D.

**RESULTS**

**Cholinephosphotransferase Activity in Absence and Presence of Phospholipid Transfer Proteins**—The synthesis of PtdCho catalyzed by isolated rat liver microsomes is summarized in Table I. Upon addition of rat liver PtdCho transfer protein, cholinephosphotransferase activity was stimulated about 10.4-fold. The combined addition of transfer protein and small unilamellar vesicles reduced the fold stimulation to 7.6; under these conditions, significant protein-mediated transfer of PtdCho to the acceptor vesicles was observed. Bovine liver PtdCho transfer protein exhibited a comparable stimulation of basal cholinephosphotransferase activity (Table I). In marked contrast, however, PtdIns transfer protein from rat liver was incapable of effecting any stimulation. A similar experiment was performed with non-radioactive CDP-choline and Ole-Gro in the presence and absence of rat liver PtdCho transfer protein. PtdCho content was chemically analyzed by extraction, thin layer chromatography, and lipid phosphorus determination. The endogenous PtdCho in 50 μg of microsomes was 22.7 ± 1.3 nmol. After 30 min of incubation, total microsomal PtdCho increased by 0.38 nmol. In the presence of PtdCho transfer protein, however, the quantity of PtdCho increased by 3.9 nmol. These data confirm the findings in Table I.

<table>
<thead>
<tr>
<th>PC-TP (rat liver) + vesicles</th>
<th>123 ± 18 (5)</th>
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<tbody>
<tr>
<td>PC-TP (rat liver) + vesicles</td>
<td>94 ± 16 (5)</td>
</tr>
<tr>
<td>PC-TP (bovine liver) + vesicles</td>
<td>82 ± 0.2 (1)</td>
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</table>

**TABLE I**

| PC-TP (bovine liver) + vesicles | 87.7 ± 0.8 (1) |

* PC-TP, PtdCho transfer protein.
* PC-TP, PtdIns transfer protein.
* ND, none detected.
Substrate Utilization by Microsomal Cholinephosphotransferase—To determine how much each substrate contributed to the synthesis of PtdCho, CDP-[\(^{14}\text{C}\)]choline, and [\(^{3}\text{H}\)]OlePGro were compared in the microsomal assay. As shown in Table II, the ratio of \[^{14}\text{C}\]/\[^{3}\text{H}\] in newly synthesized PtdCho molecule is essentially unity. The incorporation of CDP-[\(^{14}\text{C}\)]choline into PtdCho was activated from a basal level of 11.2 nmol (mg protein\(^{-1}\)) h\(^{-1}\) to 85.2 nmol (mg protein\(^{-1}\)) h\(^{-1}\) in the presence of PtdCho transfer protein; similarly, the contribution of [\(^{3}\text{H}\)]OlePGro to PtdCho synthesis was stimulated from 11.6 nmol (mg protein\(^{-1}\)) h\(^{-1}\) to 83.7 nmol (mg protein\(^{-1}\)) h\(^{-1}\) (Table II). These levels of isotope incorporation are consistent with a strong reliance on exogenous substrate utilization. In contrast, the isotopic distribution in PtdCho which was transferred to vesicles yielded a mole ratio of CDP-choline to OlePGro \(^{14}\text{C}/^{3}\text{H}\) of 1:2.2, a value which may reflect a preference of PtdCho transfer protein for transfer of certain molecular species (Welti and Helmkamp, 1984).

Kinetics of PtdCho Synthesis and Transfer—The foregoing experiments clearly describe two functions of PtdCho transfer protein: stimulation of cholinephosphotransferase and transfer of PtdCho from microsomes to vesicles. These properties were examined in greater detail by kinetic analysis (Fig. 1). In the presence of PtdCho transfer protein, the maximum synthetic activity was attained within 2–4 min and was followed by slower rate for 10 min (Fig. 1A). On the other hand, microsomes in the absence of PtdCho transfer protein reached a maximum level of PtdCho synthesis within 2–4 min and thereafter stopped. Thus, PtdCho transfer protein not only enhances cholinephosphotransferase activity but stabilizes it for an extended period of time as compared with microsomes alone. At times as early as 1 min, the fold stimulation is 2.4 (mg protein\(^{-1}\)) h\(^{-1}\) to 8.5 in the presence of PtdCho transfer protein; similarly, the stimulation of cholinephosphotransferase activity in the presence of PtdCho transfer protein became less pronounced and finally, at 200 nmol of phospholipid vesicles, approached the basal level of PtdCho synthesis observed for phospholipid vesicles in absence of PtdCho transfer protein (Table I). To pursue this further, the concentration of vesicles in the reaction mixture was varied; the results are presented in Fig. 3A. As the vesicle concentration increased, the stimulation of cholinephosphotransferase activity became less pronounced and finally, at 200 nmol of phospholipid vesicles, approached the basal level of PtdCho synthesis observed for microsomes and transfer protein. Concomitant with the loss of stimulated cholinephosphotransferase activity, there was a proportional increase in transfer of newly synthesized PtdCho to the vesicles (Fig. 3B). The reduction in transfer protein-derived activation of PtdCho synthesis in the presence of phospholipid vesicles could be due to the small
FIG. 2. Dependence of microsomal cholinephosphotransferase activity on PtdCho transfer protein concentration. Experiments are performed as described in Fig. 1, except that no vesicles are added to the assay mixtures. The indicated quantity of rat liver PtdCho transfer protein is present during the 5-min incubation period.

FIG. 3. Effect of vesicle concentration on stimulated cholinephosphotransferase activity and transfer of newly synthesized PtdCho. Experiments are carried out as described in Fig. 1 in the presence of 0.78 µg of rat liver PtdCho transfer protein. Various concentrations of phospholipid vesicles are added to the reaction mixture for a 15-min incubation. A, PtdCho synthesized and remaining with microsomes, in the presence of vesicles prepared by solvent injection (A) or sonication (Δ). B, protein-mediated transfer of newly synthesized PtdCho to vesicles.

quantity of ethanol and/or dimethyl sulfoxide employed in the preparation of vesicles. To rule out this possibility, we compared vesicles prepared by solvent injection, which contain both ethanol and dimethyl sulfoxide, with those prepared by sonication at 4 °C under N2. No major difference between the vesicles was apparent.

Protein-mediated Transfer of Newly Synthesized and Endogenous PtdCho To compare the pools of newly synthesized and endogenous PtdCho as substrates for PtdCho transfer protein, rat liver microsomes which had been labeled in vivo with [methyl-3H]choline were isolated (Wirtz et al., 1972) and subsequently used for the synthesis of PtdCho as described in Fig. 1. Each experiment was carried out in triplicate in the presence and absence of 1.56 µg of rat liver PtdCho transfer protein for a 20-min incubation period. The transfer of endogenous PtdCho (3H-labeled) and newly synthesized PtdCho (14C-labeled) to acceptor phospholipid vesicles could then be measured simultaneously. The total transfer from microsomes to acceptor membranes was 15.2 ± 1.9 nmol/mg protein/20 min, of which 7.4 ± 1.0 nmol was newly formed PtdCho. Using the endogenously labeled microsomes as donor membranes in standard transfer assay procedure (Helmkamp et al., 1974), we found that transfer of [3H]PtdCho from microsomes to small unilamellar vesicles was 14.9 ± 1.2 nmol. These results indicate that the transfer catalytic activity of PtdCho transfer protein was not altered by our synthetic reaction system. On the basis of chemical analysis, the endogenous pool of PtdCho in 1 mg of microsomal protein was 465 ± 25 nmol. In addition, newly synthesized PtdCho represented an approximately 11–16% increase over the endogenous PtdCho pool. Notably, the mole ratio of endogenous to newly formed PtdCho in microsomes is 6:1, but in the transferred population it becomes 1:1. These findings again may be attributed to the substrate selectivity of PtdCho transfer protein. This single time point assay provides an estimation of total PtdCho transferred, without adjustment for the lag seen in the transfer of newly synthesized species (Fig. 1B).

DISCUSSION

Previous studies on the regulation of de novo PtdCho synthesis strongly indicated a control point at the formation of CDP-choline. From CTP and choline phosphate (Pelech and Vance, 1984). A cytidylyltransferase-regulated pathway implies that the rate of the reaction catalyzed by cholinephosphotransferase, the next and last enzyme in the pathway, should be limited by the supply of CDP-choline, but not necessarily by the level of diacylglycerol. A variety of agents which increased the levels of CDP-choline also stimulated PtdCho synthesis (Vance et al., 1980; Sleight and Kent, 1980; Whitton et al., 1985). In this investigation the activity of cholinephosphotransferase was measured, and the incorporation of both CDP-choline and OleGro into PtdCho was monitored. The basal cholinephosphotransferase activity of rat liver microsomes measured in our assay system is low when compared with values reported in literature (Vance and Vance, 1988). This difference can most likely be attributed to the absence of detergent and anionic phospholipid in the preparation of the OleGro substrate. Miller and Weinhold (1981) have reported that cholinephosphotransferase activity was drastically reduced in rat lung when diacylglycerol was used without Tween 20 and phosphatidylglycerol. We would, in fact, suggest that the mixed micelles formed from detergent and phospholipid may function in other assay systems to release "product inhibition" in much the same way as PtdCho transfer protein.

CDP-choline and diacylglycerol are required substrates for PtdCho synthesis catalyzed by microsomal cholinephosphotransferase. In the presence of rat liver PtdCho transfer protein, the incorporation of either (radioisotopically) labeled substrate into PtdCho was approximately 10-fold higher (Table I). Activation of membrane-bound cholinephosphotransferase exhibited a linear dependence on the amount of PtdCho transfer protein added (Fig. 2), regardless of reaction incubation period (data not shown). PtdCho transfer protein from rat and bovine liver is able to stimulate de novo PtdCho synthesis (Table I). While these two proteins differ significantly in chemical properties, molecular size, isoelectric point, and immunogenecity (Poorthuis et al., 1980; Westerman et al., 1983; Teerlink et al., 1983), each catalyzes exclusively the transfer of PtdCho. Notably, rat PtdIns transfer protein failed
to stimulate PtdCho synthesis, even though this protein can also facilitate the intermembrane movement of PtdCho (Venuti and Helmkamp, 1988). Cholinephosphotransferase activation is, therefore, highly specific for PtdCho transfer protein.

Relative contributions of substrates to PtdCho synthesis in stimulated and unstimulated microsomes revealed that the ratio of CDP-choline and OleGro were equivalent and close to one (Table II). In the presence of PtdCho transfer protein, however, the transferred PtdCho displayed a content of 1 mol of CDP-choline for each 2.2 mol of OleGro. This difference may arise from acyl chain remodeling of PtdCho molecules. It has been reported that the majority of newly formed PtdCho in the hamster heart is subsequently remodeled by deacylation-reacylation processes (Arthur and Choy, 1984). Alternatively, the difference may suggest a selective acyl chain specificity of PtdCho transfer protein. Indeed, Wettl and Helmkamp (1984) have clearly demonstrated that dioleyl phosphatidylcholine was among the best substrates for bovine PtdCho transfer protein.

Kinetic analysis of de novo PtdCho synthesis and intermembrane transfer revealed distinct temporal differences. An immediate stimulation of PtdCho synthesis occurred in the presence of PtdCho transfer protein, but transfer of PtdCho from microsomes to acceptor vesicles was delayed for several minutes. The lag observed in protein-catalyzed transfer may reflect the transition of de novo synthesized PtdCho from a non-transferable to a transferable pool in the microsomal membrane. It should be stressed that it is experimentally difficult to quantitate the protein-catalyzed intra- and intermembranous transport of synthesized PtdCho.

We have routinely used small unilamellar vesicles as model membranes in phospholipid transfer systems. The activation of cholinephosphotransferase in rat liver microsomes by PtdCho transfer protein could be effectively blocked by the addition of such vesicles. We interpret this finding as a competition by a limiting amount of PtdCho transfer protein and constitutes a novel regulatory step in the synthesis of PtdCho in selected mammalian tissues.

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Stimulation of cholinephosphotransferase activity by phosphatidylcholine transfer protein. Regulation of membrane phospholipid synthesis by a cytosolic protein.

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