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COMPARISON OF THE PHOSPHOLIPID REQUIREMENTS AND MOLECULAR FORM OF CTP : PHOSPHOCHOLINE CYTIDYLYLTRANSFERASE FROM RAT LUNG, KIDNEY, BRAIN AND LIVER

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Summary

The cytidylyltransferase activity in fresh cytosol from different tissues of the rat was measured in the absence and presence of phosphatidylglycerol. In all cases addition of this lipid produced large increases in enzyme activity. Agarose gel (A-5.0) filtration profiles of the enzyme activities indicated that the L-form of the enzyme (190 000 molecular weight) predominated in liver, brain, kidney, and fetal lung. However, adult lung cytosol contained 70—80% of the activity in the H-form (molecular weight $\geqslant 5 \cdot 10^6$). Removal of phospholipid material from the alveolar spaces by lavage produced a significant reduction of the H-form of the enzyme in the cytosol fraction. The L-form of the cytidylyltransferases from fetal lung and adult liver, kidney, and brain all possess the same specificities for activation by phospholipids in vitro. In all cases, phosphatidylglycerol was the most potent activator at 0.2 mM. Lysophosphatidylethanolamine stimulated enzyme activity, whereas lysophosphatidylglycerol was a potent inhibitor. These studies implicate the role of acidic phospholipids in the regulation of cytidylyltransferase activity in vivo and the existence of a common L-form of the enzyme in several tissues of the rat.

Introduction

The enzyme CTP : phosphocholine cytidylyltransferase catalyzes the formation of CDPcholine in the pathway for the synthesis of phosphatidylcholine. Fiscus and Schneider [1] reported that total liver lipid extract and to a lesser

extent lysophosphatidylcholine stimulated the enzyme in rat liver cytosol. Lysophosphatidylcholine was also reported to stimulate cytidylyltransferase activity in cytosol preparations from rat brain [2], intestinal mucosa [3], and chicken sciatic nerve [4]. On the other hand, recent evidence by Choy and Vance [5] indicated that lysophosphatidylethanolamine is the most potent activator of the enzyme in rat liver cytosol. Furthermore, their results suggested that the production of lysophosphatidylethanolamine in the cytosol preparation may be responsible for the increased cytidylyltransferase activity that occurs during storage at 4°C. We previously reported that the cytidylyltransferase activity in the cytosol from fetal rat lung was maximally stimulated by phosphatidylglycerol [6]. Choy and Vance [7] also found phosphatidylglycerol to be a potent activator of the liver enzyme but they were unable to detect the presence of phosphatidylglycerol in liver cytosol. The activation of the cytosol enzyme in vitro appears to be a general characteristic of cytidylyltransferase isolated from mammalian tissue but the phospholipid specificity for activation remains unclear.

Detailed studies of the physical properties of cytidylyltransferase in rat lung [8] and rat liver [9] indicated that the enzyme in cytosol preparations exists in at least two forms; a low molecular weight species (190 000, L-form) and a high molecular weight species ($\geqslant 5 \cdot 10^6$, H-form). In fetal rat lung and adult rat liver, the enzyme exists predominantly in the L-form and requires added phospholipid for activity in vitro. In adult rat lung, however, the enzyme is recovered predominantly in the H-form and has considerable activity without the addition of phospholipid. Similar studies of cytidylyltransferase from other tissues have not been reported.

We analyzed the requirements for phospholipid stimulation and the patterns of the molecular forms of the enzyme from cytosol preparations of adult lung, liver, brain, kidney, and fetal lung. We also examined the enzyme activity and molecular species found in adult lung cytosol after surfactant material was removed from the alveolar spaces by a lavage procedure. The results of these studies suggest that the requirements for phospholipid activation are similar for cytidylyltransferase in different tissues and adult lung contains the highest amount of H-form enzyme.

Experimental procedures

Materials. Pregnant rats and young female rats were obtained from Holtzman Co. Phosphocholine, CTP (type V), 1,2-diacyl-*sn*-glycero-3-phospho-1-glycerol (egg yolk), 1,2-diacyl-*sn*-glycero-3-phosphoinositol (soybean), 1,2-diacyl-*sn*-glycero-3-phosphoserine (bovine brain), and monoacyl-*sn*-glycero-3-phospho-1-glycerol (egg yolk) were purchased from Sigma Chemical Co. Oleoyl-*sn*-glycero-3-phosphoethanolamine and 1,2-dioleoyl-*sn*-glycero-3-phosphate were purchased from Serdary Research Laboratory. 1-Monoacyl-*sn*-glycero-3-phosphocholine was prepared from rat lung as described previously [8]. Phospho-[*methyl*-^{14}C]choline and [^{3}H]methanol were purchased from New England Nuclear. Millipore Corp. Filters, type GS 0.22 nm pore size, were used for charcoal filtration. Agarose gel filtration media was purchased from Bio-Rad Laboratories.

Preparation of cytidylyltransferase. Female rats (180—200 g) were killed by decapitation, the tissues were removed and placed on ice. In some cases, the tissues were perfused with saline after using 5% chloral hydrate as a general anesthetic. All tissues were homogenized in 5 vol. (w/v) 50 mM Tris-HCl (pH 7.4) that contained 0.15 M NaCl. The homogenates were centrifuged at 100 000 × *g* for 60 min and the supernatants were fractionated by gel filtration on Biogel A-5.0, in the Tris/NaCl buffer. The enzyme activity eluting at a position corresponding to a molecular weight of 150 000 to 200 000 (0.55 column volume) was pooled, and concentrated with a Millipore Immersible Concentrator and stored at 4°C.

Preparation of phospholipid suspensions. The organic solvents used to dissolve the phospholipids were evaporated under nitrogen. Phospholipids were suspended in 0.25 M Tris-HCl, pH 7.4, at concentrations up to 1.5 mg/ml and sonicated for 12 min at 50 W at 4°C. The suspensions were kept on ice and used within 2 h.

Preparation and analysis of lysophosphatidylglycerol. Phosphatidylglycerol from egg yolk lecithin was suspended in 1.0 ml of a solution containing 3 mM $CaCl_2$, 100 mM NaCl and 100 mM Tris-HCl, pH 8.0. The mixture was sonicated for 12 min at 4° C. Phospholipase A_2 (100 μg) from porcine pancreas was added and the mixture incubated for 90 min at 37° C. The reaction was stopped with 0.5 ml 100 mM Na_2EDTA and phospholipids were extracted with 30 ml chloroform/methanol (2 : 1, v/v). Lysophosphatidylglycerol was isolated by thin-layer chromatography on silica gel H plates, with the solvent system chloroform/methanol/conc. NH_4OH/H_2O (70 : 30 : 2 : 3, v/v).

Lysophosphatidylglycerol migrated with an R_F value of 0.30 and was extracted from the silica gel according to the method of Arvidson [10]. Fatty acid concentration was determined by methanolysis in the presence of [^{3}H]-methanol. A maximum of 1.0 μmol lysophosphatidylglycerol was dissolved in 1.0 ml chloroform with 0.25 ml [^{3}H]methanol (spec. act. = 19 000 dpm/μmol). The reaction was initiated with 25 μl 4 N NaOH and terminated after 5 min with 50 μl 3 M HCl. Chloroform/methanol (1 : 1, v/v), 0.5 ml, was added and the methyl esters were recovered in the lower layer of the biphasic mixture. The upper layer was discarded and the samples were air-dried overnight. [^{3}H]-Methanol incorporation was measured by liquid scintillation. The molar ratio of fatty acid to phosphorus in the lysophosphatidylglycerol sample was 1.04 ± 0.05.

Enzyme assay. Cytidylyltransferase activity was measured in the 'forward' direction (CDPcholine formation) by incubation with 1.6 mM phospho[*methyl*-^{14}C]choline (1.2 Ci/mol), 2.0 mM CTP, 6.0 mM magnesium acetate and 50 mM Tris-HCl (pH 7.4), in a final volume of 0.1 ml at 37°C. The reaction was stopped by boiling 2 min and cooled, and 0.5 ml of a 5.6% charcoal suspension in 20 mM phosphocholine was added to absorb the CDPcholine. After washing the charcoal three times with 10 ml H_2O, the CDP[*methyl*-^{14}C]choline was eluted from the charcoal by boiling for 2 h in 2.0 ml of ethanol/conc. NH_4OH/H_2O (60 : 3.6 : 37, v/v). The charcoal was collected by filtration thru a Millipore filter and rinsed three times with 0.5 ml volumes of extraction solvent. The total filtrate was collected in a scintillation vial and the radioactivity was measured after addition of 10 ml PCS (Amersham Corp.).

Lung lavage procedure. Female rats were anesthetized with 2.0 ml 5% chloralhydrate. The trachea was cannulated with an 18 G animal feeding needle (Perfektum) and the aveolar spaces of the lung were washed 3 times with 5 ml aliquots of 50 mM Tris-HCl buffer (pH 7.4), containing 0.25 M sucrose kept at 37°C. In some experiments, the lung vascular system was perfused with saline prior to the lavage treatment.

Analytical procedures. Phospholipids were extracted from tissue cytosol preparations according to the method of Folch et al. [11]. Lipid phosphorus was determined as described previously [12]. Protein was measured according to Sedmak and Grossberg [13], using ovalbumin as a standard.

Results and Discussion

Comparison of cytidylyltransferase activity and gel filtration profiles

The total enzyme activity in fresh cytosol (100 000 × g · 60 min supernatant) from the different tissues ranged from 36 to 127 nmol/min/g tissue (Table I). In all cases, the addition of phosphatidylglycerol produced a large increase in enzyme activity. The amount of stimulation varied from 5-fold for adult lung to 20-fold for brain.

The gel filtration profiles of the enzyme activities indicated that in liver, brain, kidney and fetal lung the low molecular weight form (L-form) predominated (Fig. 1). Fetal lung and kidney appeared to have almost all of the enzyme activity as the L-form. Brain and liver preparations contained 10—30% high molecular weight form (H-form). The profile for adult lung was strikingly different with most of the activity (70—80%) eluting as the H-form.

Previously, we reported an apparent correlation during lung development between the phospholipid concentrations in cytosol preparations and the phospholipid requirement for cytidylyltransferase [8]. However, the phospholipid concentrations of the cytosol preparations from the various tissues varied considerably and no correlation between the total phospholipid concentration

TABLE I

CYTIDYLYLTRANSFERASE ACTIVITY IN CYTOSOL OF RAT TISSUES

Enzyme assays were performed in the presence (+) and absence (—) of 0.26 mM phosphatidylglycerol.

	Phosphatidyl-glycerol	Activity	
		nmol · min^{-1} · g^{-1} tissue	nmol · min^{-1} · mg^{-1} protein
Fetal lung	—	11	0.3
	+	78	3.6
Adult lung	—	17	0.3
	+	84	1.5
Adult liver	—	8	0.09
	+	127	1.4
Adult kidney	—	12	0.3
	+	56	1.4
Adult brain	—	1	0.06
	+	36	1.2

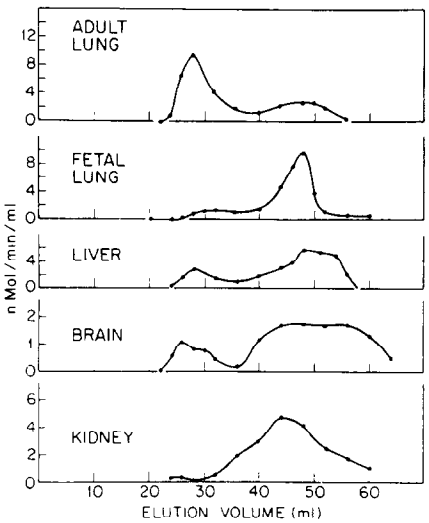

Fig. 1. Biogel column elution profiles of cytidylyltransferase activity in fresh cytosol preparations. A Biogel A-5.0 column (1.5 X 56 cm) was equilibrated with 50 mM Tris-HCl (pH 7.4), containing 0.15 M NaCl. Enzyme activity was measured in the presence of 0.25 mM phosphatidylglycerol. Cytosol samples were applied as follows: adult lung, 100 units of activity at 1.5 units/mg protein; fetal lung, 170 units at 3.6 units/mg protein; adult liver, 120 units at 1.4 units/mg protein; adult brain, 30 units at 1.2 units/mg protein; and adult kidney, 80 units at 1.4 units/mg protein. The recovery of enzyme activity from the column was 97, 47, 88, 95 and 90% for adult lung, fetal lung, liver, brain and kidney, respectively.

and the form of cytidylyltransferase was apparent, Table II. Non-perfused liver had a relatively high content of phospholipid. Perfusion reduced the amount of phospholipid about 8-fold but did not significantly change the distribution of cytidylyltransferase activity. Similarly, perfused lung contained less phospholipid than non-perfused but the distribution of cytidylyltransferase did not change. The phospholipid content of perfused lung and kidney are essentially the same but the distribution of cytidylyltransferase is very different. However, removal of blood components by perfusion did lead to slightly less recovery of total enzyme activity.

TABLE II

PHOSPHOLIPID CONCENTRATION AND CYTIDYLYLTRANSFERASE SPECIES IN CYTOSOL OF RAT TISSUES

Data are presented as the mean values ± S.D. with the number of determinations given in parenthesis. Cytidylyltransferase activities were measured in the presence of phosphatidylglycerol (0.26 mM).

Tissue	Cytosol phospholipid (μmol/g tissue)	Cytidylyltransferase (nmol/min/g tissue)	
		H-form	L-form
Brain	0.2	7.0	27
Kidney	0.9 ± 0.2 (3)	1.0	49
Liver	5.8	21.0	90
Liver, vascular perfused	0.7 ± 0.2 (3)	13.0	35
Fetal lung	0.6 ± 0.04 (16)	7 ± 5 (5)	32 ± 5 (5)
Adult lung	2.4 ± 0.3 (7)	43 ± 11 (5)	16 ± 4 (5)
Adult lung, perfused	1.1 ± 0.6 (9)	31 ± 6 (4)	8 ± 3 (5)

Our previous studies of cytidylyltransferase suggested that the H-form of the enzyme in adult lung may be bound to intracellular membranes that contain phosphatidylglycerol and other anionic lipids [6]. Since phosphatidylglycerol is present in relatively high amounts in the surface-active material lining the alveolar spaces of the lung [14,15], we entertained the possibility that cytidylyltransferase in adult lung was binding to material containing phosphatidylglycerol in the alveolar surfactant. We analyzed the gel filtration profile of a cytosol preparation from a lung previously lavaged to remove alveolar surfactant. The activity profile from lavaged lung (Fig. 2) was significantly different from that obtained with non-lavaged lung (Fig. 1). This experiment was repeated six times. The enzyme activity as nmol/min per g lung in non-lavaged vs. lavaged lung was; 43 ± 11 H-form, 16 ± 5 L-form in non-lavaged; and 12 ± 8 H-form, 25 ± 11 L-form in lavaged. The recovery from the column was the same for both types of preparation (60—70%). The decrease in H-form was significant, $P < 0.001$. These results are consistent with the possibility that cytidylyltransferase has a special affinity for some component of the surfactant complex and could partially explain differences between adult lung and the other tissues. However, the results are only circumstantial and not conclusive. Although the drop in H-form observed after lavage suggested that the association of cytidylyltransferase with material in the alveolar space occurred after homogenization of the tissue, the H-form which remained after lavage may reflect similar interactions that occurred intracellularly prior to homogenization. Further studies are necessary to resolve this question.

Comparison of phospholipid specificities for activation of cytidylyltransferase

The L-form of cytidylyltransferase from the various tissues was prepared by chromatography on Biogel 5. Phosphatidylglycerol was the most potent activator for cytidylyltransferase from liver, kidney, brain and fetal lung, see Fig. 3. Maximal enzyme activity was obtained with 0.26 mM phosphatidylglycerol. 1-Oleoyl-lysophosphatidylethanolamine also gave maximal activity but a much higher concentration was required. Our results substantiate the results of Choy and Vance [5], which showed that oleoyl lysophosphatidylethanolamine was a potent activator for liver cytidylyltransferase. Our data also show that the

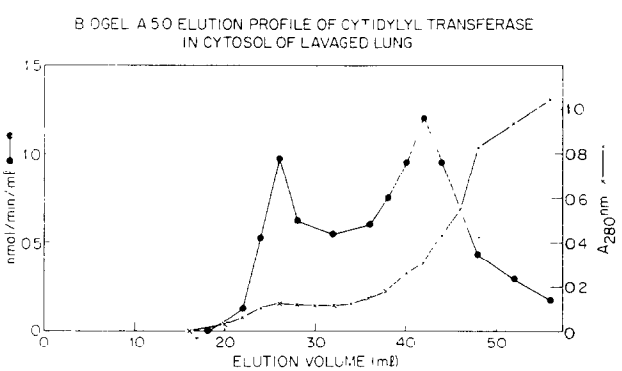

Fig. 2. Biogel A-0.5 column elution profile of cytidylyltransferase in cytosol from lavaged rat lung. The column characteristics were the same as described under Fig. 1.

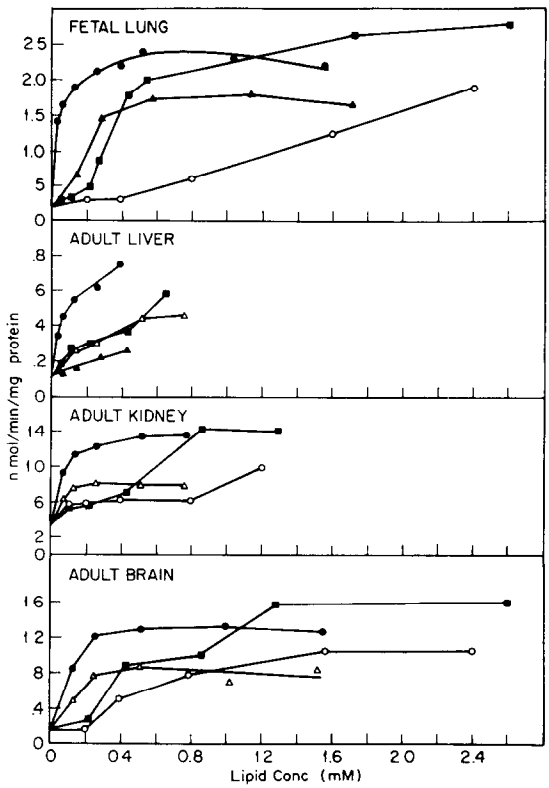

Fig. 3. Phospholipid activation of cytidylyltransferase. Enzyme was isolated from tissue cytosol preparations by agarose gel column chromatography, as described in Experimental procedures. Phospholipid samples were suspended in 0.25 M Tris-HCl (pH 7.4) by sonication. The lipids were mixed with the enzyme at the beginning of the assay without preincubation. ■———■, oleoyl lysophosphatidylethanolamine; ●———●, phosphatidylglycerol (egg); ○———○, lysophosphatidylcholine (rat lung); ▲———▲, dioleoylphosphatidic acid; △———△, phosphatidylserine (bovine).

L-form of the enzymes from the four tissues examined all possess the same specificity for activation by phospholipids in vitro. As we have demonstrated previously [6,8], the enzyme can be activated in vitro by a wide variety of phospholipids, many of which are anionic at physiological pH. These results resolve the apparent discrepancies which have occurred in previously published studies of cytidylyltransferase in animal tissues.

Choy and Vance [5] reported that 1-oleoyl-lysophosphatidylethanolamine produced greater stimulation of liver cytidylyltransferase than either the mixed-acyl species from pig liver or the 1-palmitoyl species. A similarly striking difference between the stimulation activity of 1-oleoyl-lysophosphatidylethanolamine and lysophosphatidylethanolamine from egg was observed with the L-form of enzyme from fetal lung (Fig. 4). Lysophosphatidylglycerol stimulated cytidylyltransferase at low concentrations but at high concentrations produced a large inhibition. Thus, both lysophosphatidylethanolamine and phosphatidylglycerol at 1.0 mM produced nearly maximal activity whereas lysophosphatidylglycerol at the same concentration caused almost complete loss of activity. Furthermore, 0.26 mM phosphatidylglycerol did not cause a

stimulation of activity in the presence of 0.80 mM lysophosphatidylglycerol. Although the physiological significance of these observations is uncertain, a conversion of phosphatidylglycerol by intracellular phospholipase A could lead to a decrease in cytidylyltransferase activity.

Choy and Vance [5] proposed that lysophosphatidylethanolamine may be a physiological activator of cytidylyltransferase in liver cytosol. We are more inclined to interpret the phospholipid activations as indications of the type of phospholipid environment within intracellular membranes necessary for the interaction and subsequent activation of cytidylyltransferase. Presumably, these associations are easily disrupted during homogenization of most tissues resulting in most of the enzyme activity becoming localized in the supernatant fraction after centrifugation. The unique situation with adult lung may result from the relatively higher concentration of phosphatidylglycerol in specific cellular membranes causing a subsequent tighter association of the enzyme to fragments of these membranes.

The role of acidic phospholipids as structural components in membrane receptor sites has been implicated for a wide variety of enzymes and hormones. Phosphatidylserine is required for adenylate cyclase activation by thyrotropin in thyroid plasma membrane [16]. The addition of phosphatidylglycerol to preparations of particulate membrane from *Escherichia coli* produces activation of glycerol-3-phosphate acyltransferase [17]. The rate of synthesis of *N*-acetylglucosaminylpyrophosphoryl dolichol from dolichol phosphate by rat lung endoplasmic reticulum was stimulated 18-fold by phosphatidylglycerol [18]. Pyruvate oxidase, a peripheral plasma membrane enzyme of *E. coli*, is readily released by sonication and the activity is increased 25-fold by sonicated suspensions of phosphatidylglycerol [19]. Phosphatidylglycerol will also activate the detergent solubilized forms of lactate dehydrogenase [20], NADH oxidoreductase [21], and isoprenoid alcohol phosphokinase [22].

Perhaps, the asymmetric distribution of certain phospholipids into specific domains within a membrane may be an important feature for the regulation of biosynthetic processes in the cell. In the case of cytidylyltransferase, the presence of acidic phospholipid domains within certain intracellular membranes may provide the proper environment for enzyme activity. The reversible association of cytidylyltransferase with membrane surfaces could provide a readily

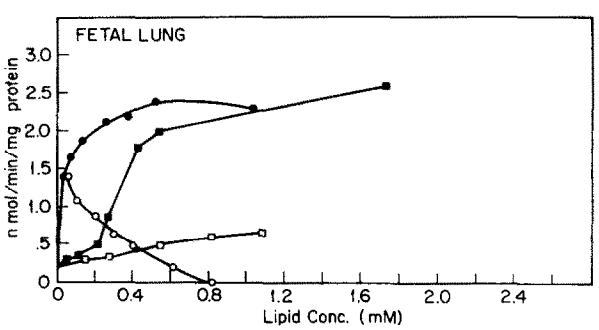

Fig. 4. Lysophospholipid activation of cytidylyltransferase. Cytidylyltransferase was isolated from fetal lung cytosol by agarose gel chromatography. ■———■, oleoyl lysophosphatidylethanolamine; □———□, lysophosphatidylethanolamine (egg); ●———●, phosphatidylglycerol; ○———○, lysophosphatidylglycerol.

available source of CDPcholine to react with membrane bound diacylglycerol. Association with membranes could thus permit an efficient coordination of the enzymes required for the de novo synthesis of phosphatidylcholine and provide a mechanism for the coordination of phospholipid synthesis with the dynamics of membrane function. The physiological significance of enzyme-membrane interaction as a potential regulatory process in metabolism is well recognized and has been extensively pursued as an important component in the control of glycolysis [23].

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