Head-group specificity for feedback regulation of CTP:phosphocholine cytidyltransferase

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The specificity of the phospholipid head-group for feedback regulation of CTP: phosphocholine cytidyltransferase was examined in rat hepatocytes. In choline-deficient cells there is a 2-fold increase in binding of cytidyltransferase to cellular membranes, compared with choline-supplemented cells. Supplementation of choline-deficient cells with choline, dimethylethanolamine, monomethylethanolamine or ethanolamine resulted in an increase in the concentration of the corresponding phospholipid. Release of cytidyltransferase into cytosol was only observed in hepatocytes supplemented with choline or dimethylethanolamine. The apparent EC_{50} values (concn. giving half of maximal effect) for cytidyltransferase translocation were similar for choline and dimethylethanolamine (25 and 27 μ M respectively). The maximum amount of cytidyltransferase released into cytosol with choline supplementation (1.13 m-units/mg membrane protein) was twice that (0.62) observed with dimethylethanolamine. Supplementation of choline-deficient hepatocytes with *NN'*-diethylethanolamine, *N*-ethylethanolamine or 3-aminopropanol also did not cause release of cytidyltransferase from cellular membranes. The translocation of cytidyltransferase appeared to be mediated by the concentration of phosphatidylcholine in the membranes and not the ratio of phosphatidylcholine to phosphatidylethanolamine. The results provide further evidence for feedback regulation of phosphatidylcholine biosynthesis by phosphatidylcholine.

INTRODUCTION

The major route for PC synthesis is via the CDP-choline pathway and may account for 60–80% of PC synthesis in rat liver [1]. The rate-limiting step for PC synthesis by the CDP-choline pathway is catalysed by CT (EC 2.7.7.15), which is localized in both cytosol and microsomes [2]. Evidence has accumulated that the cytosolic form of CT is an inactive reservoir which can be translocated reversibly to the ER, where it becomes activated by interaction with phospholipids in the membrane [2]. Recently we provided the first evidence that the movement of CT activity was due to translocation of CT protein [3]. Several possible mechanisms for CT translocation have been identified. These include changes in CT distribution by an increase in unesterified fatty acid concentrations [4–6], diacylglycerol concentrations [6,7] or phosphorylation/dephosphorylation [8].

A feedback-regulatory mechanism for PC biosynthesis in choline-deficient hepatocytes has been recently recognized in our laboratory [9]. The amount of CT recovered in choline-deficient rat liver microsomes is approx. 2-fold higher than in microsomes of choline-supplemented rats [3]. Supplementation of the medium of hepatocytes prepared from choline-deficient livers with choline, methionine or lyso-PC causes CT to move from membranes to cytosol [9]. There was a highly significant correlation between the increase in cytosolic CT, the decrease in membrane CT and an increase in PC concentration.

PC is also synthesized from PE by sequential formation of PMME and PDME catalysed by PE methyltransferase (EC 2.1.1.17) [10]. These metabolic intermediates, PMME and PDME, are generally present in very small amounts [1]. However, in the absence of choline and methionine, a significant amount of PMME and PDME can be synthesized from their corresponding bases *N*-monomethylethanolamine and *NN'*-dimethylethanolamine [1]. It has been suggested that the formation of the CDP-derivatives of monomethylethanolamine and dimethylethanol-

amine is catalysed by different enzymes, probably by CTP:phosphoethanolamine cytidyltransferase and CT respectively [11]. Incorporation of these bases into membrane phospholipids has been studied in mouse fibroblasts. Accumulation of PMME and PDME did not affect the activities of seven membrane-bound enzymes [12] or the characteristic transition temperature of plasma or intracellular membranes [13]. However, supplementation of only dimethylethanolamine, not monomethylethanolamine, can partially correct impaired lipoprotein secretion in choline-deficient hepatocytes [14].

To gain further insight into the mechanisms of CT translocation, we examined the effect of modification of the head-group of various phospholipids. The results provide further evidence for a feedback regulation of PC biosynthesis and show that the polar head group plays a specific role in translocation of CT from membranes to cytosol.

EXPERIMENTAL

Materials

Male Sprague–Dawley rats, initially weighing 50–60 g, were fed on a choline-deficient diet (ICN Biochemicals, Montreal, Canada) and water *ad libitum* for 3 days. [*Me*-³H]Choline chloride (15 Ci/mmol) was purchased from Amersham International, Amersham, Bucks., U.K. Phospho[*Me*-³H]choline (5–7 mCi/mmol) was synthesized enzymically from [*Me*-³H]choline and ATP with choline kinase as described previously [3]. All other reagents were obtained as previously stated [3] or from Sigma.

Treatment of hepatocytes and CT translocation measurements

Hepatocytes were obtained from a single choline-deficient rat by a collagenase-perfusion technique [15] and plated (3×10^6 cells/dish) in a choline- and methionine-free medium + 17%

Abbreviations used: PC, phosphatidylcholine; CT, CTP:phosphocholine cytidyltransferase; ER, endoplasmic reticulum; PMME, phosphatidylmonomethylethanolamine; PDME, phosphatidylmethylethanolamine; PE, phosphatidylethanolamine; EC_{50} , concentration at which half of the maximal effect is observed.

(v/v) delipidated fetal-bovine serum as described previously [16]. At 4 h after plating, the medium was replaced with a serum-free medium containing different precursors. At various time intervals the medium was removed, the cell monolayer was washed with 2 ml of ice-cold phosphate-buffered saline, and dishes were placed on an ice-cold metal tray [9]. Cold digitonin buffer (0.8 ml/dish), containing 10 mM-Tris/HCl, pH 7.4, 250 mM-sucrose, 0.5 mM-phenylmethanesulphonyl fluoride and 0.5 mg of digitonin/ml, was carefully added to each dish. The cells were occasionally swirled during the incubation period. After 8 min the digitonin solution was transferred to plastic micro-centrifuge tubes, and the cell debris in the solution was removed by centrifugation at 16000 *g*/min for 2 min in a micro-centrifuge. After the digitonin treatment, ghosts of cells were scraped with 1 ml of buffer R (10 mM-Tris/HCl, pH 7.4, 0.25 M-sucrose and 0.1 mM-phenylmethanesulphonyl fluoride) and sonicated for 7 × 1 s. CT activity was measured in the digitonin solution and in cell ghosts. Cell ghosts were also used to measure the concentration of phospholipids.

CT assay

CT activity was measured by the method of Weinhold *et al.* [17] as described previously [3], with phospho[Me-³H]choline as substrate. [Me-³H]CDP-choline was separated from phosphocholine on plastic backed silica-gel 60 t.l.c. plates (10 cm × 20 cm) developed in methanol/0.6% NaCl/28% NH₄OH (10:10:1, by vol.).

Chemical analysis

Phospholipids were determined by phosphorus assay as described previously [18]. Protein was determined as described by Lowry *et al.* [19], with bovine serum albumin as a standard. Lipids were fractionated and analysed as described previously [14].

RESULTS

Head-group specificity of CT translocation

Addition of 100 μ M-choline to choline-deficient hepatocytes in culture increases the specific activity of CT in the cytosol, and decreases CT activity in the microsomes when fractionated after homogenization [3]. Similar results were obtained in digitonin-release assays [9]. Thus addition of choline to the choline-deficient hepatocytes translocates CT from membranes to cytosol [9]. Evidence was provided that this mass translocation was due to an increase in the concentration of PC in the ER [3,9]. To investigate the head-group specificity for CT translocation, choline-deficient hepatocytes were incubated with 100 μ M-choline, 200 μ M-*N*-monomethylethanolamine, -*NN*-dimethylethanolamine or -ethanolamine. Cytosolic enzyme activity was measured in the digitonin-released samples, and membrane-associated enzyme activity was measured in the cell ghosts. CT activity is presented as m-units of enzyme activity released/mg of membrane protein to compare the increase in cytosolic activity with the decrease in membrane activity by the addition of phospholipid bases. Maximum translocation of CT from membranes to cytosol was obtained by the addition of 100 μ M-choline to choline-deficient hepatocytes. CT activity in digitonin-released samples was increased from 0.25 to 1.14 m-units/mg of membrane protein and decreased in membranes from 3.6 to 1.85 m-units/mg of membrane protein after 4 h (Fig. 1). PC concentration was increased at the same time from 94 to 112 nmol/mg of protein measured in cell membrane samples (Table 1). There was also a small decrease in concentration of PE, from 81 to 70 nmol/mg of protein, after addition of choline to the choline-deficient hepatocytes. The increase in PC concentration has a

Fig. 1. Effect of choline, dimethylethanolamine, monomethylethanolamine or ethanolamine on the distribution of CT in choline-deficient hepatocytes

Hepatocytes were prepared from a single liver of a rat fed on a choline-deficient diet for 3 days and plated in choline- and methionine-free medium + 17% delipidated serum. At 4 h after plating, the medium was replaced with a serum-free medium containing no addition (●), 100 μ M-choline (○), 200 μ M-dimethylethanolamine (▲), 200 μ M-monomethylethanolamine (■) or 200 μ M-ethanolamine (□). At 1, 2 and 4 h the cells were treated with digitonin to measure release of cytosolic CT (a). After the digitonin treatment, the ghosts of cells were collected and sonicated in buffer R (10 mM-Tris/HCl, pH 7.4, 0.25 M-sucrose and 0.1 mM-phenylmethanesulphonyl fluoride) for 7 × 1 s, and CT activity was measured. Cytosolic activity is plotted as the amount of enzyme activity released per mg of membrane protein. Membrane activity was measured as nmol of CDP-choline formed/mg of membrane protein (b). Each point is the average of four separate dishes from two different experiments, $\pm$ S.D. (*n* = 4), assayed in duplicate.

linear relationship to the increase in CT activity in cytosol or the decrease in activity on membranes after the addition of choline.

Incubation of *NN*-dimethylethanolamine with choline-deficient hepatocytes increased the cytosolic activity in digitonin-released samples from 0.25 to 0.76 m-unit/mg of protein, and concurrently the CT activity was decreased in the membranes from 3.5 to 2.3 m-units/mg of protein after 4 h (Fig. 1). From Fig. 1(b), it is possible that the release of CT from membranes may not be complete. However, no additional release of CT was observed when the time was extended to 8 h. The increase in activity of cytosol was approx. 59% of that observed in the presence of 100 μ M-choline in the same length of incubation (Fig. 1). This treatment also decreased the concentration of PC from 94 to 82 nmol/mg of protein and the PE concentration from 81 to 63 nmol/mg of protein. There was no detectable amount of PDME in choline-deficient hepatocytes. When the concentration of PDME with time (Table 1) was re-plotted against the increase in cytosolic CT activity and the decrease in membrane CT activity (Fig. 2), there was a linear relationship. The correlation coefficient for the PDME concentration increase was 0.97 versus the increase in cytosolic CT activity and 0.99 versus the decrease in membrane CT activity (Fig. 2). These results showed that an increase in PDME concentration in membranes can also translocate from membranes to cytosol, though to a lesser extent in comparison with PC.

Incubation of *N*-monomethylethanolamine with choline-deficient hepatocytes showed similar changes in PC and PE concentrations as observed with *NN*-dimethylethanolamine. The PC concentration was decreased from 94 to 83 nmol/mg of protein, and the PE concentration was lowered from 81 to 63 nmol/mg of protein. This treatment accumulated 26 nmol of PMME/mg of protein in 4 h (Table 1). Incubation of cells with *N*-monomethylethanolamine showed no significant differences in cytosolic or membrane CT activity (Fig. 1). Treatment of choline-

Table 1. Changes in phospholipid composition in choline-deficient hepatocytes supplemented with choline, dimethylethanolamine, monomethylethanolamine or ethanolamine

Experimental conditions were the same as Fig. 1. Phospholipids were extracted from cell ghosts and quantified as described in the Experimental section. Percentage CT translocation was calculated from Fig. 1(a), by assuming that 100% translocation was obtained after the addition of 100 μ M-choline to choline-deficient hepatocytes for 4 h. Each point is the average of four dishes from two different experiments, $\pm$ S.D.: * $P < 0.025$; † $P < 0.01$; ‡ $P < 0.0005$.

	Time (h)	Cytosol CT activity (munits/mg of membrane protein)	Membrane CT activity (munits/mg of protein)	Percentage of CT activity translocation	Phospholipid (nmol/mg of protein)				PC/PE ratio
					PC	PE	PDME	PMME	
No addition	0	0.25 $\pm$ 0.1	3.6 $\pm$ 0.13	0	93.7 $\pm$ 4.6	80.7 $\pm$ 7.5			1.16
	1	0.23 $\pm$ 0.1	3.61 $\pm$ 0.28	0	93.7 $\pm$ 2.8	81.2 $\pm$ 6.2			1.15
	2	0.20 $\pm$ 0.06	3.4 $\pm$ 0.14	0	90.4 $\pm$ 4.8	78.3 $\pm$ 8.3			1.15
	4	0.22 $\pm$ 0.09	3.4 $\pm$ 0.25	0	91.4 $\pm$ 3.7	82.5 $\pm$ 9.0			1.11
100 μ M-Choline	1	0.63 $\pm$ 0.03	2.8 $\pm$ 0.05	43	99.8 $\pm$ 3.0*	79.2 $\pm$ 4.5			1.26
	2	0.85 $\pm$ 0.1	1.89 $\pm$ 0.12	71	103.5 $\pm$ 3.0†	75.9 $\pm$ 2.6			1.36
	4	1.14 $\pm$ 0.2	1.85 $\pm$ 0.26	100	111.7 $\pm$ 5.7‡	70.4 $\pm$ 6.9			1.58
200 μ M- <i>NN</i> -Dimethylethanolamine	1	0.51 $\pm$ 0.15	3.2 $\pm$ 0.4	30	90.5 $\pm$ 4.4	75.7 $\pm$ 2.8	16.9 $\pm$ 1.0		1.2
	2	0.64 $\pm$ 0.15	2.76 $\pm$ 0.35	48	86.6 $\pm$ 3.0	71.2 $\pm$ 4.0	30.7 $\pm$ 2.3		1.22
	4	0.76 $\pm$ 0.3	2.3 $\pm$ 0.35	59	81.8 $\pm$ 2.4	63.0 $\pm$ 1.9	46.6 $\pm$ 2.5		1.3
200 μ M- <i>N</i> -Monomethylethanolamine	1	0.34 $\pm$ 0.1	3.54 $\pm$ 0.2	12	82.0 $\pm$ 5.3	77.7 $\pm$ 1.0		10.7 $\pm$ 1.5	1.06
	2	0.35 $\pm$ 0.14	3.34 $\pm$ 0.23	16	83.8 $\pm$ 3.8	73.0 $\pm$ 4.9		19.5 $\pm$ 0.1	1.15
	4	0.3 $\pm$ 0.15	3.23 $\pm$ 0.39	11	82.9 $\pm$ 2.9	63.3 $\pm$ 7.8		25.9 $\pm$ 2.3	1.31
200 μ M-Ethanolamine	1	0.1 $\pm$ 0.01	3.5 $\pm$ 0.1	0	87.6 $\pm$ 0.3	80.8 $\pm$ 1.1			1.08
	2	0.12 $\pm$ 0.01	3.54 $\pm$ 0.1	0	88.0 $\pm$ 2.9	83.2 $\pm$ 0.9			1.06
	4	0.18 $\pm$ 0.04	3.28 $\pm$ 0.04	0	89.2 $\pm$ 6.8	92.2 $\pm$ 8.7			0.97

Fig. 2. Correlation between increase in PDME concentration and increase in cytosolic CT activity (a) and decrease in membrane CT activity (b)

Data for PDME concentrations (Table 1) with increase in cytosolic CT activity (a) and decrease in membrane CT activity (b) (Fig. 1) were replotted and correlation coefficients calculated.

deficient hepatocytes with 200 μ M-ethanolamine did not significantly change the cellular PC concentration. The PE concentration was increased from 81 to 92 nmol/mg of membrane protein in 4 h. The ethanolamine had no effect on CT translocation (Fig. 1). The lack of CT translocation after the incubation of cells with ethanolamine or monomethylethanolamine showed a specific head-group requirement of phospholipids for CT translocation from membranes to cytosol.

Concentration-dependence of phospholipid bases for CT translocation

CT translocation was studied at different concentrations of choline and *NN*-dimethylethanolamine. Hepatocytes were prepared from choline-deficient rat liver and incubated in the presence of 0–200 μ M-choline or -dimethylethanolamine. After 2 h, CT activity was measured in the digitonin-released sample (Fig. 3). In both cases the amount of CT activity released into cytosol increased with the increase in the concentration of phospholipid base, and reached a plateau at 60 μ M. The increase in the cytosolic activity in the presence of choline was higher compared with dimethylethanolamine. Semi-logarithmic plots showed that the concentration of phospholipid base required for half of the maximal CT translocation was the same for both choline and dimethylethanolamine (Fig. 3). The apparent EC_{50} (concn. at which half of maximal effect observed) and maximum CT activity released with phospholipid bases were calculated from semi-logarithmic plots (Fig. 3). The apparent EC_{50} values for CT translocation from choline and dimethylethanolamine were 25 μ M and 27 μ M, and the maximum CT activity released was 0.93 and 0.52 m-unit/mg of protein, respectively. This 2-fold difference in maximum rate of translocation probably relates to the structural difference in the head-group of the phospholipid that was increased by the addition of choline or dimethylethanolamine.

Effect of other choline and ethanolamine analogues on CT translocation

It appears from the above results that there is a specific requirement for at least two methyl groups in the phospholipid amino head-group for CT translocation. We next determined if *N*-ethylethanolamine, *NN*-diethylethanolamine or 3-amino-1-

Fig. 3. Concentration-dependence of choline and dimethylethanolamine on CT translocation

Hepatocytes from a single choline-deficient rat liver were prepared and plated as described in Fig. 1. Cells were incubated with different concentrations of choline (○) or dimethylethanolamine (□) for 2 h. After 2 h the cells were treated with digitonin to measure cytosolic CT activity. Cytosolic CT activity is plotted versus the log of the precursor concentration. Each point is the average of four dishes from two separate experiments. This experiment was repeated three times with similar results.

propanol would affect CT translocation. Choline-deficient hepatocytes were incubated with 200 μ M concentrations of the above analogues for up to 4 h. At various times the cells were treated with digitonin to measure cytosolic released enzyme activity, and cell ghosts were used to measure membrane CT activity plus phospholipid concentrations (Table 2). In choline-deficient hepatocytes there were no detectable amounts of phosphatidyl-*N*-ethylethanolamine or phosphatidyl-*NN*-diethylethanolamine. Incubation of cells with *N*-ethylethanolamine and *NN*-diethylethanolamine accumulated 32 nmol of phosphatidyl-*N*-ethylethanolamine and 27 nmol of phosphatidyl-*NN*-diethylethanolamine/mg of membrane protein respectively. Concomitantly, the PC concentration was decreased from 94 to 79 nmol/mg of protein with *N*-ethylethanolamine and to 80 nmol/mg of protein with *NN*-diethylethanolamine. The cellular PE concentration also decreased from 81 to 73 nmol/mg of protein on incubation with either analogue. These changes in PE and PC concentrations were similar to those observed when hepatocytes were incubated with monomethylethanolamine or dimethylethanolamine (Table 1). However, no increase in CT activity in cytosol or decrease in CT activity in the membranes were observed during the incubations of cells with *N*-ethylethanolamine or diethylethanolamine. Incubation of cells with aminopropanol for up to 4 h did not affect the PC or PE concentrations, and CT distribution was not affected, either. These results show that ethyl groups cannot be substituted for methyl groups to promote CT translocation.

Changes in the PC/PE ratio are not responsible for CT translocation

It has been suggested that the PC/PE ratio might be maintained as constant as possible *in vivo* and that changes in the PC/PE ratio might regulate PC synthesis or CT translocation [20]. The PC/PE ratio was altered by supplementation of hepatocytes with choline and related choline analogues (Table 1), and also by supplementing the cells with lyso-PC, methionine or ethanolamine+methionine (Table 3). However, when the changes in PC/PE ratio were plotted versus release of CT into

Table 2. CT activity and phospholipid concentrations in choline-deficient hepatocytes supplemented with *N*-ethylethanolamine, *NN*-diethylethanolamine and 3-amino-1-propanol

Experimental conditions were the same as Fig. 1, except that hepatocytes were supplemented with ethylethanolamine, diethylethanolamine or 3-aminopropanol. At various time intervals the cells were treated with digitonin to measure cytosolic activity. After the digitonin treatment, the ghosts of the cells were scraped and sonicated for 7 $\times$ 1 s. CT activity was measured in cell ghosts to measure membrane CT activity. Cell ghosts were also used for quantification of phospholipids. Cytosolic CT activity is presented as the amount of enzyme activity released per mg of membrane protein. Each point is the average of four dishes $\pm$ S.D. from two separate experiments. Abbreviations: PEAE, phosphatidyl-ethylethanolamine; PDEA, phosphatidyl-diethylethanolamine.

Conditions	Incubation time (h)	Cytosol CT activity (munits/mg of protein)	Membrane CT activity (munits/mg of protein)	Phospholipid (nmol/mg of protein)				PC/PE ratio
				PC	PE	PEAE	PDEA	
200 μ M- <i>N</i> -Ethylethanolamine	0	0.25 $\pm$ 0.1	3.6 $\pm$ 0.13	93.7 $\pm$ 4.6	80.7 $\pm$ 7.5			1.16
	1	0.15 $\pm$ 0.03	3.48 $\pm$ 0.1	99.1 $\pm$ 3.9	82.7 $\pm$ 2.1	11.8 $\pm$ 2.3		1.2
	2	0.2 $\pm$ 0.05	3.17 $\pm$ 0.02	89.4 $\pm$ 1.5	80.1 $\pm$ 1.0	19.6 $\pm$ 0.3		1.12
	4	0.18 $\pm$ 0.05	3.31 $\pm$ 0.2	79.4 $\pm$ 4.6	72.5 $\pm$ 3.9	31.5 $\pm$ 1.8		1.1
200 μ M- <i>NN</i> -Diethylethanolamine	1	0.17 $\pm$ 0.01	3.41 $\pm$ 0.2	82.9 $\pm$ 1.5	77.9 $\pm$ 4.2		10.4 $\pm$ 1.1	1.06
	2	0.19 $\pm$ 0.01	2.98 $\pm$ 0.3	82 $\pm$ 1.1	74.6 $\pm$ 2.4		17.0 $\pm$ 0.3	1.1
	4	0.33 $\pm$ 0.04	3.26 $\pm$ 0.13	79.7 $\pm$ 1.2	72.5 $\pm$ 5.9		26.6 $\pm$ 2.8	1.1
200 μ M-3-Aminopropanol	2	0.17 $\pm$ 0.03	3.66 $\pm$ 0.2	87.8 $\pm$ 3.0	75.9 $\pm$ 1.0			1.16
	4	0.14 $\pm$ 0.02	3.31 $\pm$ 0.1	87.1 $\pm$ 1.5	81.9 $\pm$ 1.2			1.06

Table 3. CT translocation and change in phospholipid composition after the addition of lyso-PC, methionine or methionine + ethanolamine to choline-deficient hepatocytes

Experimental conditions were the same as Fig. 1. Cytosolic CT activity was measured in digitonin-released samples and is presented as the amount of enzyme released per mg of membrane protein. Membrane CT activity was measured in cell ghosts and is presented as munits/mg of membrane protein. Phospholipid concentrations were also measured in cell ghosts as described in the Experimental section. Each point is the average of four dishes from two separate experiments, $\pm$ S.D.

Conditions	Incubation time (h)	Cytosolic CT activity (munits/mg of protein)	Membrane CT activity (munits/mg of protein)	Phospholipid (nmol/mg of protein)		PC/PE ratio
				PC	PE	
100 μ M-Lyso-PC*	0	0.25 $\pm$ 0.1	3.6 $\pm$ 0.13	93.7 $\pm$ 4.6	80.7 $\pm$ 7.5	1.16
	1	0.38 $\pm$ 0.07	3.43 $\pm$ 0.29	98.06 $\pm$ 2.9	77.9 $\pm$ 2.9	1.26
	2	0.6 $\pm$ 0.06	3.09 $\pm$ 0.18	104 $\pm$ 1.7	75.0 $\pm$ 6.1	1.38
	4	0.87 $\pm$ 0.12	2.78 $\pm$ 0.07	108.4 $\pm$ 3.3	74.9 $\pm$ 6.4	1.45
200 μ M-Methionine*	4	0.24 $\pm$ 0.03	2.8 $\pm$ 0.6	104.7 $\pm$ 3.1	67.6 $\pm$ 4.9	1.55
	8	0.37 $\pm$ 0.02	2.19 $\pm$ 0.12	110.5 $\pm$ 0.1	55.7 $\pm$ 1.2	1.98
	12	0.52 $\pm$ 0.3	1.89 $\pm$ 0.12	116.2 $\pm$ 2.3	54.2 $\pm$ 1.0	2.15
200 μ M-Methionine + 200 μ M-ethanolamine	4	0.32 $\pm$ 0.06	2.7 $\pm$ 0.1	107.7 $\pm$ 3.4	84.6 $\pm$ 1.8	1.27
	8	0.41 $\pm$ 0.05	2.1 $\pm$ 0.2	110.5 $\pm$ 2.3	79.9 $\pm$ 2.9	1.36
	12	0.57 $\pm$ 0.02	1.85 $\pm$ 0.1	118.3 $\pm$ 1.0	75.9 $\pm$ 1.0	1.56

* Data on CT translocation and PC concentrations have been reported in ref. [9].

Fig. 4. Lack of correlation between increase in PC/PE ratio and increase in cytosolic CT activity and decrease in membrane CT activity

Experimental conditions were the same as Fig. 1. Data on PC/PE ratios for supplementation of hepatocytes with 100 μ M-choline (●), 100 μ M-lyso-PC (○), 200 μ M-methionine (▲) and 200 μ M-methionine + 200 μ M-ethanolamine (□) from Tables 1 and 3 were plotted against increase in cytosolic CT activity and decrease in membrane CT activity.

the cytosol from the membrane (Fig. 4), there was no relationship between amount of CT released and the PC/PE ratio.

DISCUSSION

Head-group specificity for translocation of CT

Feedback regulation between cytosol and membranes has been demonstrated for translocation of CT in choline-deficient rat hepatocytes [3,9]. When rats were fed with a choline-deficient diet for 3 days, the concentration of PC decreased to 89 % of the control value with accumulation of CT on the ER [3]. Addition of choline, methionine or lyso-PC to hepatocytes prepared from these choline-deficient rats restored the concentration of PC to normal levels, with a concomitant translocation of CT from membranes to cytosol [9]. The present study shows that if

PDME, but not PC, was increased by supplementation of the hepatocytes with dimethylethanolamine, translocation of CT from membranes to cytosol still occurred (Fig. 1). However, an increase in the concentrations of PMME or PE had no significant effect on CT translocation.

The number of methyl groups in the phospholipid head-group is decisive for CT translocation. Very large increases in the concentration of PDME (from 0 to 47 nmol/mg of membrane protein) are only 60 % as effective for CT translocation as are much smaller increases in PC (from 94 to 112 nmol/mg of membrane protein) (Table 1). An increase in the concentration of PMME or PE had no effect on CT translocation. Hence there is a minimal requirement for at least two methyl groups in the phospholipid head-group for CT translocation. Moreover, this requirement could not be met by supplementation of the hepatocytes with *N*-ethylethanolamine.

Supplementation of dimethylethanolamine leads to the synthesis of PDME via a CDP pathway [21]. When the concentration-dependence of choline or dimethylethanolamine for CT translocation was evaluated, the concentration of either phospholipid base required for maximum CT translocation (approx. 60 μ M) was apparently the same (Fig. 3). However, much of the choline will be utilized for the biosynthesis of betaine [1]. Thus the effective concentration of choline required for maximum CT translocation is probably much lower, even though the enzyme activity involved in the formation of betaine was decreased by 40 % in the livers from choline-deficient rats [22]. It is not known if dimethylethanolamine might also be oxidized (to dimethylglycine) in the hepatocytes.

The concentration-dependence of CT translocation on PDME could not be evaluated, because an increase in PDME concentration is accompanied by a decrease in the concentration of PC in the membrane (Table 1). This decrease in PC would presumably counteract the promotion of CT release by PDME. The translocation of CT from membrane to cytosol might have been larger if PC levels had not been decreased. Similarly, some translocation of CT with an increase in PMME might have been observed if the level of PC had not decreased concomitantly.

Recovery of CT activity in the cytosolic fraction was only 50 % of the total enzyme activity lost from the membrane

fraction after the addition of choline or dimethylethanolamine to hepatocytes (Fig. 1). Similar results of low recoveries of CT activity in the cytosolic fraction have been reported previously [9]. The reason for this low recovery is not known. Possibly partial degradation of membrane-bound CT might have occurred. This postulate is reminiscent of increased degradation of HMG-CoA reductase on ER when the cholesterol content was increased [23]. We require a good precipitating antibody to test this hypothesis.

Studies with Type II pneumonocytes [24] or CHO cells and LM cells [25] have shown increased binding of CT to membranes in the absence of choline. Supplementation with dimethylethanolamine, but not monomethylethanolamine, partially prevented the increased CT binding [25]. These results are in qualitative agreement with our studies in hepatocytes. However, they [25] did not report on the kinetics of the translocation of CT or the effects of other structural analogues, or evaluate the importance of the PC/PE ratio.

An increase in the concentration of PC seems to mediate CT translocation

Before the completion of these experiments, we were unsure whether the translocation of CT was due to an increase in the concentration of PC in the membrane or to a change in the PC/PE ratio. The latter has been shown to change during different physiological conditions. In choline deficiency the PC/PE ratio decreased from 1.95 to 1.27 in rat liver [3]. Addition of choline or lyso-PC to choline-deficient hepatocytes increased this ratio, with concurrent translocation of CT from membranes to cytosol. Similarly, in livers from 48 h-fasted rats the PC/PE ratio decreased (from 1.7 to 1.5), with an increase in membrane-bound CT activity. If the rats were fasted for 48 h and refed for 72 h, the PC/PE ratio (2.2) increased with a decrease in membrane-bound CT [20]. These results suggest that a change in the PC/PE ratio might regulate CT translocation. However, when choline-deficient hepatocytes were supplemented with methionine or methionine plus ethanolamine, identical changes in the level of CT translocation were obtained, even though for methionine supplementation the PC/PE ratio was 2.15 and for methionine/ethanolamine supplementation the ratio was 1.56. Moreover, supplementation of hepatocytes with dimethylethanolamine or monomethylethanolamine altered the PC/PE ratio to 1.3, but only dimethylethanolamine caused CT translocation (Table 1). Thus CT translocation occurred over a wide range of values for the PC/PE ratio (Fig. 4). In contrast, there was a linear relationship between an increase in cytosolic CT activity or a decrease in membrane CT activity and either an increase in PC [9] or PDME (Fig. 2) concentrations. It therefore seems that the concentration of PC (or PDME) is the critical factor in mediating release of CT from membranes.

Conclusion

The results of this study further elaborate on a feedback regulation of PC biosynthesis by CT translocation. PDME can partially substitute for PC for CT translocation, whereas PMME is ineffective. In studies on PC turnover, supplementation of choline to choline-deficient hepatocytes restored PC catabolism to normal rates as the concentration of PC increased (L. Tjburg & D. E. Vance, unpublished work). PDME, but not PMME, could also be substituted for PC in restoring PC catabolism. Thus there are distinct similarities between the regulation of PC biosynthesis and PC catabolism. In both anabolism and cat-

abolism the concentration of PC appears to be a major regulatory factor. Limited incubation of cells in culture with phospholipase C [26] or phospholipase A₂ [27] causes an increase in CT binding to membranes, specifically demonstrated to be the ER in one instance [28]. It has been proposed that the phospholipase C effect is due to a decrease in the concentration of PC in the membranes [29]. If this were proved to be true, the result would provide further support for the idea that the concentration of PC is very important for regulation of PC biosynthesis and catabolism. The molecular mechanism by which the PC concentration in membranes mediates CT binding is also an important question for future investigations.

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