Kinetic selectivity of cholinephosphotransferase in mouse liver: the K_m for CDP-choline depends on diacylglycerol structure

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The effects of different 1,2-diacyl-*sn*-glycerols on the kinetic properties of CDP-choline: 1,2-diacylglycerol cholinephosphotransferase (EC 2.7.8.2) from mouse liver microsomes have been studied. Initial-velocity experiments were carried out with various concentrations of several species of diacylglycerol at different fixed concentrations of CDP-choline. Kinetic analysis of these data showed a family of intersecting lines consistent with a sequential kinetic mechanism of catalysis. The K_m and V_{max} values derived from rate data revealed a pronounced effect of diacylglycerol species utilization on the K_m value for CDP-choline. There was a biphasic relationship between diacyl-

glycerol chain length and the K_m for CDP-choline. Substitution of an unsaturated fatty acid in the *sn*-2 position of distearin also dramatically increased the CDP-choline K_m value as well as the V_{max} . 1,2-Dipalmitoyl-*sn*-glycerol was the preferred substrate over other disaturated species, but 1,2-dihexanoyl-*sn*-glycerol could not be utilized. These results demonstrate the kinetic mechanism of *in vitro* catalysis and suggest a regulatory role for CDP-choline concentration in the diacylglycerol species selectivity of cholinephosphotransferase resulting in the *de novo* biosynthesis of different molecular species of phosphatidylcholine.

INTRODUCTION

Phosphatidylcholine (PC) is the most abundant phospholipid in all mammalian tissues. It is also the major lipid in all eukaryote cell membranes. Because of its important structural role, the fatty acyl groups comprising different molecular PC species have been strongly implicated in helping to determine the physicochemical properties of the membrane bilayer [1,2], as well as playing a role in membrane–protein interactions [3,4] and influencing the mobility and conformation of proteins that are associated with the bilayer [5]. Recent evidence also suggests that PC biosynthesis and transfer may have a role in secretion, membrane trafficking and membrane fusion [6,7]. Because its precursor molecules and catabolic products are important second-messenger molecules in cell-surface signal transduction, PC metabolism has recently been suggested as being involved in the transmission of these signals [8–13] and as having a role in oncogenic transformation [14–20].

The enzyme pathway responsible for the *de novo* biosynthesis of PC is the CDP-choline or Kennedy pathway. PC is synthesized by a three-step process involving the phosphorylation of choline (choline kinase; EC 2.7.1.32) to phosphocholine, then addition of a nucleotide [CTP: phosphocholine cytidyltransferase (PCCT); EC 2.7.7.15] to form CDP-choline, and finally condensation of this substrate with a diacylglycerol (DAG) [cholinephosphotransferase (CPT); EC 2.7.8.2] to form PC (reviewed in refs. [21] and [22]).

Different molecular PC species arise primarily either by the ‘retailoring’ of existing PC by the sequential actions of phospholipase A and lyso-PC acyltransferase or by the selection of specific DAG species to be condensed with CDP-choline by CPT. Other processes such as base exchange and transphosphatidyl transfer also contribute to the PC content of membranes. The

individual participation and regulation of each of these processes in determining the final PC species content of various tissues is poorly understood. However, evidence for a selective mechanism comes from studies of the specific content of DAGs compared with PC species content [23]. These studies showed that clear differences in species ratios for these two molecular pools exist. Previous studies of the DAG species selectivity of CPT have shown some differences in kinetic specificity [24–30], but to date no clear picture of the regulation and mechanism of selection has emerged.

On the basis of these considerations, we investigated PC species biosynthesis by CPT from mouse liver and demonstrate the *in vitro* kinetic mechanism of catalysis of the enzyme. A role for CDP-choline concentration in regulation of the biosynthesis of different molecular PC species is proposed and the significance of these findings within the context of other cellular processes is developed.

EXPERIMENTAL

Preparation of microsomes

Microsomes were prepared essentially as described previously [30]. Briefly, livers from female C3H/HeJ mice (Jackson Laboratories, Bar Harbor, ME, U.S.A.) were removed after cervical dislocation and homogenized in 10 times the tissue volume in a buffer containing 250 mM sucrose, 5 mM EDTA and 50 mM Tris/HCl, pH 7.4. The homogenate was centrifuged at 1000 *g* for 25 min to remove unbroken cells, cell debris and nuclei. The 1000 *g* supernatant was centrifuged at 16000 *g* for 40 min and this supernatant was centrifuged at 250000 *g* for 60 min. The microsomal pellet was resuspended in 10 mM Tris/HCl, pH 8.2, containing 250 mM sucrose and adjusted to 1 mg of protein/ml as measured by the Bio-Rad protein assay (Richmond, CA,

Abbreviations used: di-C_{6:0}, 1,2-dihexanoyl-*sn*-glycerol; di-C_{20:0}, 1,2-diarachidonoyl-*sn*-glycerol; C_{20:4}, arachidonic acid; di-C_{18:0}, 1,2-distearoyl-*sn*-glycerol; di-C_{16:0}, 1,2-dipalmitoyl-*sn*-glycerol; di-C_{18:1}, 1,2-dioleoyl-*sn*-glycerol; di-C_{14:0}, 1,2-dimyrystoyl-*sn*-glycerol; PC, phosphatidylcholine; PCCT, CTP:phosphocholine cytidyltransferase; DAG, diacylglycerol; CPT, cholinephosphotransferase; PG, phosphatidylglycerol; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol.

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U.S.A.), with BSA as the standard. The microsomal suspension was stored at -20°C and used within 4 weeks, although it could be stored for up to 6 weeks without loss of activity.

Preparation of DAG substrates

DAGs (Sigma, St. Louis, MO, U.S.A.) were used as purchased for the preparation of the substrates which was essentially by the methods of Miller and Weinhold [30]. (All DAGs contained less than 2% of the 1,3-isomer.) Briefly, the DAGs and phosphatidylglycerol (PG) were dissolved in chloroform at 5 mg/ml, and an appropriate volume of each was combined and evaporated to dryness under a stream of N_2 . Then 167 mM Tris/HCl, pH 8.2, and Tween-20 were added to 1 ml and the mixture was sonicated on maximum for 10 min at 65°C with a Fisher model 330 sonic dismembrator equipped with a microtip. The final proportions during sonication were 3.33 mM DAG/2.7 mM PG/0.33 mg/ml Tween-20. (The concentrations of DAG solutions listed assume their dissolution under ideal conditions; however, the true physical form of these suspensions is unknown.) In some cases the substrate mixture was sonicated at different proportions of DAG, and in others the mixture was sonicated without DAG. All mixtures were prepared immediately before use.

CPT assay

Assay conditions were based on those previously described [30]. Briefly, each reaction mixture contained 0.15 ml of various dilutions of the DAG mixture, 0.15 ml of 167 mM Tris/HCl, pH 8.2, 0.05 ml of microsomal suspension and various dilutions of CDP-[methyl- ^{14}C]choline (NEN Research Products, Boston, MA, U.S.A.; 150 d.p.m./nmol) added in 0.05 ml. The final reaction mixture contained 100 mM Tris/HCl, pH 8.2, 0.01–2.0 mM CDP-choline, 0.015–1.0 mM DAG, 5 mM EDTA, 5 mM dithiothreitol, 25 mM MgCl_2 , 0.8 mM PG, 0.1 mg/ml Tween-20 and 50 μg of microsomal protein in a final volume of 0.5 ml. The reaction was started by the addition of microsomes and incubated at 37°C for 30 min. The reaction was stopped by the addition of 3 ml of chloroform/methanol (1:2, v/v). This was followed by the addition of 1 ml of chloroform, after which the reaction mixture was mixed vigorously. The chloroform phase was partitioned by addition of 1 ml of 1% KCl. It was then washed three times with 2 ml of 1% KCl, and 1 ml of the final chloroform layer was evaporated to dryness in a liquid-scintillation vial. Then 3 ml of Beckman (Fullerton, CA, U.S.A.) Ready Protein + liquid-scintillation cocktail was added and the sample was counted on a Beckman LS6000IC liquid-scintillation counter at an efficiency of 90%. Radioactive counts were converted into nmol of PC formed and expressed as nmol/min per mg of microsomal protein (initial velocity; v). The reaction rate was constant for at least 40 min and linearly proportional to microsomal protein at least up to 100 μg (results not shown).

Characterization of the reaction products

More than 95% of the radioactivity found in the chloroform phase co-migrated with authentic PC when analysed by two-dimensional t.l.c. on 20 cm $\times$ 20 cm silica-gel 60A plates (Whatman International Ltd., Maidstone, Kent, U.K.) with chloroform/methanol/ammonium hydroxide (65:25:4, by vol.) as solvent in the first dimension and butan-1-ol/acetic acid/water (6:1:1, by vol.) as solvent in the second dimension.

Data analysis

Initial-velocity (v) measurements were analysed by the computer-driven non-linear enzyme-kinetics curve-fitting program Enz-

fitter [31]. K_m and $V_{\max}$ values as well as standard errors were calculated directly from v measurements. Constants derived from secondary plots of these data were analysed by least-squares analysis. All regression coefficients were greater than 0.9. Steady-state kinetics were calculated and described in the nomenclature of Cleland [32]. All calculations were based on the most general mathematical approach with the fewest assumptions (i.e. ordered addition of substrates and release of products). Under these conditions the rate equation describing this reaction is as follows:

$$v = \frac{V_{\max} [A][B]}{K_d^A K_m^B + K_m^B [A] + K_m^A [B] + [A][B]}$$

where [A] and [B] represent the concentration of each substrate. All data presented are the result of a series of experiments using the same microsomal preparation. Different assay conditions were sometimes repeated three times with different microsomal preparations. The maximum variation in initial rate with different microsomal preparations was 15%.

RESULTS

Initial-velocity studies of kinetic mechanism

Microsomal CPT activity from mouse liver was studied using exogenously added substrates. Activity resulting from only the

Figure 1 Double-reciprocal plots of initial-rate data for mouse liver CPT activity

Mouse liver microsomes were incubated in the presence of various fixed concentrations of CDP-[methyl- ^{14}C]choline and selected DAGs as described in the Experimental section. Labelled PC was extracted, quantified and expressed as initial velocity (v). (a) 1,2-Dioleoyl-*sn*-glycerol; (b) 1-oleoyl-2-acetyl-*sn*-glycerol; (c) 1,2-dipalmitoyl-*sn*-glycerol.

Figure 2 Double-reciprocal replots of initial-rate data shown in Figure 1

CPT activity is expressed as initial rate (v) at different fixed concentrations of DAG (see Figure 1). (a) 1,2-Dioleoyl-*sn*-glycerol; (b) 1-oleoyl-2-acetyl-*sn*-glycerol; (c) 1,2-dipalmitoyl-*sn*-glycerol.

addition of the CDP-choline substrate (1.0 mM) was less than 0.03 nmol of PC formed/min per mg of microsomal protein (initial velocity; v) for three independent microsomal preparations. This representative activity due to endogenous DAG and was

Table 1 Steady-state kinetic constants of mouse liver CPT for selected substrates

Mouse liver microsomes were incubated in the presence of CDP-[methyl- 14 C]choline and DAG as described in the Experimental section. Labelled PC was extracted and quantified to obtain initial-rate data from which the kinetic constants ($\pm$ S.E.M.) were mathematically derived.

DAG	K_d (μ M)	K_m (μ M)		V_{max} (nmol/min per mg)
		DAG	CDP-choline	
Di-C _{6:0}				0
Di-C _{8:0}	67 $\pm$ 6	189 $\pm$ 21	889 $\pm$ 95	2.57 $\pm$ 0.51
Di-C _{12:0}	14 $\pm$ 2	219 $\pm$ 35	297 $\pm$ 17	0.91 $\pm$ 0.08
Di-C _{14:0}	46 $\pm$ 4	84 $\pm$ 5	230 $\pm$ 11	2.99 $\pm$ 0.14
Di-C _{16:0}	79 $\pm$ 7	86 $\pm$ 6	41 $\pm$ 2	2.36 $\pm$ 0.05
Di-C _{18:0}	31 $\pm$ 2	45 $\pm$ 8	70 $\pm$ 2	0.59 $\pm$ 0.06
Di-C _{20:0}	80 $\pm$ 6	97 $\pm$ 8	400 $\pm$ 36	0.40 $\pm$ 0.05
Di-C _{18:1(cis-9)}	21 $\pm$ 1	1680 $\pm$ 39	1000 $\pm$ 141	11.8 $\pm$ 0.08
1-C _{18:0} -2-C _{20:4}	29 $\pm$ 2	1280 $\pm$ 36	650 $\pm$ 33	25.0 $\pm$ 2.2
OAG	402 $\pm$ 72	3900 $\pm$ 488	746 $\pm$ 72	20.0 $\pm$ 1.2

considered negligible. The reaction was therefore completely dependent on the presence of exogenously added DAG. If the substrate was prepared in the absence of DAG (PG and Tween-20 only), v was again less than 0.03. When exogenous 1,2-dipalmitoyl-*sn*-glycerol was added to the reaction mixture, the incorporation of CDP-[methyl- 14 C]choline into the chloroform-phase-soluble material increased dramatically. When the DAG concentration was varied at different fixed CDP-choline concentrations, a family of v measurements was obtained that gave rise to linear double-reciprocal plots. Figure 1 shows the kinetic analysis of data obtained as outlined above for three different DAG substrates. The same data when plotted with CDP-choline as the variable substrate at different fixed DAG concentrations were also linear (Figure 2). The data plotted in Figures 1 and 2 indicate that the slopes and intercepts of the Lineweaver-Burk plots vary as a function of the concentration of the non-varied substrate. This is consistent with the hypothesis that the enzyme reaction sequence is Bi-Bi sequential [33].

Figure 3 Secondary plots of initial-rate data shown in Figure 1

μ -intercepts and slopes are replotted as a function of CDP-choline concentration. Michaelis constants for DAG (K_m^A), CDP-choline (K_m^B), V_{max} , and DAG dissociation constant (K_d^A) were mathematically calculated from slopes and intercepts of these plots as previously described [33]. (a) 1,2-Dioleoyl-*sn*-glycerol; (b) 1-oleoyl-2-acetyl-*sn*-glycerol; (c) 1,2-dipalmitoyl-*sn*-glycerol.

The effect of preparing the DAG substrate at different DAG/PG/Tween-20 proportions was also studied. When the mixture was sonicated with different proportions of a disaturated (di-C_{18:0}) or a diunsaturated (di-C_{18:1}) DAG and assayed, Lineweaver-Burk plots were still linear and the calculated kinetic constants were similar to those already obtained. This demonstrates that the reaction velocity was dependent on the amount of exogenously added DAG present in the reaction mixture until saturation and that the kinetics of this system can be approximated by the Michaelis-Menten model.

The DAG concentrations used resulted in v values from lower first-order to saturation. Michaelis constants and maximum velocities were obtained by analysis of the secondary plots shown in Figure 3. The values for these three representative, as well as those for additional, substrates can be found in Table 1 and will be discussed in detail below, but when calculations were based on rapid-equilibrium (apparent) kinetics at a single subsaturating CDP-choline concentration they were in the same range as those reported earlier from different tissues when defined DAGs were utilized [26,27,34].

DAG specificity of CPT

As previously stated, the secondary plots of intercept and slope from initial-velocity data were linear and allowed calculation of true K_m and V_{max} values. These data are presented in Table 1. As noted, di-C_{6:0} could not be utilized by CPT as a substrate in this system. This is at variance with that reported by Coleman and Bell [26]. However, the activity due to di-C_{6:0} was among the lowest that they observed. Values for K_d for all DAGs tested ranged from 14 to 80 μ M except for 1-oleoyl-2-acetyl-*sn*-glycerol (OAG), a strong activator of protein kinase C. There appeared to be no clear relationship between chain length or degree of unsaturation and DAG K_d . There were, however, obvious differences in V_{max} and DAG K_m values for the different DAGs, with di-C_{20:0} having the lowest V_{max} and a mixed DAG containing *sn*-2-C_{20:4} the highest; di-C_{18:0} had the lowest DAG K_m and OAG the highest. There also appeared to be a relationship between increasing unsaturation and higher V_{max} and DAG K_m values.

The most salient result was the observation of different K_m values for CDP-choline when different DAG substrates were utilized. The difference was as much as 24 times when di-C_{16:0} and di-C_{18:1} were compared. As with V_{max} and DAG K_m , there appeared to be a relationship between CDP-choline K_m and chain length and degree of unsaturation, but it was much more pronounced. Di-C_{14:0}, di-C_{16:0} and di-C_{18:0} appeared to have the preferred chain lengths with the lowest K_m for CDP-choline, which increased as the length became longer or shorter. The addition of a *cis*-double bound at carbon number 9 of di-C_{18:0} (i.e. di-C_{18:1}) resulted in a more than 14-fold increase in K_m for CDP-choline. Also, exchanging arachidonic acid for stearic acid (i.e. 1-C_{18:0}-2-C_{20:4}) resulted in an increase of nearly one order of magnitude in CDP-choline K_m .

The ratio of V_{max}/K_m has been used to relate overall enzyme specificity for a substrate [35]. Table 2 shows the relationship between apparent specificity ($V_{max}/\text{CDP-choline } K_m$) and DAG chain length and unsaturation. There was a biphasic relationship between chain length and overall specificity, with a C₁₆ chain being preferred. Also, increasing the unsaturation at the *sn*-2-position increased the apparent substrate specificity. The $V_{max}/\text{DAG } K_m$ ratio, however, did not show any clear relationship to DAG substrate structure, whereas $V_{max}/\text{DAG } K_d$ showed very high specificities for di-C_{18:1} and 1-C_{18:0}-2-C_{20:4} (0.56 and 0.86 respectively).

Table 2 Effect of DAG chain length and unsaturation on substrate specificity of CPT

Substrate specificity is expressed as the ratio of V_{max} divided by K_m for CDP-choline when different DAGs are utilized. The number of carbons per fatty acid chain was 6–20. The saturated fatty acid in the *sn*-2 position of di-C_{18:0} is also exchanged for a monoenoic species (*cis*-9) or a tetraenoic species (arachidonic acid).

DAG	$10^3 \times (V_{max}/K_m)$
Di-C _{6:0}	0
Di-C _{8:0}	2.9
Di-C _{12:0}	3.1
Di-C _{14:0}	13.0
Di-C _{16:0}	57.6
Di-C _{18:0}	8.4
Di-C _{20:0}	1.0
Di-C _{18:1}	11.8
1-C _{18:0} -2-C _{20:4}	38.5

These data indicate that, at least in this microsomal system, the concentration of CDP-choline has a very pronounced effect on the utilization of different substrate species.

DISCUSSION

Previous studies of the DAG species specificity of CPT have been largely carried out with near-saturating CDP-choline concentrations (0.5–1.0 mM) and by calculation of 'apparent' kinetics at a single subsaturating CDP-choline concentration. By using these conditions some comparative differences in DAG species selectivity have been observed. However, no concise examination of the *in vitro* kinetic mechanism using different DAGs at various CDP-choline concentrations has been performed. Usually, highly purified enzyme preparations are used for characterization of enzyme kinetics, but previous attempts to purify native CPT have been hampered by the apparent requirement for a membrane bilayer for activity [34,36]. Hence most kinetic analyses have been performed using microsomal preparations. In addition, the interpretations of absolute enzyme specificities using insoluble substrates (i.e. DAG) should be considered with caution because of the unknown physical state of these reactants and the nature of presentation to the enzyme (i.e. micellar, membrane-bound, etc.). However, the biphasic response to substrate chain length (Tables 1 and 2) strongly argues against the differences in specificity being solely due to differing solubility or micellar concentration. Nonetheless, information from the *in vitro* kinetics may suggest important inferences about *in vivo* catalysis. We have therefore conducted initial-velocity studies of CPT from mouse liver using different DAG species at various fixed CDP-choline concentrations.

When the data were analysed by the method of Lineweaver and Burk [37], a family of intersecting lines was obtained for each DAG species tested. This pattern is characteristic of a Bi-Bi sequential mechanism of catalysis where both substrates must be present on the enzyme before release of any products. Pontoni et al. [38] also observed evidence of a sequential mechanism for CPT using a mixed DAG preparation. One product of the reaction, CMP, was inhibitory in this system, but the other product, PC, was not inhibitory at any concentration tested (results not shown). Because of this, it may be difficult to differentiate conclusively between a steady-state compulsory-order ternary-complex mechanism and a rapid-equilibrium random-order ternary-complex mechanism [33,39]. However, because of the differences in K_m for CDP-choline when different

DAGs were utilized, a logical choice is the compulsory-ordered system which allows the hypothesis that the DAG binds first, and, depending on the species structure, either the conformation of the active site is slightly modified or the orientation of presentation of the DAG to the CDP-choline is different enough to affect the rate of catalysis, which is observed as different K_m values for CDP-choline. Although isotope-exchange studies should be able to differentiate between the two mechanisms conclusively, the elucidation of binding order (if any) or reaction mechanism is not critical to the demonstration of a relationship between CDP-choline concentration and different DAG species utilization *in vitro*.

The observation of different K_m values for CDP-choline when different DAG species are utilized suggests important aspects of regulation of PC species biosynthesis. Although choline kinase and PCCT have both been implicated in the regulation of PC biosynthesis [10], a large body of evidence has accumulated that suggests that PCCT may contribute significantly to the control of flux through the Kennedy pathway [21]. Hence the formation of CDP-choline appears to be not only rate-limiting for overall PC synthesis, but, considering the notions advanced in this paper, may also participate in the regulation of biosynthesis of different molecular species of PC as well.

It could be argued that the reported cytosolic concentration of CDP-choline [40] is too low and varies by too little to affect the selection of DAG by CPT. However, evidence presented by George et al. [41,42] strongly suggests that there is channelling of intermediates in the CDP-choline pathway. As Vance [43] points out, the localized concentration of intermediates, especially CDP-choline, may be much higher than previously thought, and so the notion that the PCCT forward reaction is essentially irreversible *in vivo* should be reconsidered. In this vein, it is interesting to note that the K_m for CDP-choline of the PCCT reaction (reverse direction) is reported to be 640 μ M [44], which is in the same range of CDP-choline K_m values reported here for the CPT forward direction. This concentration is much higher than the reported cytosolic concentration and is the reason why the PCCT reaction was assumed to be irreversible *in vivo*. However, if CDP-choline is compartmentalized as George's data suggest, this concentration range could be high enough to effect the selection of DAG species by CPT.

These findings may also help explain the non-linear relationship of CPT activity and CDP-choline concentrations previously reported [45]. These studies used a mixed DAG preparation from soy beans, which contained both saturated and unsaturated species. When the concentration of CDP-choline was increased above 280 μ M, double-reciprocal plots of initial-velocity data became concave downward. The authors suggested negative co-operativity or the presence of two or more enzyme isoforms as being responsible. However, in the light of our data, it is possible that the earlier observation was due to substrate species 'switching' to DAG species with higher V_{max} and CDP-choline K_m values (i.e. diolein).

It is intriguing to speculate on the potential linkage of our findings to mitogenic signal transduction. Mitogenic stimulation of several cell lines and tissues has been shown to increase intracellular CDP-choline concentrations [9–11]. These observations have been linked to increased PC turnover and synthesis. PC catabolic products (i.e. DAGs) have recently received much attention due to their proposed involvement in mitogenic signal transduction (i.e. activation of protein kinase C).

It is especially interesting to note that the data reported here predict that, as the intracellular (localized) CDP-choline concentration increases, the PC synthesized via the Kennedy pathway in mouse liver may contain altered species ratios. DAG generated

from these PC species may have different downstream effects from those otherwise generated from PC. This could have profound effects on PC-derived DAG signals in mouse liver cells. Although these data implicate CDP-choline concentration in DAG species selection *in vitro*, further studies with more appropriate DAG species (i.e. 1-C_{18:0}-2-C_{18:1}, etc.) are required before any potential relationship to normal PC species distribution *in vivo* can be advanced. Also, 1,2-DAG can be utilized by the ethanolamine phosphotransferase, and since PC and phosphatidylethanolamine have different species ratios, it will be of interest to determine if similar selective mechanisms are involved in this enzyme pathway as well.

These observations may lend new insights into the nature of regulation of PC species biosynthesis, and for the first time provide a potential mechanism of regulated DAG selection by CPT that may be linked to the mitogenic state of the cell.

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