Evidence that binding of CTP:phosphocholine cytidyltransferase to membranes in rat hepatocytes is modulated by the ratio of bilayer- to non-bilayer-forming lipids

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The mechanism by which phospholipase C (PLC) digestion of cultured cells mediates binding of CTP:phosphocholine cytidyltransferase to cellular membranes was investigated. Incubation of choline-depleted rat hepatocytes with PLC caused a translocation of enzyme from cytosol to membranes concomitant with a decrease in the concentration of phosphatidylcholine with no effect on the concentration of other phospholipids. Removal of PLC and supplementation with choline restored the amount of phosphatidylcholine in the cells and translocated cytidyltransferase to the cytosol. However, when phosphatidylcholine levels were decreased by incubation with phospholipase A₂ (PLA₂), there was no significant redistribution of cytidyltransferase activity. With PLA₂ the concentration of phospho-

tidylethanolamine, as well as of phosphatidylcholine, was significantly decreased. Since PLC, but not phospholipase A₂, raised the cellular concentration of diacylglycerol, possibly diacylglycerol mediated the binding of cytidyltransferase to membranes. This possibility was examined, but is unlikely, since addition of lysophosphatidylcholine to PLC-treated cells restored the concentration of phosphatidylcholine and released cytidyltransferase into the cytosol, but did not lower diacylglycerol levels to normal values. Studies *in vitro*, incubations of cells with choline analogues and a survey of the literature suggested that the over-riding common factor in regulation of cytidyltransferase binding to membranes may be the ratio of bilayer to non-bilayer lipids in that membrane.

INTRODUCTION

CTP:phosphocholine cytidyltransferase (CT) catalyses the rate-limiting and regulated reaction for phosphatidylcholine (PC) biosynthesis via the CDP-choline pathway under a variety of different metabolic conditions [1–3]. CT appears to exist in the cytosol as an inactive reservoir and can be translocated reversibly to cellular membranes, where CT becomes activated by interactions with phospholipids. The major mechanism identified for the control of PC biosynthesis involves the regulation of the subcellular distribution of CT. Several changes in the lipid composition of cellular membranes that modulate the translocation of CT have been identified.

Addition of fatty acids to cultured cells [4–6] causes translocation of CT from cytosol to membranes with concomitant stimulation of PC biosynthesis. This process can be readily reversed by incubation of cells with albumin [7]. The mechanism for CT translocation under these conditions has not been clearly defined. An increase in cellular diacylglycerol (DG) levels may be important in mediating CT translocation to membranes in hepatocytes incubated with oleate [8]. On the other hand, CT does bind to and is activated by lipid vesicles containing PC and fatty acid, suggesting that fatty acid is potentially a regulator itself [7].

The modulation of CT translocation by DG has been recently demonstrated [9]. Incubation of HeLa cells with phorbol 12-myristate 13-acetate increased the concentration of DG, followed by translocation of CT to membranes and its activation. Subsequently PC biosynthesis was stimulated [9]. Studies *in vitro* [7], and the addition of DiC₈ to pituitary cells [10] or HeLa cells

[9], support the possible role of DG in translocating CT to membranes.

Feedback regulation of PC biosynthesis by the concentration of PC was recently demonstrated in our laboratory [11,12]. In choline-deficient hepatocytes there is enhanced binding of CT to membranes, which is reversed when the concentration of PC is restored to normal levels after supplementation of the cells with choline, methionine or lyso-PC. These results suggested that the amount of PC in cellular membranes regulates the level of CT binding.

Phospholipase C (PLC) has been extensively used as a tool by Kent and co-workers to investigate the mechanism of regulation of PC biosynthesis; incubation of cells under controlled conditions with exogenous PLC stimulates PC biosynthesis and causes translocation of CT from cytosol to membranes [13–16]. The organelle membrane to which CT binds has been identified as endoplasmic reticulum (ER) in Krebs II cells [17] and nuclear membrane in CHO cells [18]. The mechanism for this translocation in response to PLC has been investigated previously and attributed to both a decrease in PC content [15] and an increase in DG levels [19]. Treatment of rat hepatocytes with phospholipase A₂ (PLA₂) also caused a translocation of CT from cytosol to membranes, but the mechanism was not investigated [20]. Thus, potentially either a decrease in PC or an increase in DG, or both, could cause the translocation of CT to membranes in cells treated with phospholipases.

PC is the major bilayer-forming lipid in eukaryotic membranes. DG tends to favour the hexagonal H_{II} phase [21] and fatty acids form micelles. We hypothesized that when the ratio of bilayer- to non-bilayer-forming lipids was decreased, the cell would attempt

Abbreviations used: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; DG, diacylglycerol; CT, CTP:phosphocholine cytidyltransferase; PMME, phosphatidylmonomethylethanolamine; PDME, phosphatidyl dimethylethanolamine; PLC, phospholipase C; PLA₂, phospholipase A₂; ER, endoplasmic reticulum.

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to restore the optimal balance of these two types of lipids in order to avoid catastrophe (membrane leakage). The results of the studies reported in this paper support this proposal and show that the binding of CT to membranes is sensitive to the ratio of bilayer- to non-bilayer-forming lipids in cellular membranes.

EXPERIMENTAL

Materials

[methyl- ^3H]Choline chloride (15 Ci/mmol) was purchased from Amersham International. Phospho[methyl- ^3H]choline (5–7 mCi/mmol) was synthesized enzymically from [methyl- ^3H]choline and ATP with choline kinase as described [22]. Cell culture medium, Hanks' balanced salt solution and fetal-bovine serum were obtained from GIBCO. Primary culture dishes (60 mm diam.) were obtained from Becton, Dickinson and Co. (Oxnard, CA, U.S.A.) and coated with collagen. PLC from *Clostridium welchii*, PLA₂ from *Naja mocambique mocambique* and all other chemicals were obtained from Sigma.

Preparation of hepatocytes

Hepatocytes were isolated from male Sprague–Dawley rats (80–100 g) by the collagenase-perfusion technique [23] and were plated (2.6×10^6 cells/dish) in a medium containing 17% fetal-calf serum. The hepatocytes were incubated at 37 °C under an atmosphere of air/CO₂ (19:1). After 4 h, the cultured hepatocytes were washed free of serum and floating dead cells and incubated in serum-, choline- and methionine-free medium for 16 h before use.

Preparation of samples for measurement of CT activity and lipid extraction

After incubation of cells under the indicated conditions, the cell monolayer was washed with 2 ml of ice-cold PBS and the dishes were placed on a glass tray on ice. Cold digitonin buffer (0.8 ml) containing 10 mM Tris/HCl, pH 7.4, 250 mM sucrose, 0.5 mM phenylmethanesulphonyl fluoride and 0.5 mg/ml digitonin was carefully added to each dish and incubated on ice for 8 min with occasional swirling. Subsequently the digitonin solution was transferred to plastic micro-centrifuge tubes and centrifuged at 14000 rev./min for 2 min in a micro-centrifuge. The resulting pellet was sonicated (7 × 1 s) in buffer R (10 mM Tris/HCl, pH 7.4, 250 mM sucrose and 0.1 mM phenylmethanesulphonyl fluoride). Digitonin-released samples (soluble fraction) and cell ghosts were assayed for CT activity. Cell ghosts were used for measurement of phospholipid, DG and fatty acid concentration after lipid extraction as described [11].

Assay of CT activity

CT activity was determined as described previously [8].

Chemical analysis

Phospholipids were extracted, separated and quantified as described previously [12,24]. Protein was measured by the Bio-Rad assay based on the method of Bradford [25], with BSA as a standard. Non-esterified fatty acid concentrations were measured with the NEFA C kit [11]. DG concentration was measured with

a sensitive DG kinase assay [8,26]. Statistical analysis was performed by Student's *t* test.

RESULTS

Effect of PLC on the subcellular distribution of CT and cellular PC concentrations

We first investigated the effect of PLC on the subcellular distribution of CT and cellular PC concentration in a dose- and time-dependent manner. Rat hepatocytes were incubated in a choline- and methionine-deficient medium for 16 h, after which the medium was replaced with fresh medium containing various amounts of PLC. At 1, 2 and 3 h the cells were treated with digitonin, which permeabilized the cells, and the soluble and membrane fractions were isolated as described in the Experimental section. CT activity was measured in both soluble and membrane fractions. The concentration of PC in the membrane ghosts was also measured. The results are shown in Figure 1.

Membrane PC concentration was decreased in the presence of PLC in a concentration- and time-dependent manner (Figure

Figure 1 Dose-dependent effect of PLC on subcellular distribution of CT and cellular PC concentrations

Rat hepatocytes were cultured for 4 h in the presence of 17% fetal-calf serum, then incubated for 16 h in medium free of choline and methionine. Subsequently the hepatocytes were incubated with 0.005 (○), 0.01 (●), 0.02 (■) or 0.05 (□) unit of PLC/ml. At various times the hepatocytes were treated with digitonin and CT activity was measured in soluble (a) and membrane (b) fractions. The lipids were extracted from membrane fractions and PC concentration (c) was measured. Each point represents the mean of three measurements. This experiment was repeated once with similar results.

Figure 2 Reversal of PLC-mediated binding of CT to membranes

Rat hepatocytes were prepared as described in Figure 1. After incubation for 16 h in a choline- and methionine-free medium, the cells were incubated in a medium with no addition (○), with 0.02 unit of PLC/ml (●) or with 200 μ M choline (▲). In some dishes at 0 h the medium containing PLC was removed and replaced with medium containing 0 (●) or 200 μ M choline (□). At various times, the cells were treated with digitonin and CT activity was measured in soluble (cytosol) (a) and membrane (b) fractions. PC concentration was also measured in the membrane fraction (c). Each point represents the mean $\pm$ S.D. of three different measurements.

1c). PLC also had an effect on the subcellular distribution of CT. CT activity was decreased in cytosolic (digitonin-released) samples (Figure 1a) and increased in membrane samples as a function of time of PLC treatment (Figure 1b). The largest decrease in cytosolic CT or increase in the membrane CT activity was observed in the presence of 0.05 unit of PLC/ml and the smallest effect with 0.005 unit/ml (Figures 1a and 1b). These results, as observed in many studies of non-hepatic cells [13,14,19], demonstrated the translocation of CT from cytosol to membranes in a time- and dose-dependent manner when hepatocytes were incubated with PLC. There was a positive correlation between the decrease in PC concentration and the decrease in cytosolic CT activity. The membrane CT activity increased concomitantly. These results suggest that a decrease in membrane PC concentration may be responsible for trans-

location of CT from cytosol to membranes. This phenomenon was observed previously in choline-deficient rats [27] and is consistent with an earlier proposition by Sleight and Kent [15].

Effect of PLC treatment on concentrations of PC, phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) in rat hepatocytes

Hepatocytes were prepared as described in Figure 1 and incubated in a medium containing 0.02 unit of PLC/ml. At various times the cells were washed with PBS and the lipids extracted. The concentrations of PC, PE, PS and PI were measured. The results showed a time-dependent decrease in the concentration of PC in the presence of PLC from 114 to 77.3 nmol/mg of protein (68 % of the zero-time value) in 2 h, with no further decrease in cellular PC observed at 3 and 4 h. The values for PC are lower than in Figure 1, since the data are expressed per mg of total cellular protein, whereas the data in Figure 1 are expressed per mg of membrane protein. PLC treatment did not have a major effect on the concentrations of PE, PS or PI. Hence, PLC from *C. welchii* caused a specific degradation of PC, without an effect on the other cellular phospholipids analysed.

Reversal of PLC-mediated binding of CT to membranes by addition of choline

Since a decrease in PC concentration resulted in the translocation of CT from cytosol to membranes in PLC-treated hepatocytes (Figures 1a and 1b), it was expected that an increase in PC levels would reverse this process. Rat hepatocytes were prepared and incubated in a choline- and methionine-free medium as described in Figure 1. Subsequently, the hepatocytes were incubated under one of the following conditions: +200 μ M choline, without choline, +PLC for 2 h and then +200 μ M choline, or +PLC for 2 h and then without choline. After 6 h, CT activity in soluble and membrane fractions and PC concentration in membrane fractions were measured (Figure 2).

The cytosolic CT activity was slightly increased and membrane CT activity was slightly decreased when the choline-depleted hepatocytes were incubated with 200 μ M choline compared with controls (Figures 2a and 2b). This treatment did not have any significant effect on membrane PC concentration (Figure 2c). However, when hepatocytes were incubated with PLC for 2 h, the membrane PC concentration decreased from 182 to 131 nmol/mg of membrane protein, similar to results shown in Figure 1(c). PLC treatment also decreased cytosolic CT activity (Figure 1a), with a concomitant increase in CT activity in the membrane fraction (Figure 2b). The PC concentration correlated positively with the decrease in cytosolic CT activity ($r^2 = 0.99$) and negatively with the increase in membrane CT activity ($r^2 = -0.91$). Addition of 200 μ M choline to the PLC-treated hepatocytes at the time of removal of PLC released CT from membranes to cytosol, with a concomitant increase in cellular PC concentration (Figures 2a–2c) compared with control. There were strong correlations between the cellular PC concentration and the increase in CT activity in cytosol ($r^2 = 1$) or a decrease in membrane CT activity ($r^2 = -1$). These results provided additional evidence that the concentration of PC in membranes might regulate CT translocation.

Decrease in the membrane PC concentration by PLA₂ does not affect the subcellular distribution of CT

Since PLC treatment of hepatocytes caused translocation of CT from cytosol of membranes, we reasoned that a decrease in

Table 1 Effect of PLC and PLA₂ on subcellular distribution of CT and on PC, PE, fatty acid and DG concentrations

Rat liver hepatocytes were cultured in a choline- and methionine-deficient medium for 16 h, after which the medium was replaced with medium containing no addition, PLC or PLA₂ for 2 h at 37 °C. CT activity was measured in the cytosol and membrane fractions after treatment of hepatocytes with digitonin. Lipids were extracted from membrane fractions, and the concentrations of PC, PE, non-esterified fatty acids and DG were measured. Each value is the mean $\pm$ S.D. of three measurements. This experiment was repeated three times with similar results. *P* values relative to control: **P* < 0.0005; †*P* < 0.025; ‡*P* < 0.005.

Addition	CT activity (nmol/min per mg of protein)		Concn. (nmol/mg of protein)			
	Cytosol	Membranes	PC	PE	DG	Fatty acid
Control	1.63 $\pm$ 0.09	1.43 $\pm$ 0.28	178.6 $\pm$ 15.3	53.4 $\pm$ 2.6	2.7 $\pm$ 0.2	24.5 $\pm$ 2.3
0.02 unit of PLC/ml	0.51 $\pm$ 0.03*	3.84 $\pm$ 0.24*	143.1 $\pm$ 4.8†	51.8 $\pm$ 2.9	5.4 $\pm$ 0.5*	26.4 $\pm$ 1.5
0.05 unit of PLC/ml	0.44 $\pm$ 0.03*	4.04 $\pm$ 0.06*	141.4 $\pm$ 1.5†	58.1 $\pm$ 2.9	5.7 $\pm$ 0.6*	29.1 $\pm$ 0.2
0.5 unit of PLA ₂ /ml	1.94 $\pm$ 0.06	1.18 $\pm$ 0.08	105.7 $\pm$ 4.9‡	36.9 $\pm$ 2.9‡	2.7 $\pm$ 0.1	35.2 $\pm$ 7.0‡
1.0 unit of PLA ₂ /ml	1.80 $\pm$ 0.11	1.34 $\pm$ 0.06	68.9 $\pm$ 7.2‡	25.4 $\pm$ 3.3*	3.2 $\pm$ 0.3	39.0 $\pm$ 4.7*

cellular PC by exogenous PLA₂ treatment should have a similar effect on the subcellular distribution of CT. Hepatocytes were incubated with two different concentrations of PLC or PLA₂ for 2 h at 37 °C. Compared with controls, the membrane PC concentration decreased from 178 to 143 or 141 nmol/mg protein in the presence of 0.02 or 0.05 unit of PLC/ml (Table 1) respectively, similar to the results shown in Figures 1 and 2. The concentration of PC was also measured in microsomal membranes, and decreased from 282 to 207 nmol/mg of protein (73 % of control; Table 2), similar to the decrease in PC in the total membrane fractions (Figures 1 and 2; Table 3).

Incubation of cells with PLA₂ also resulted in a decrease in the membrane PC concentration (Table 1). (The PLA₂-treated cells remained impermeable to Trypan Blue.) In contrast with treatment with PLC, there was no significant change in cytosolic CT activity or membrane CT activity in PLA₂-treated cells. The surprising implication of this experiment was that the changes in membrane PC concentrations may not be solely responsible for CT translocation.

The concentrations of PE, DG and non-esterified fatty acids were also measured in PLC- and PLA₂-treated hepatocytes. PLC did not have a significant effect on PE (Table 1) or non-esterified fatty acid concentrations, whereas the concentration of DG increased in the presence of PLC (Table 1). The DG concentration in microsomal membranes increased 2.7-fold (Table 2) after PLC treatment. PLA₂ treatment did not significantly affect DG levels (Table 1).

In contrast with PLC treatment, PE concentration was decreased in hepatocytes incubated with exogenous PLA₂ (Table 1). Hence, exogenous PLA₂ was not specific for degradation of PC. In addition, PLA₂ treatment produced a small increase in fatty acids (Table 1).

In a previous study [20] we saw a partial translocation of CT from cytosol to membranes in hepatocytes that had been in culture for 4 h in a serum-containing medium and then treated with PLA₂. We attempted to resolve this apparent discrepancy with the above results. First we treated hepatocytes exactly as previously described [20], and were able to reproduce the small increase in membrane-associated CT (1 nmol/min per mg) with a corresponding decrease in cytosolic CT. Since the studies reported in the present paper were done with cells incubated for 16 h in a medium depleted of choline, methionine and serum, we wondered if the lack of choline and methionine might explain the difference. Thus, we performed an experiment in which the cells were incubated for 16 h with methionine and choline and then

incubated for 2 h with PLC (0.02 unit/ml of medium) or PLA₂ (0.5 unit/ml of medium). Under these conditions, PLC caused the usual translocation of CT, and PLA₂ had no effect. The differences in the results with PLA₂ are apparently due to some unknown effect of the length of incubation of the hepatocytes without serum. Nevertheless, the results of the present studies show clearly that a decrease in PC will not in itself cause a translocation of CT.

Although the decrease in membrane PC levels was significant after PLC or PLA₂ treatment of hepatocytes, DG levels were increased only when the cells were incubated with PLC. Thus, in PLC-treated hepatocytes only an increase in DG concentration correlated with the increased association of CT with membranes. Therefore it seemed possible that a change in DG concentration after PLC treatment might be responsible for the increased translocation of CT to the membranes.

Decrease in DG concentration is not responsible for CT translocation from membranes to cytosol when PC levels are restored in PLC-treated hepatocytes

If an increase in DG concentration increased the association of CT to membranes, a corollary would be that a decrease in the concentration of DG would displace CT from membranes to cytosol. A decrease in DG concentration correlated with translocation of CT from membranes to cytosol when choline was added to the PLC-treated cells, compared with controls (Table 3). DG concentration in microsomal membranes was also decreased after addition of choline to PLC-treated hepatocytes (Table 2). These results suggested that changes in DG concentration may be responsible for CT translocation.

In previous experiments lyso-PC was shown to replace choline in reversing the effects of choline deficiency on PC biosynthesis and CT translocation from cytosol to membranes [11]. Thus we incubated the PLC-treated hepatocytes with 100 μ M lyso-PC. CT was released from membranes to cytosol similarly to the addition of choline (Table 3), and there was a rapid conversion of lyso-PC into PC as previously observed in choline-deficient hepatocytes [11]. However, in contrast with choline addition, lyso-PC did not lower the membrane DG levels (Table 3). Thus the translocation of CT to cytosol by addition of lyso-PC corresponded to an increase in the PC concentration, but not to a change in DG levels. Consequently, the changes in the membrane lipid composition, rather than the fluctuation in

Table 2 Changes in the concentrations of PC, PE and DG in the microsomes of rat hepatocytes after PLC treatment

Rat hepatocytes were cultured for 4 h in a medium containing 17% fetal-calf serum and subsequently incubated in a choline- and methionine-deficient medium. After 16 h, hepatocytes were incubated in the presence of 0.02 unit of PLC/ml or were first incubated for 2 h in the presence of 0.02 unit of PLC/ml and then treated with a medium containing 200 μ M choline for 2 h. Cells from 10 dishes were combined and homogenized in 2 ml of buffer [50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 2 mM dithiothreitol, 1 mM phenylmethane-sulphonyl fluoride, 0.25% (w/v) NaN₃] with 50 strokes in a tight-fitting Potter–Elvehjem homogenizer. The homogenate was centrifuged at 1000 *g* for 5 min to remove unbroken cells. Mitochondria were removed by centrifugation of the supernatant at 10000 *g* for 10 min. Microsomes were obtained after centrifugation of the post-mitochondrial supernatant at 350000 *g* for 15 min and lipids were extracted. Each value is the mean $\pm$ S.D. of three different measurements: * $P < 0.0005$ compared with control values.

Conditions	Concn. (nmol/mg of protein)		
	PC	PE	DG
Control	282.1 $\pm$ 17.5	147.8 $\pm$ 3.6	8.96 $\pm$ 0.58
2 h PLC treatment	207.6 $\pm$ 10.1*	162.8 $\pm$ 9.1	24.3 $\pm$ 3.4*
2 h PLC treated, then incubated with 200 μ M choline	276.7 $\pm$ 4.2	176.9 $\pm$ 3.6	10.1 $\pm$ 2.6

concentration of a single lipid, were responsible for the regulation of CT distribution between cytosol and membranes.

The above results on CT translocation may be related to lipid polymorphism as follows. (1) CT translocated from cytosol to membranes during PLC treatment when there was an increase in DG level (a lipid which promotes hexagonal H_{II} phase rather than bilayer formation [21]), and a decrease in membrane PC, which preferentially forms bilayers [28]. (2) Addition of choline to PLC-treated hepatocytes resulted in translocation of CT from membranes to cytosol, concomitant with an increase in the concentration of bilayer-forming PC and a decrease in the concentration of DG, which promotes hexagonal H_{II} phases. (3) Supplementation of PLC-treated cells with lyso-PC mediated release of CT from the membrane. Under these conditions the PC concentration increased, with no effect on the concentration of DG. (4) In PLA₂-treated hepatocytes, the concentration of both a bilayer-forming lipid (PC) and a hexagonal-H_{II}-phase-pro-

moting lipid (PE) decreased without any significant change in DG concentration or distribution of CT. The subcellular distribution of CT in response to changes in lipid concentration may therefore relate to the ratio of bilayer- to non-bilayer-forming lipids.

Use of phospholipid head-group modifications to test the effect of the ratio of bilayer- to non-bilayer-forming lipid on subcellular distribution of CT

The release of CT from membranes of choline-deficient hepatocytes is observed when the concentration of PC or phosphatidylmethylethanolamine (PDME) is increased by supplementation with choline or dimethylethanolamine respectively [12]. In contrast, an increase in the concentrations of phosphatidylmonomethylethanolamine (PMME) or PE has no significant effect [12]. We concluded from those results that the number of methyl groups on the phospholipid head-group would determine whether or not CT translocation would be induced. Similar results were obtained when we investigated the effect of choline analogues on the translocation of CT in PLC-treated hepatocytes. Addition of choline or dimethylethanolamine to PLC-treated cells induced the translocation of CT from membranes to cytosol compared with control (Table 4). Supplementation with choline or dimethylethanolamine also resulted in an increase in the concentration of bilayer-forming lipids (Table 4). In contrast, addition of ethylaminoethanol or monomethylethanolamine did not significantly affect the subcellular distribution of CT, although these treatments increased the concentrations of their respective phospholipids, phosphatidylethylaminoethanol or PMME, with a concomitant decrease in membrane DG (Table 4). Phosphatidylethylaminoethanol and PMME are hexagonal-H_{II}-phase-forming, rather than bilayer-forming, lipids [29]. Thus translocation of CT to cytosol was only observed when there was an increase in the concentration of lipids which promote bilayer formation. These results support the notion that the ratio of bilayer- to non-bilayer-forming lipids may be responsible for the control of CT translocation.

Concordant results were obtained in experiments with subcellular fractions. Cytosol was incubated with membranes containing different compositions of bilayer-forming and hexagonal-H_{II}-phase-forming lipids, and the distribution of CT was measured. Total membrane fractions were prepared either from hepatocytes treated with 0.02 unit of PLC/ml for 2 h or from

Table 3 Addition of lyso-PC or choline reverses CT translocation caused by PLC treatment of rat hepatocytes

Hepatocytes were incubated for 16 h in a choline- and methionine-free medium, after which the cells were incubated in a medium with or without 0.02 unit of PLC/ml for 2 h. In some dishes of cells, the medium containing PLC was removed after 2 h and replaced with fresh medium containing 200 μ M choline or 100 μ M lyso-PC, and cells were incubated for 2 h. CT activity was measured in soluble and membrane fractions. The concentrations of PC, PE and DG were measured. Each value is the mean of three measurements. This experiment was repeated twice with similar results.

Conditions	CT activity (nmol/min per mg of protein)		Lipids (nmol/mg of protein)		
	Cytosol	Membrane	PC	PE	DG
No addition	2.32 $\pm$ 0.25	1.75 $\pm$ 0.24	184.7 $\pm$ 47	95.9 $\pm$ 18.5	2.6 $\pm$ 0.1
0.02 unit of PLC/ml for 2 h	0.58 $\pm$ 0.1	3.98 $\pm$ 0.52	132.0 $\pm$ 8.6	102.4 $\pm$ 7.4	7.0 $\pm$ 0.75
Hepatocytes were first incubated with 0.02 unit of PLC/ml for 2 h and then replaced for 2 h with medium that contained:					
200 μ M choline	1.97 $\pm$ 0.14	2.36 $\pm$ 0.34	153.0 $\pm$ 12.9	102.1 $\pm$ 9.2	4.65 $\pm$ 0.4
100 μ M lyso-PC	2.23 $\pm$ 0.09	2.0 $\pm$ 0.25	205.3 $\pm$ 15.7	135.9 $\pm$ 7.3	7.3 $\pm$ 0.4

Table 4 Translocation of CT from membranes to cytosol depends on the structure of the head-group of the phospholipid

Hepatocytes, previously incubated for 16 h in a choline-methionine deficient medium, were incubated with 0.02 unit of PLC/ml at 37 °C. After 2 h, the medium containing PLC was removed and replaced for 2 h with one containing 200 μ M ethylaminoethanol (EAE), monomethylethanolamine (MME), dimethylethanolamine (DME) or choline. CT activity was measured in cytosol and membrane fractions after treatment of hepatocytes with digitonin. The concentrations of PC, PE, phosphatidylethylaminoethanol (PEAE), PMME, PDME and DG were measured. Each value is the mean $\pm$ S.D. of three measurements. This experiment was repeated once with similar results.

Additive	CT activity (nmol/min per mg of protein)		Phospholipid (nmol/mg of protein)					DG (nmol/mg of protein)
	Cytosol	Membrane	PC	PE	PEAE	PMME	PDME	
Control	2.16 $\pm$ 0.36	1.56 $\pm$ 0.16	194.3 $\pm$ 22.8	88.9 $\pm$ 8.9				2.50 $\pm$ 0.50
Hepatocytes first treated with 0.02 unit of PLC/ml for 2 h and then incubated for 2 h with:								
No addition	0.14 $\pm$ 0.02	4.60 $\pm$ 0.38	119.7 $\pm$ 3.2	78.9 $\pm$ 3.0				4.84 $\pm$ 0.20
200 μ M EAE	0.40 $\pm$ 0.2	3.80 $\pm$ 0.30	110.4 $\pm$ 2.9	78.0 $\pm$ 2.5	26.0 $\pm$ 2.7			2.48 $\pm$ 0.02
200 μ M MME	0.44 $\pm$ 0.04	3.62 $\pm$ 0.02	114.3 $\pm$ 3.0	75 $\pm$ 4.8		33.9 $\pm$ 4.8		2.33 $\pm$ 0.25
200 μ M DME	1.44 $\pm$ 0.38	2.38 $\pm$ 0.17	118.7 $\pm$ 3.6	76.8 $\pm$ 3.6			43.9 $\pm$ 2.9	2.54 $\pm$ 0.15
200 μ M choline	1.62 $\pm$ 0.30	2.60 $\pm$ 0.20	147.6 $\pm$ 9.9	75.7 $\pm$ 3.1				3.36 $\pm$ 0.04

Table 5 Incubation of cytosol with membranes of different lipid compositions

Hepatocytes were prepared as described for Table 4. Cytosolic and membrane fractions were prepared by homogenization of 7 dishes of cells in 2 ml of buffer A (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1.0 mM EDTA, 2.0 mM dithiothreitol, 1.0 mM phenylmethanesulphonyl fluoride, 0.025% NaN₃) with 50 strokes in a tight-fitting Potter–Elvehjem homogenizer. The homogenate was centrifuged at 1000 *g* for 5 min to remove unbroken cells. The supernatant was centrifuged at 350 000 *g* for 15 min to separate cytosolic and membrane fractions. Membranes were suspended in 1 ml of buffer A. Cytosolic samples containing CT activity of 0.33 nmol/min were incubated for 1 h at 37 °C with 0.45 mg of membrane protein from control cells, or from cells first treated with PLC (0.02 unit/ml) for 2 h and then incubated for 2 h with no addition (PLC), or 200 μ M choline, dimethylethanolamine (DME), monomethylethanolamine (MME) or ethylaminoethanol (EAE). Samples were centrifuged at 350 000 *g* for 15 min, and CT activity was measured in the supernatant. The results are presented as the amount of CT activity remaining in the supernatant. Each value is the average of two measurements. This experiment was repeated twice with similar results.

Membranes prepared from cells	CT activity remaining in supernatant (nmol/min)
(a) Cells treated with:	
Control	0.33
PLC	0.09
(b) Cells first treated with PLC and then incubated with:	
Choline	0.21
DME	0.17
MME	0.1
EAE	0.1

hepatocytes first treated with PLC, then incubated for 2 h with one of the following: no addition, 200 μ M choline, 200 μ M dimethylethanolamine, 200 μ M monomethylethanolamine or 200 μ M ethylaminoethanol (for 2 h as described in the legend to Table 5). These treatments provided membranes containing different lipid composition (Table 4). Cytosol from control cells was incubated with different membranes for 1 h at 37 °C, followed by centrifugation at 350 000 *g* for 15 min to separate supernatants and pellets. CT activity was measured in the supernatant. The results are presented in Table 5 as the amount of CT that did not translocate to membranes and was recovered in the supernatant.

In control incubations there was negligible translocation of

CT to membranes. In contrast, 73 % of the cytosolic CT activity was translocated to membranes in PLC-treated cells. The results of this incubation *in vitro* correlated well with translocation of CT to membranes from PLC-treated intact cells containing decreased concentrations of PC and increased levels of DG (Table 4). When the cytosol was incubated with membranes containing higher levels of PC (PLC, then choline; Table 5) or PDME (PLC, then dimethylethanolamine) and lower levels of DG, the amount of CT translocated to membranes was decreased from 73 % of the total cytosolic activity (PLC) to only 36 % (PLC, then choline) or 48 % (PLC, then dimethylethanolamine). In contrast, in incubations with membranes containing high levels of PMME or phosphatidylethylaminoethanol, there was no significant change in binding of CT compared with the membranes from PLC-treated cells (Table 5). It appears from these results that the lipid composition of membranes plays an important role in the association of CT with membranes and that the ratio of bilayer-forming to hexagonal-H_{II}-phase-forming lipids may control CT translocation to membranes.

DISCUSSION

Previous studies in cells and tissues support the hypothesis

The data presented in this paper are entirely consistent with the conclusion that the ratio of bilayer- to non-bilayer-forming lipids in a membrane is responsible for the distribution of CT between the membrane and cytosolic compartments. Moreover, this hypothesis appears to fit all previous reports in which lipid modifications have altered CT translocation in intact cells.

Kent and co-workers have studied the translocation of CT to membranes in cultured cells treated with PLC [13–15]. The results reported herein are in agreement with their studies. In addition, we show that a decrease in the content of bilayer-forming PC and an increase in non-bilayer-forming DG correlate with CT translocation from cytosol to membranes. We have decreased PC in hepatocytes by another approach (choline deficiency) [11], which caused translocation of CT to membranes. This was reversed when the levels of PC were restored by supplementation of the cells with choline, lyso-PC or methionine (which stimulated the conversion of PE into PC). CT also translocated from membranes to cytosol when the cells were supplemented with dimethylethanolamine, but not with ethanolamine or monomethylethanolamine [12]. Interestingly, the

Table 6 Correlations in intact cells between CT translocation and the ratio of bilayer- to non-bilayer-forming lipids and charged to uncharged lipids

Abbreviations specific to this Table are: def., deficiency; DME, dimethylethanolamine; MME, monomethylethanolamine.

Treatment	Changes in lipid content	CT on membrane	Bilayer/non-bilayer ratio	Charged/uncharged ratio	Reference
PLC	PC↓ DG↑	↑	↓	↑	This paper
Choline def.	PC↓	↑	↓	↑	[27]
3-Deaza-adenosine	PE↑	↑	↓	↓	[33]
Phorbol ester	DG↑	↑	↓	↓	[9]
Diocanoylglycerol	Diocanoylglycerol↑	↑	↓	↓	[9,10,19]
Oleate	Oleate↑	↑	↓	↑	[4-7]
Oleoyl alcohol	Oleoyl alcohol↑	↑	↓	↓	[7]
Choline def.					
+ choline	PC↑	↓	↑	↓	[11,12]
+ DME	PDME↑	↓	↑	↓	[12]
+ MME	PMME↑	—	↓*	↓	[12]

* Although there is a decrease in the bilayer to non-bilayer ratio, there is no further binding of CT to membranes, since most of the CT is already bound to membranes (see Table 4).

lipid derived from dimethylethanolamine supplementation is PDME, which is considered to be a bilayer-forming lipid [29], whereas the lipids derived from monomethylethanolamine (PMME) and ethanolamine (PE) prefer the hexagonal H_{II} phase [29]. Similarly, in CHO and LM cells that were deficient in PC, less translocation of CT to membranes occurred when the cells were supplemented with dimethylethanolamine as compared with supplementation with monomethylethanolamine or ethanolamine [16].

PE in hepatocytes is composed mostly of molecular species [30] that are considered to prefer the hexagonal H_{II} phase [28]. PE can be converted into PC via methylation, and this reaction is generally restricted to hepatocytes [31]. When the conversion of PE into PC in hepatocytes was blocked by a specific methylation inhibitor, 3-deaza-adenosine, the ratio of PC to PE decreased from ~ 5 to ~ 3 after 18 h [32]. When rats were injected with 3-deaza-adenosine and microsomes were harvested after 4.5 h, there was a 1.5-fold increase in CT associated with microsomes [33]. The results are consistent with the hypothesis that a decrease in the ratio of bilayer- to non-bilayer-forming lipids could be responsible for the increased association of CT with membranes after treatment with 3-deaza-adenosine.

Fatty acids have been shown to cause translocation of CT from cytosol to membranes in a variety of different types of cells in culture [4-6]. For example, in HeLa cells incubated with 1 mM oleate, virtually all of the cytosolic CT was translocated to the membranes [4], and this was rapidly reversible when the fatty acids were removed by incubation with albumin [7]. Fatty acids form micelles and do not form bilayers *in vitro*. When intercalated into membranes, fatty acids would decrease the ratio of bilayer- to non-bilayer-forming lipids [28]. These results are thus consistent with this ratio of bilayer- to non-bilayer-forming lipids dictating the amount of CT binding to membranes.

DG, by virtue of its conical shape, does not form bilayers [21]. Incubation of cells with DG species [10,19], or endogenous generation of DG by incubation of HeLa cells with phorbol 12-myristate 13-acetate [9], promotes the translocation of CT from cytosol to membranes. These studies also support the hypothesis that the ratio of bilayer- to non-bilayer-forming lipids in a membrane dictates the translocation of CT to membranes in intact cells.

We cannot eliminate the possibility that factors other than the ratio of bilayer- to non-bilayer-forming lipids might be important

for CT binding. For example, CT binding to membranes might be more powerfully influenced by the ratio of PC to DG than by the ratio of bilayer- to non-bilayer-forming lipids. In HeLa cells incubated with phorbol 12-myristate 13-acetate an increase in the level of DG from 2.8 to 6.8 nmol/mg of microsomal protein appears to cause a 3-fold increase in CT binding to membranes [9]. Assuming that the PC concentration in HeLa cells is similar to that in hepatocytes (260 nmol/mg of microsomal protein), the ratio of PC to DG would be decreased from 93 to 38. However, under these circumstances the ratio of bilayer- to non-bilayer-forming lipids would have decreased only slightly. For example, we could assume that the membrane lipid composition of ER in HeLa cells resembles that of ER in rat liver [34]. Secondly, we could assume that under physiological conditions the major bilayer-forming lipids in ER would be sphingomyelin, PC, PS and PI (together the concentration would be 350 nmol/mg of ER protein) and the major non-bilayer-forming lipids in the ER would be PE, DG and cholesterol (together the concentration would be 220 nmol/mg of ER protein) [28]. In such circumstances the ratio of bilayer- to non-bilayer-forming lipids would normally be approx. 1.59 and would decrease to 1.52 when the concentration of DG doubles. Clearly, the ratio of PC to DG has changed much more dramatically than the ratio of bilayer- to non-bilayer-forming lipids.

On the other hand, the studies with choline-deficient hepatocytes indicate that a small change in the ratio of bilayer- to non-bilayer-forming lipids can have a major effect on CT translocation [11]. In these studies the membrane PC decreases by only 15%. When the same assumptions are made about lipid composition and which lipids are bilayer- and non-bilayer-forming lipids, the ratio of bilayer- to non-bilayer-forming lipids changes from 1.59 to 1.41 in the choline-deficient cells. This suggests that a small change in the ratio of bilayer- to non-bilayer-forming lipids may be sufficient to cause translocation.

Although the nature of these studies is correlative, most results over the past decade are consistent with the ratio of bilayer- to non-bilayer-forming lipids being a key factor in modulating the binding of CT to cellular membranes.

Most studies *in vitro* support the hypothesis

In 1979 DG was recognized as a potential modulator of CT binding to lipids [35]. Fatty acids were also identified as potential

activators of CT in extracts from lung microsomes [36]. Fatty acids were shown to stimulate CT translocation from cytosol to microsomes *in vitro* [5,6]. Subsequent studies *in vitro* with cytosol and membranes from HeLa cells demonstrated that translocation of CT to membranes was stimulated by fatty acids, fatty alcohols, monoacylglycerols and DG [7]. A recent study on the binding of pure CT to lipid vesicles identified two features of vesicles that facilitated binding: a high degree of curvature, and induction of the phase transition from gel to liquid-crystalline [37]. They also showed activation of CT by lipids with small polar head-groups such as DG [37]. The common feature identified in these studies was a disruption of the packing of the lipid bilayer. The experiments *in vitro* in the present manuscript (Table 4) are in agreement with the above studies, since a change in the ratio of bilayer- to non-bilayer-forming lipids would alter the packing of lipids in the bilayer.

Studies *in vitro* have shown that charged lipids such as phosphatidylglycerol and PI activate CT. Thus PC vesicles are poor activators of CT, whereas inclusion of 9% oleic acid, PI or phosphatidylglycerol in the vesicles promotes the binding and maximal activation of CT [38]. In studies with pure enzyme and Triton micelles, the negative charge of lipids can apparently play a role in the activation of CT [39]. Furthermore, sphingosine and other positively charged lipids antagonize the activation of CT by negatively charged lipids *in vitro* [40]. Phosphatidylglycerol and PI are considered to be bilayer-forming lipids [28]. Thus *in vitro*, factors other than the ratio of bilayer- to non-bilayer-forming lipids can also be important for the binding of CT to lipids. Dependence of the modulation of CT translocation in intact cells on the negative charge of lipids is less certain.

To distinguish between the relative importance of the bilayer-/non-bilayer-forming lipid ratio versus charged/uncharged lipid ratio, we have summarized in Table 6 correlations taken from several different studies. It is apparent that in every instance there is an inverse relationship between the binding of CT to membranes and the ratio of bilayer- to non-bilayer-forming lipids. When the ratio decreases there is enhanced binding of CT to membranes; when the ratio increases, there is a release of CT from membranes. In contrast, the correlation between CT binding to membranes and the ratio of charged/uncharged lipids is variable. In some instances there is increased charge and increased binding (e.g. treatment with PLC or choline deficiency), whereas under other conditions there is increased binding but a decrease in the charged-to-uncharged ratio (treatment with DG, oleoyl alcohol) (Table 6). A correlation between the subcellular distribution of CT and the proportion of charged versus uncharged lipids would be complicated by the presence of charged proteins in the membranes. Thus we conclude that the key factor dictating the binding of CT to membranes is the ratio of bilayer- to non-bilayer-forming lipids in the membrane.

Bilayer-/non-bilayer-forming lipid ratios affect the binding of other proteins to membranes

It appears from the literature that the activities of several membrane bound-enzymes may also be regulated by changes in the ratio of bilayer- to non-bilayer-forming lipids [21]. Of the examples cited, only protein kinase C activity appears to be regulated by translocation of the enzyme between cytosolic and membrane fractions [21]. A membrane-bound PLA_2 is also activated by an increase in the content of hexagonal- H_{II} -phase lipids in membranes [41]. A recent report suggests that the activity of a cytosolic PLA_2 may be regulated by its movement between soluble and particulate fractions [42]. Thus it is possible that several enzymes may share a common mechanism in which

enzyme activity is regulated by changes in the ratio of bilayer- to non-bilayer-forming lipids.

We have noted one other membrane-associated enzyme, dolichyl-phosphomannose synthase, that is activated when the ratio of bilayer- to non-bilayer-forming lipids in a membrane is decreased [43]. Monogalactosyl diacylglycerol, a non-bilayer-forming lipid, activated the synthase, whereas digalactosyl diacylglycerol, a bilayer-forming lipid, did not activate the synthase.

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