Volume 214, number 1, 149–152 FEB 04587 April 1987

De novo CDP-choline-dependent glycerophosphorylcholine synthesis and its involvement as an intermediate in phosphatidylcholine synthesis

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Received 30 January 1987

The activity of CDP-choline-dependent glycerophosphorylcholine synthetase (CDP-choline:*sn*-3-glycerophosphate cholinetransferase), a newly discovered enzyme involved in the recently proposed pathways of acyl-specific phosphatidylcholine synthesis, is reported in rat liver. Endogenous CDP-choline, synthesized via the CMP-driven back reaction of phosphorylcholine transferase, is also shown to be a choline donor for this glycerophosphorylcholine synthetase. The function of glycerophosphorylcholine as an intermediate in phosphatidylcholine synthesis is demonstrated by specific isotope trapping whereby unlabelled glycerophosphorylcholine inhibited label incorporation from *sn*-[^{14}C]glycerol-3-phosphate into phosphatidylcholine in mouse gastrocnemius, a tissue that is essentially devoid of the cytidine pathway for phosphatidylcholine synthesis and uses a non-allelic glycerophosphorylcholine synthetase (exogenous PC:*sn*-3-glycerophosphate cholinetransferase) in the synthesis of glycerophosphorylcholine.

Glycerophosphorylcholine; Phosphatidylcholine; CDP-choline; Phosphatidylethanolamine; (Muscle)

1. INTRODUCTION

New biosynthetic pathways for acyl-specific phospholipids have been postulated [1]. In these new sequences, de novo synthesized glycerophosphodiesters are proposed to be intermediates in the synthesis of acyl-specific PC, PE, PG, PI and PS. A GPC-dependent pathway of PC synthesis has also been proposed to operate in the synthesis of di-C16:0-PC, the principal phospholipid component of lung surfactant, and in the synthesis of highly unsaturated PC in other tissues, such as brain, muscle and spermatozoa [1,2]. Two non-allelic GPC synthetases have been postulated to occur in various tissues [1]. In tissues which have an active cytidine pathway for PC synthesis (such as liver, lung and brain) GPC has been proposed to be synthesized by the transfer of choline from CDP-choline to *sn*-glycerol-3-phosphate (CDP-choline:*sn*-glycerol-3-phosphate cholinetransferase). In tissues such as fully differentiated skeletal muscle, in which the cytidine pathway for PC synthesis is essentially absent [3], exogenous PC has been proposed as a choline donor for *sn*-3-glycerophosphate to form GPC (i.e. exogenous PC:*sn*-3-glycerophosphate cholinetransferase). Experimental evidence of de novo GPC and GPE synthesis has been obtained in mouse brain, liver [3] and muscle [3,4]. In addition, evidence for a proposed primary defect in GPC synthetase in Duchenne and murine *dy* muscular dystrophies [5] has been obtained in the mouse [3,4].

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Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; G3P, *sn*-glycerol-3-phosphate; GPC, *sn*-glycerol-3-phosphorylcholine; C22:6n–3, all-*cis*-4,7,10,13,16,19-docosahexaenoate

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

This paper reports for the first time de novo GPC synthesis via the CDP-choline:*sn*-glycerol-3-phosphate cholinetransferase, using rat liver, and the involvement of GPC as an intermediate in the synthesis of PC, in mouse gastrocnemius.

2. MATERIALS AND METHODS

2.1. *Animals*

Adult male (3–4 months old) Sprague-Dawley rats were obtained from Blue Spruce Farms (Altamont, NY) as weanlings and fed Purina rat chow ad libitum until use. Adult male (2–3 months old) C3H/HeN mice were obtained from a breeding colony originating from the National Institutes of Health. The Center for Research Animal resources' guide for the care and use of laboratory animals of Cornell University was followed.

2.2. *Chemicals*

ATP, UTP, CMP, CDP-choline, L-carnitine, CoA-SH, DL-α-tocopherol, palmitoyl-CoA, and all-*cis*-4,7,10,13,16,19-docosahexaenoic acid were obtained from Sigma (St. Louis, MO). $MgCl_2$ (1.0 M solution) was obtained from Fisher Scientific (Fair Lawn, NJ). [U-^{14}C]Glycerol-3-phosphate (140 mCi/mmol) and cytidine-5'-diphospho[*Me*-^{14}C]choline (49 mCi/mmol) were obtained from New England Nuclear (Boston, MA). All common chemicals were of reagent grade or of the highest purity available.

2.3. *Homogenate preparation*

Animals were killed by cervical dislocation; livers and muscle were immediately excised, blotted and homogenized in 5 vols of ice-cold 0.25 M sucrose. Post-nuclear and connective tissue-free supernatants were obtained by centrifugation at 600 × *g* for 10 min, at 4°C.

2.4. *Enzyme assays*

The complete assay medium for incorporation of *sn*-[^{14}C]glycerol-3-phosphate into GPC contained: 0.54 mM *sn*-[U-^{14}C]glycerol-3-phosphate (2.6 mCi/mmol), 3.0 mM $MgUTP^{2-}$, 1.0 mM CDP-choline, 2.3 mM free Mg^{2+} and 80 mM Hepes, pH 7.0, with 3.1–3.5 mg of homogenate protein. In assay protocols where CMP substituted for CDP-choline, the substitution was equimolar. The complete assay medium for CDP-[^{14}C]choline incorporation into GPC was identical to that indicated above for *sn*-[^{14}C]glycerol-3-phosphate incorporation except that unlabeled *sn*-glycerol-3-phosphate replaced its labeled counterpart and CDP-[*Me*-^{14}C]choline (3.8 mCi/mmol) replaced its unlabeled counterpart. Initial velocities were obtained with 15–30 min incubations at 37°C. Reaction rates were first order with respect to protein concentrations at the above protein levels. Other assay procedures have been described [3,4]. Incorporation of *sn*-[^{14}C]glycerol-3-phosphate into PC and PE was assayed in a medium containing: 0.54 mM [U-^{14}C]glycerol-3-phosphate (2.6 mCi/mmol), 2.3 mM free Mg^{2+}, 3.0 mM $MgUTP^{2-}$, 1.3 mM L-carnitine, 0.030 mM DL-α-tocopherol, 0.040 mM C22:6*n*–3, 0.030 mM palmitoyl-CoA, 0.40 mM CoASH, 80 mM Hepes, pH 7.0 and 1.3–1.7 mg homogenate protein; assay media were sonicated before addition of homogenate. Some assay protocols included 1.0 mM GPC or CDP-choline. Incubations were at 37°C for 30 min. Reactions were stopped with 1.1 ml of 2:1 (v/v) chloroform-methanol containing 0.05% (w/v) butylated hydroxytoluene: phospholipids were subsequently extracted in the lower phase of the resulting Folch [6] solvent system. All assays were carried out in a volume of 0.375 ml. $MgUTP^{2-}$ was included in the assay protocols as an alternative substrate for competing endogenous nucleotidases (EC 3.1.3.5) and other phosphoryl-hydrolases; these hydrolytic activities have previously been detected in muscle and other tissues [3,11,12].

2.5. *Analytical*

GP and GPC were resolved by ion-exchange paper chromatography as previously described [4]. The same system was used to resolve CDP-choline, GPC and choline: R_f values were 0.30, 0.58 and 0.85, respectively. Identification of choline and the various phosphoryl-substituted compounds has been described [3]. PC and PE were resolved by silica gel TLC [7]; spots were scraped for radioactivity determination. Radioactivity was measured by liquid scintillation spectrometry as described before [3].

2.6. *Miscellaneous*

Concentrations of various ligands and Mg species were calculated from solutions of the cor-

responding multiple equilibria equations as indicated before [4]. Protein was determined by the Folin phenol reagent [8] with crystalline bovine serum albumin as a standard. Statistical analysis was performed with a computerized version (MINITAB, Statistics Dept, Pennsylvania State University) of the *t*-test [9] and analysis of variance [10].

3. RESULTS AND DISCUSSION

The requirements for GPC synthesis in liver as measured by the *sn*-[^{14}C]glycerol-3-phosphate assay are shown in table 1. The enzyme shows a strong dependence on exogenous CDP-choline, since in its absence the rate of GPC synthesis is about half that of the complete assay. However, the sizable activity without an exogenous choline donor suggests that the homogenate has a significant concentration of CDP-choline or that it can be generated from endogenous sources. The observation that CMP can substitute for CDP-choline is consistent with the proposed role of the reverse reaction of the CDP-choline:1,2-diacylglycerol cholinephosphotransferase (EC 2.7.8.2) in supplying CDP-choline for the GPC-dependent pathway of PC synthesis [1]. This reaction, operating with endogenous PC and CMP, may account for the substantial rate of GPC synthesis in the absence of exogenous CDP-choline as observed above. At the same time, the increased synthesis of GPC by CMP (with respect to the rate in the absence of CDP-choline) rules out the possibility that GPC was being made from PC synthesized by the cytidine pathway, since CMP as a product of the last step of this pathway (i.e. catalyzed by the phosphorylcholine transferase) would inhibit the synthesis of PC via this pathway, as observed by others [13,14]. The direct synthesis of GPC from CDP-choline and G3P is also substantiated by the observed initial velocity condition in these assays; otherwise, a catabolic origin of GPC would produce a long lag period in the appearance of label in GPC, since there are six intermediate steps between G3P and GPC if this glycerophosphodiester was being synthesized via the cytidine pathway followed by PC catabolism.

Table 1

CDP-choline:*sn*-glycerol-3-phosphate cholinetransferase activity in rat liver

Labeled substrate	nmol GPC/mg protein per min[a]
Complete *sn*-[^{14}C]glycerol-3-phosphate assay	0.352 ± 0.025
− CDP-choline	0.185 ± 0.027
− CDP-choline, + CMP	0.301 ± 0.020
Complete CDP-[^{14}C]choline assay	0.263 ± 0.018
− *sn*-glycerol-3-phosphate	0.031 ± 0.005

[a] Significant differences: 0.352 > 0.185 at $P < 0.0001$; 0.301 > 0.185 at $P = 0.0003$; 0.352 > 0.301 at $P = 0.02$; 0.263 > 0.031 at $P < 0.0001$; 0.352 > 0.263 at $P = 0.0013$

Assay conditions as indicated in section 2 ($n = 4$)

The rate of GPC synthesis as measured by the CDP-[^{14}C]choline incorporation assay is also indicated in table 1. This biosynthetic rate is somewhat less than from *sn*-[^{14}C]glycerol-3-phosphate, i.e. 0.263 vs 0.352 units. Since the complete assay protocols provided identical reactant concentrations for both radioassays, observed reaction rates would be expected to be similar according to the postulated stoichiometry [1]. However, this deviation from the predicted stoichiometry in this crude homogenate is expected if there is substantial endogenous synthesis of CDP-choline, which would lead to label dilution; that this is the case is shown by the only partial requirement for exogenous CDP-choline in the synthesis of GPC from labeled G3P (table 1). Purification of this CDP-choline-dependent GPC synthetase will be required to confirm the expected stoichiometry. The strong dependence of GPC synthesis on exogenous G3P is consistent with the previously proposed reaction scheme [1]. However, the residual activity without exogenous G3P suggests that the homogenate contains a small but detectable endogenous G3P concentration.

The experiment for which data are shown in table 2 was designed to test the previous proposition that GPC is indeed an intermediate in the synthesis of PC [1]. The entry of labeled G3P into PC via the classic cytidine pathway should be precluded in this experiment since fully differentiated mouse skeletal muscle is essentially devoid of the enzymes of this cytidine pathway [3,4]. These data

show that *sn*-[^{14}C]glycerol-3-phosphate is incorporated into muscle PC and PE in an assay medium containing acyl donors. Addition of exogenous unlabeled GPC produced a significant and specific inhibition (73%) of label incorporation into PC, whereas label incorporation into PE remained unchanged. These data indicate that GPC acts as a specific isotope trap for labeled G3P incorporation into PC, suggesting that GPC is a required intermediate in the synthesis of PC from G3P in this tissue. The lack of inhibition of labeled G3P incorporation into PE precludes the effect of GPC as a source of unlabeled G3P via a residual phosphodiesterase activity. To confirm the functional absence of the cytidine pathway for PC synthesis, the same experiment was repeated with the addition of 1.0 mM CDP-choline. The presence of CDP-choline did not increase the rate of PC synthesis from labeled G3P when muscle homogenates were used for this assay (table 2). These data confirm the previous conclusion that skeletal muscle PC is not synthesized via the cytidine pathway in the mouse [3,4], and shows that G3P is a common substrate for both the cytidine and glycerophosphodiester-dependent pathways of phospholipid synthesis.

The proposed CDP-choline-dependent synthesis of PC [1], via a pathway not involving the forward reaction of the phosphorylcholine transferase of the classic cytidine pathway, offers an alternative mechanism for the lack of coupling of CDP-choline and diacylglycerol incorporation into PC observed in liver and other tissues [15–19], other than the suggested compartmentalization of diacylglycerol pools. A high rate of de novo GPC synthesis has been reported in these tissues [3,20], which is consistent with the operation of GPC-dependent PC synthesis.

Table 2

Incorporation of *sn*-[^{14}C]glycerol-3-phosphate into mouse gastrocnemius PC and PE, in the presence or absence of GPC or CDP-choline

Reactants	PC[a]	PE
− GPC	0.088 ± 0.007	0.021 ± 0.004
+ GPC	0.023 ± 0.004	0.020 ± 0.004
+ CDP-choline	0.084 ± 0.009	0.023 ± 0.004

[a] Significant differences: 0.088 > 0.023 at $P < 0.0001$; 0.084 not significantly different from 0.088 at $P = 0.10$

Data in nmol phospholipid/mg protein per min ($n = 4$). Assay conditions as indicated in section 2

ACKNOWLEDGEMENTS

The challenging and helpful discussions with my colleague Virginia Huszagh (who also edited the manuscript) are gratefully acknowledged. My thanks also to Dr W.T. Watson of the National Institutes of Health for the gift of the C3H/HeN mice, and to Joan Tirado for typing the manuscript. This work was supported in part by a grant from the K.O. Scott Research Trust.

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