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Regulation of Cardiolipin Biosynthesis in H9c2 Cardiac Myoblasts by Cytidine 5′-Triphosphate*

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Grant M. Hatch‡ and Grant McClarty

From the Departments of Pharmacology and Therapeutics, Biochemistry and Molecular Biology, Internal Medicine and Medical Microbiology, University of Manitoba, Winnipeg, Manitoba R3E OW3, Canada

The regulation of cardiolipin biosynthesis by CTP in H9c2 cardiac myoblasts was investigated. H9c2 cells were incubated in the presence of cyclopentenylcytosine which is converted to cyclopentenylcytosine-triphosphate, a potent and specific inhibitor of CTP synthetase. Incubation of cells for 12 h with cyclopentenylcytosine reduced the cellular pool size of CTP to less than 10% of control cells but did not influence the pool size of other nucleotides. The *de novo* biosynthesis of phosphatidylcholine from [*methyl*-^{3}H]choline, phosphatidylethanolamine from [1-^{3}H]ethanolamine, and biosynthesis of all glycerol containing phospholipids from [U-^{14}C]glycerol or [1,3-^{3}H]glycerol were reduced approximately 50% after preincubation of the cells with cyclopentenylcytosine. In contrast, radioactive glycerol accumulated in phosphatidic acid, diacylglycerol, and triacylglycerol in cyclopentenylcytosine-treated cells compared with controls suggesting a re-routing of phospholipid biosynthesis away from CTP utilizing reactions toward neutral lipid synthesis. The *de novo* biosynthesis of all phospholipids was restored to control levels by addition of cytidine to the medium which elevated CTP levels. Cyclopentenylcytosine did not affect the *in vitro* enzyme activities involved in cardiolipin biosynthesis in these cells. In addition, the resynthesis of cardiolipin and most phospholipids from [1-^{14}C]linoleic acid was not affected by cyclopentenylcytosine. Our findings indicate that the cellular CTP level may regulate cardiolipin biosynthesis in H9c2 cardiac myoblasts and support the notion that the cellular CTP level may be a universal signal/switch for all phospholipid biosynthesis in eukaryotic cells.

Cardiolipin (CL)[1] was the first polyglycerophospholipid discovered and was isolated from beef heart in 1942 by Mary Pangborn (for comprehensive review see Ref. 1). In mammalian tissues, CL is characteristically associated with the inner mitochondrial membrane (2) and is required for the activity of, or intimately associated with, a number of key mitochondrial enzymes including cytochrome *c* oxidase (3). Recently, the biosynthesis of PG and CL were shown to be essential for cell growth and function of the electron transport chain (4). Regulation of the enzymes and some of the mechanisms that govern CL biosynthesis in bacteria (5, 6) and in yeast (7) have been documented. In mammalian tissues biosynthesis of CL occurs via the CDP-DG pathway. In the first step of this pathway PA and CTP are converted to CDP-DG, catalyzed by PA:CTP cytidylyltransferase (EC 2.7.7.41) (8). The committed step of CL biosynthesis involves the conversion of CDP-DG and glycerol 3-phosphate to PG by sequential action of PGP synthase (EC 2.7.8.5) and PGP phosphatase (EC 3.1.3.27) (8). PGP synthase and PGP phosphatase have been partially purified from mammalian liver mitochondria (9, 10). In the final step of the pathway, PG is then converted to CL via condensation with CDP-DG catalyzed by CL synthase (11). The CL synthase was recently purified to homogeneity from rat liver mitochondria, but the low protein yield did not allow for the production of an antibody (12). The enzyme was localized exclusively to the inner mitochondrial membrane (13, 14). All eukaryotic CL synthases exhibited similar properties in that they were all inhibited by lysophosphatidylglycerol and were insensitive to *N*-ethylmaleimide (15).

In the heart, CL represents a major phospholipid component comprising approximately 8–15% of the entire cardiac phospholipid mass of that organ (1, 16). Recently, the biosynthesis of CL in the isolated perfused rat heart was characterized (17). It was postulated that the rate-limiting step of CL biosynthesis in the rat heart was the conversion of PA to CDP-DG catalyzed by the PA:CTP cytidylyltransferase (17). Reduction in cardiac ATP and CTP level during hypoxic perfusion of rat hearts resulted in a decreased *de novo* biosynthesis of CL from [1,3-^{3}H]glycerol and [^{32}P]orthophosphate (18). However, since ATP is required for many cellular functions, including the amination reaction in CTP production, it could not be directly determined in that study if reduction in the cellular CTP pool was itself limiting for CL biosynthesis.

CTP is required for the biosynthesis of all membrane phospholipids via the provision of CDP-DG (19), CDP-ethanolamine, and CDP-choline (20). In mammalian tissues, CDP-DG provides PA for the biosynthesis of PG, PI, and CL (for review see Refs. 21, 22). CDP-choline and CDP-ethanolamine provide the phosphorylated head groups for PC and PE, respectively (21, 22). In this study, we have solved the problem of specifically reducing cellular CTP levels, but not ATP levels, in H9c2 cardiac myoblasts via incubation of these cells with CPEC. It is well documented that upon addition to cells CPEC is rapidly converted to CPEC-triphosphate which is a specific and potent inhibitor of CTP synthetase (23). We now demonstrate that incubation of H9c2 cardiac myoblasts with CPEC inhibits CL biosynthesis and that the mechanism for this inhibition is a

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‡ Heart and Stroke Foundation of Canada Scholar. To whom correspondence should be addressed: Dept. of Pharmacology and Therapeutics, Faculty of Medicine, University of Manitoba, Rm. 307, Chown Bldg., 770 Bannatyne Ave., Winnipeg, Manitoba R3E OW3, Canada. Tel.: 204-789-3405; Fax: 204-783-6915; E-mail: ghatch@bldghsc.Lan1.umanitoba.ca.

[1] The abbreviations used are: CL, cardiolipin; PG, phosphatidylglycerol; PA, phosphatidic acid; CDP-DG, cytidine-5′-diphosphate-1,2-diacyl-*sn*-glycerol; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; CPEC, cyclopentenylcytosine, PGP, phosphatidylglycerolphosphate.

reduction in CTP level for the CTP utilizing reaction of the CDP-DG pathway of CL biosynthesis, the PA:CTP cytidylyltransferase. In addition, we demonstrate that the biosynthesis of all other phospholipids is inhibited when the cellular CTP pool is reduced and as a result phospholipid biosynthesis is re-routed to the biosynthesis of neutral lipids. The results support the notion that CTP may be a universal signal/switch for all phospholipid biosynthesis in eukaryotic cells.

EXPERIMENTAL PROCEDURES

Materials—[5-^{3}H]Cytidine 5′-triphosphate, [*methyl*-^{3}H]choline, [1-^{3}H]ethanolamine, [1-^{14}C]linoleic acid, [1,3-^{3}H]glycerol, [U-^{14}C-]glycerol 3-phosphate, [2,8-^{3}H]adenine, and [*methyl*-^{3}H]thymidine were obtained from Amersham Corp. or Du Pont Canada Inc., Missasauga, Ontario, Canada. Phosphatidyl[^{14}C]glycerol was synthesized as described previously (17). Thin-layer plates (silica gel 60, 0.25-mm thickness) were obtained from Baxter CanLab, Winnipeg, Manitoba. Ecolite Scintillant was obtained from ICN Biochemicals, Costa Mesa, CA. Fetal bovine serum and Dulbecco's modified Eagle's medium were obtained from Life Technologies, Inc. All other biochemicals were of analytical grade and obtained from either Sigma or Fisher, Winnipeg, Manitoba, Canada or Baxter CanLab, Winnipeg, Manitoba, Canada.

Rat heart H9c2 myoblasts were obtained from the American Type Culture Collection. The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (by volume) fetal bovine serum. Cell cultures were maintained at 37 °C saturated with humidified air, 5% CO_2. Each dish of cells was subcultured at a 1:5 ratio and confluency was usually obtained after 4 days of incubation. Cell viability was assessed by trypan blue exclusion and protein concentration. Treatment of cells with CPEC, CPEC plus cytidine, or cytidine for up 16 h did not affect trypan blue exclusion or the total cellular protein concentration when compared with controls.

Preparation of H9c2 Homogenates and Mitochondrial Fractions—All isolation procedures were performed at 4 °C. Cells were harvested from flasks (1.8×10^7 cells/flask) with 10 ml of medium using glass beads. The suspension was centrifuged at $500 \times g$ for 5 min and the medium removed. For assay of PA:CTP cytidylyltransferase activity and [U-^{14}C]glycerol 3-phosphate and CDP-DG conversion to lipids, a 10% homogenate (by volume) was prepared in 50 mM Tris maleate, pH 8.5, 0.1 M KCl, 10 mM $MgCl_2$, 0.5% Triton X-100, and 10% glycerol. Cells were homogenized with 10 strokes of a tight fitting Dounce homogenizer. The homogenate was used for the above assays. In other experiments a 10% homogenate was prepared in 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, 0.145 M NaCl. Cells were homogenized with 50 strokes of a tight fitting Dounce homogenizer. The homogenate was then centrifuged at $1,000 \times g$ (Sorvall RC-5 Superspeed Refrigerated Centrifuge with SS-34 rotor). The resulting supernatant was centrifuged at $10,000 \times g$ for 15 min. The resulting pellet was resuspended in 1 ml of homogenizing buffer by 15 strokes of a hand-held tissue grinder and used as the source of mitochondrial fraction for assay of CL synthase. For the synthesis of [^{14}C]PG, a 10% homogenate of rat liver (typically 1–3 g) was prepared in 0.25 M sucrose, 0.1 mM EDTA, pH 7.4, using a hand tissue grinder. The homogenate was centrifuged at 2,000 rpm (SS-34 rotor) for 10 min. The resulting supernatant was centrifuged at 6,000 rpm for 10 min and the speed increased to 9,300 rpm for another 2 min. The resulting mitochondrial pellet was washed three times and finally resuspended in 1 ml of the homogenizing buffer. This resuspended mitochondrial fraction was the enzyme source for synthesis of [^{14}C]PG.

Preparation of Phosphatidyl[^{14}C]glycerol—[^{14}C]PG was prepared by a modification of the procedure of Macdonald and McMurray (9). The incubation mixture contained 150 mM Tris-HCl, pH 7.4, 1.0 mM CDP-DG, and 0.5 mM [U-^{14}C]glycerol 3-phosphate (40 μCi/μM), and 10 mg of rat liver mitochondrial protein in a final volume of 0.7 ml. The mixture was incubated for 4.5 h at 37 °C and terminated by the addition of 5.6 ml of chloroform:methanol:HCl (100:100:0.6, v/v/v). This mixture was centrifuged at 2,000 rpm for 10 min to pellet protein and the supernatant transferred to 16 × 100-mm screw cap tubes. 2 ml of chloroform and 0.7 ml of 0.9% NaCl was added to cause phase separation. The mixture was recentrifuged and the aqueous phase removed by suction. The lower phase was washed three times with 5 ml of theoretical upper phase (chloroform:methanol:sodium chloride, 1:45:47, v/v/v) except that the first wash contained 0.1 mM glycerol 3-phosphate. [^{14}C]PG was eluted from the silicic acid column in the 25% fraction of methanol in chloroform. An aliquot of the final column fraction was separated on thin-layer plates using the two-dimensional system described (16) and on plates that had been previously soaked, then air-dried overnight, in 0.4 M oxalic acid and developed in a solvent system containing chloroform:methanol:HCl (87:13:0.2, v/v/v). Using both one-dimensional and two-dimensional thin-layer systems, only [^{14}C]PG was detected and co-migrated with PG markers. Final purification of [^{14}C]PG involved thin-layer chromatography on the oxalate-treated plates with a separate lane for standard PG. The plate was covered with cellophane, except for the standard lane, and stained with iodine vapor. [^{14}C]PG was eluted from the silica gel using the method of Arvidson (24).

Assay of PA:CTP Cytidylyltransferase, Cardiolipin Synthase, and Conversion of Glycerol 3-Phosphate and CDP-DG to Lipids—CL synthase was assayed exactly as described by Schlame and Hostetler (12) except that the assay contained 0.05–0.1 mg of mitochondrial protein; the pH of the assay was 8.5, and the samples were sonicated for 10 s in a Branson model 1200 sonicator prior to incubation. Incubation was at 37 °C for 60 min with [^{14}C]PG (specific activity, 45,000 dpm/nmol). CL synthase activity was linear for up to at least 70 min with 0.1 mg of protein. PA:CTP cytidylyltransferase was assayed by a modification of the method of Carman and Kelley (25). To a 16 × 100-mm test tube (all test tubes were treated with dimethyldichlorosilane, 2% in 1,1,1-trichloroethane) was added in this order, 0.05–0.1 mg of homogenate protein and 0.145 M NaCl to a volume of 60 μl, 10 μl of 0.5 M Tris maleate, pH 6.5 (Tris-buffered with 1.0 M maleic acid solution), 10 μl of 10 mM [5-^{3}H]CTP (specific activity, 12,000 dpm/nmol), 10 μl of 0.15 M Triton X-100, 5 mM PA (prepared by sonicating for 20 min, a weighed aliquot of PA in the Triton solution in a silated 16 × 100-mm tube). The reaction was started by the addition of 10 μl of 0.2 M $MgCl_2 \cdot 6H_2O$. The mixture was incubated at 30 °C for 5 min and terminated by the addition of 0.5 ml of 0.1 M HCl in methanol. Subsequent steps were performed exactly as described (25). Enzyme activity was linear with time up to at least 10 min with 0.1 mg of protein. For conversion of [^{14}C]glycerol 3-phosphate and CDP-DG to lipids the incubation mixture contained enzyme (50–100 μg) and 0.145 M NaCl added to a volume of 50 μl, 10 μl of 0.5 M Tris-HCl, pH 7.0, 10 μl of β-mercaptoethanol (prepared fresh), 10 μl of 5.0 mM [U-^{14}C]glycerol 3-phosphate (specific activity, 12,500 dpm/nmol). The reaction was initiated by the addition of 10 μl of 10 mM $MgCl_2 \cdot 6H_2O$. The mixture was incubated at 30 °C for 10 min and terminated by addition of 0.5 ml of 0.1 M HCl in methanol and subsequent steps performed exactly as described (17). Enzyme activity was linear with time up to at least 15 min with 0.1 mg of protein.

Isolation and Analysis of Radioactive Phospholipids—Cells were grown to confluence and incubated for 12 h in the absence or presence of 5 μM CPEC, 5 μM CPEC plus 0.1 mM cytidine, or 0.1 mM cytidine alone. A 5 μM concentration of CPEC was the optimum concentration for reducing CTP level, and higher concentrations did not reduce CTP levels further. Cells remained viable for at least 16 h in the presence of CPEC. We chose 12 h as our starting incubation time since it is documented that CPEC-triphosphate levels reach a maximum by 12 h in cells incubated with CPEC (23). After 12 h of incubation tracer levels of [1,3-^{3}H]glycerol (10 μCi/dish) or [^{14}C]glycerol (10 μCi/dish) or [1-^{14}C]linoleic acid (2 μCi/dish) were added to the incubation medium, and the cells were further incubated for up to 4 h. Subsequently, the medium was removed, and the cell layer was washed with 2 ml of ice-cold phosphate-buffered saline. The phosphate-buffered saline was removed, and 2 ml of methanol:water (1:1, v/v) was added, and the cells were harvested with a rubber policeman into 16 × 25-mm screw cap tubes. The tubes were mixed, and a 50-μl aliquot was taken for the determination of total radioactivity associated with the cells, and a 50-μl aliquot was taken for protein determination. Two ml of chloroform was then added to the tubes followed by 0.5 ml of 0.73% NaCl to initiate phase separation. The tubes were shaken for 5 min and then centrifuged at 2,000 rpm (model TJ-6 Bench Top Centrifuge) for 20 min. The aqueous phase was removed, and 5 ml of theoretical upper phase (4 ml chloroform, 48 ml methanol, 47 ml 0.9% NaCl) was added to wash the organic phase. The tubes were mixed and centrifuged as described above and the aqueous phase removed. The organic phase was dried under a stream of N_2 gas and resuspended in 100 μl of chloroform:methanol (2:1, v/v). A 50-μl aliquot of the organic phase was placed on a thin-layer plate and phospholipids separated by the two-dimensional thin-layer chromatography procedure described (16). The thin-layer plates were prepared by spraying the plate lightly with 0.4 M boric acid, blotting dry the excess boric acid, and then air drying the plates overnight. Phospholipid standards were placed on plates prior to chromatography. A 10–25-μl aliquot was placed on a thin-layer plate for DG and TG isolation. DG and TG were separated in a solvent system containing diethyl ether:benzene:absolute ethanol:acetic acid (45:50:2:0.2, v/v/v/v). The lipids were visualized with iodine vapor and removed

TABLE I
The nucleotide pools of H9c2 cardiac myoblasts incubated with CPEC

H9c2 cardiac myoblasts were incubated for 12 h in the absence or presence of 5 μM CPEC, 5 μM CPEC plus 0.1 mM cytidine, or 0.1 mM cytidine and the pool size of ATP, CTP, GTP, and UTP determined. Results represent the mean of two separate experiments.

	Nucleotide			
	ATP	CTP	GTP	UTP
	μM			
Control	3666	333	517	708
+5 μM CPEC	3833	25[a]	575	700
+5 μM CPEC +0.1 mM cytidine	3417	835	585	792
+0.1 mM cytidine	3583	758	533	825

[a] In most experiments the CTP pool was less than 10% of controls.

into 7-ml scintillation vials. 0.5 ml of water was added, and the vials were sonicated for 5 min to disperse the silica gel. Finally, 5 ml of Ecolite scintillation mixture was added. The radioactivity was determined using a Beckman model LS 3801 Scintillation Counter with internal standards.

Other Procedures—DNA and RNA synthesis were determined in cells by addition of 0.3 μM [2,8-^{3}H]adenine (12 μCi/dish) or 0.3 μM [*methyl*-^{3}H]thymidine (20 μCi/dish) to cells that were preincubated for 12 h in the absence or presence of 5 μM CPEC. Two h after [2,8-^{3}H]adenine or [*methyl*-^{3}H]thymidine addition, radioactivity incorporated into DNA and RNA was determined as described (26). Cellular nucleotide pools were extracted, neutralized, and then analyzed by high performance liquid chromatography exactly as described (26). Radioactivity incorporated into CPEC liponucleotide was determined by incubation of cells for 12 h with 5 μM [^{3}H]CPEC (10 μCi/dish). Subsequently, the cells were harvested as described above, and the organic fraction was isolated and separated on thin-layer plates in a solvent system containing chloroform:methanol:acetic acid:water (50:24:4:8, by volume) with authentic CDP-DG standard. One-cm fractions of silica gel from the origin were removed, and the radioactivity in these fractions was determined. H9c2 cells treated with CPEC were analyzed for P_i present in phospholipids. The lipid extraction procedure was the same as described above. The organic fraction was dried under N_2 and resuspended in 100 μl of chloroform:methanol (2:1, v/v). A 10-μl aliquot of the organic phase was taken for the determination of total phospholipid phosphorus by the method of Rouser *et al.* (27). In addition, the percent phospholipid phosphorus of individual phospholipids was determined after separation on two-dimensional thin-layer plates. For the enzyme assays protein concentration was determined by the method of Bradford (28). Cell protein content was measured by the method of Lowry *et al.* (29).

RESULTS

Treatment of H9c2 Cardiac Myoblasts with CPEC Specifically Reduces the Intracellular CTP Level, DNA, and RNA Synthesis—We studied phospholipid biosynthesis in rat heart H9c2 myoblast cells since they are a clonal line derived from embryonic rat heart tissue and exhibit many characteristics similar to that of striated muscle (30). In addition, these cells readily incorporated glycerol and linoleic acid into CL. To determine if cellular CTP level could be specifically reduced in H9c2 cardiac myoblasts, we treated the cells with 5 μM CPEC. After a 12-h incubation with CPEC, the pool size of CTP in H9c2 cells was reduced to less than 10% of the control values (Table I). The presence of CPEC did not significantly affect the pool sizes of the other major nucleotides ATP, UTP, or GTP. When control or cells treated with CPEC were incubated with cytidine, which should reverse the CPEC-induced reduction in CTP level (31), the pool size of CTP was not only restored but increased to 2.5-fold that of the control values. The pool sizes of ATP, UTP, and GTP were not significantly affected by the presence of cytidine in the incubation. Thus, the presence of CPEC specifically reduces the intracellular CTP level, and the presence of cytidine reverses this effect without influencing the level of other cellular nucleotides. CPEC addition to cancer cells was shown to cause a reduction in DNA and RNA synthesis (32). As a control, H9c2 cardiac myoblast cells were incubated in the absence or presence of 5 μM CPEC for 12 h and then incubated with [2,8-^{3}H]adenine or [*methyl*-^{3}H]thymidine, and the radioactivity incorporated into DNA and RNA was determined. Incubation with CPEC resulted in an 86% decrease in the formation of DNA and RNA from [2,8-^{3}H]adenine and a 91% decrease in the formation of DNA from [*methyl*-^{3}H]thymidine (Table II). Thus, CPEC inhibited DNA and RNA synthesis in these cells. The phospholipid phosphorus concentration of these cells was determined to be 124 ± 14 nmol/mg protein and was not affected by a 12-h incubation with CPEC or CPEC plus cytidine. The percent phospholipid phosphorus of individual phospholipids in these cells were determined to be as follows: PC, 42.3%; PE, 26.9%; sphingomyelin, 6.9%; CL, 4.2%; PS, 4.0%; PI, 3.2%; PG, 2.9%; PA, 2.0%; lyso-PC, 3.2% and were unaltered by treatment of cells with CPEC.

TABLE II
DNA and RNA synthesis in H9c2 cardiac myoblasts treated with CPEC

H9c2 cardiac myoblasts were incubated for 12 h in the absence or presence of 5 μM CPEC and subsequently incubated with [2,8-^{3}H]adenine or [*methyl*-^{3}H]thymidine for 2 h, and the radioactivity incorporated into DNA and RNA was determined. Results represent the mean of two separate experiments.

	[2,8-^{3}H]Adenine		[*methyl*-^{3}H]Thymidine
	DNA	RNA	DNA
	dpm/10^6 cells		
Control	110,381	543,617	601,453
CPEC-treated	15,161	75,258	55,063

TABLE III
The biosynthesis of lipids from [U-^{14}C]glycerol in H9c2 cardiac myoblasts treated with CPEC

H9c2 cardiac myoblasts were incubated for 12 h in the absence or presence of 5 μM CPEC, 5 μM CPEC plus 0.1 mM cytidine, or 0.1 mM cytidine and subsequently incubated with [U^{14}C]glycerol acid for 4 h, and the radioactivity incorporated into neutral lipids and phospholipids was determined. Results represent the mean of two separate experiments.

	Treatment			
	Control	CPEC	CPEC + cytidine	Cytidine
	dpm × 10^3/mg protein			
Phospholipids				
Cardiolipin	0.61	0.30	0.66	0.64
Phosphatidylglycerol	1.43	0.63	1.32	1.30
Phosphatidylethanolamine	4.40	3.20	4.23	4.44
Phosphatidylcholine	37.2	29.0	36.9	37.4
Phosphatidylserine	0.98	0.52	0.89	0.90
Phosphatidylinositol	1.36	0.89	1.29	1.26
Neutral lipids				
Diacylglycerol	2.78	7.36	2.67	2.62
Triacylglycerol	2.39	11.8	2.25	2.14

Cardiolipin Biosynthesis Is Regulated by the Intracellular CTP Level in H9c2 Cardiac Cells—We investigated if such a dramatic reduction in CTP level in these cells would affect CL biosynthesis. Cells were incubated for 12 h in the absence or presence of CPEC or CPEC plus cytidine or cytidine and subsequently incubated for 4 h with [U-^{14}C]glycerol. In [U-^{14}C]glycerol-labeled cells the total amount of radioactivity incorporated was 2.96 × 10^5 dpm/mg and was unaltered by the presence of CPEC in the incubation. Thus, CPEC did not affect glycerol uptake into these cells. When [U-^{14}C]glycerol was used to label phospholipids, biosynthesis of CL, PG, PI, PC, PE, and PS was inhibited by the presence of CPEC (Table III). In contrast, radioactivity incorporated into DG and TG was increased in CPEC-treated cells compared with controls. When cells were incubated with CPEC plus cytidine, radioactivity

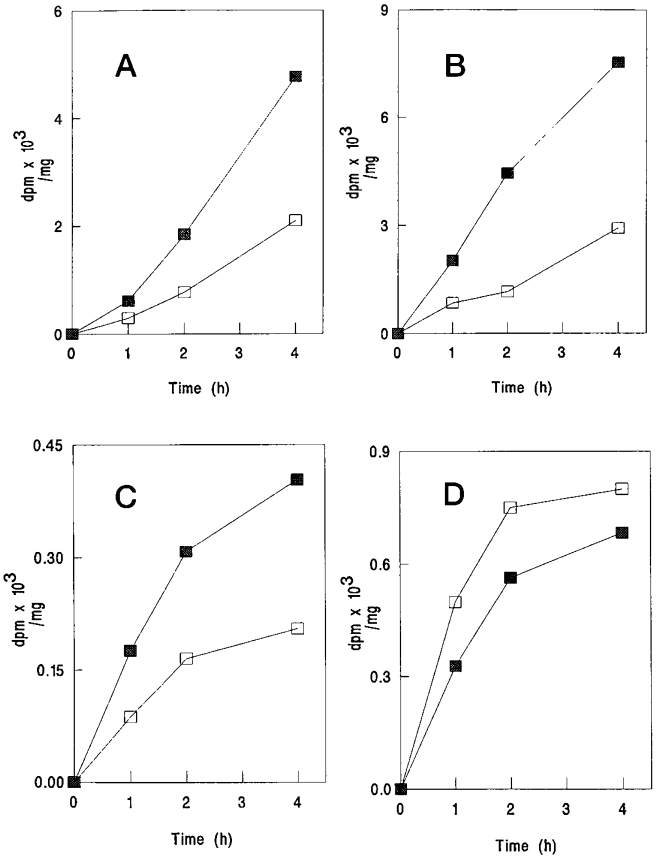

FIG. 1. **The biosynthesis of PA, CDP-DG, PG, and CL from [1,3-^{3}H]glycerol in H9c2 cardiac myoblasts treated with CPEC.** H9c2 cardiac myoblasts were incubated for 12 h in the absence or presence of 5 μM CPEC and subsequently incubated with [1,3-^{3}H]glycerol for up to 4 h, and the radioactivity incorporated into CL (*A*), PG (*B*), CDP-DG (*C*), and PA (*D*) was determined. *Shaded symbols*, control; *open symbols*, CPEC-treated. Results represent the mean of two separate experiments.

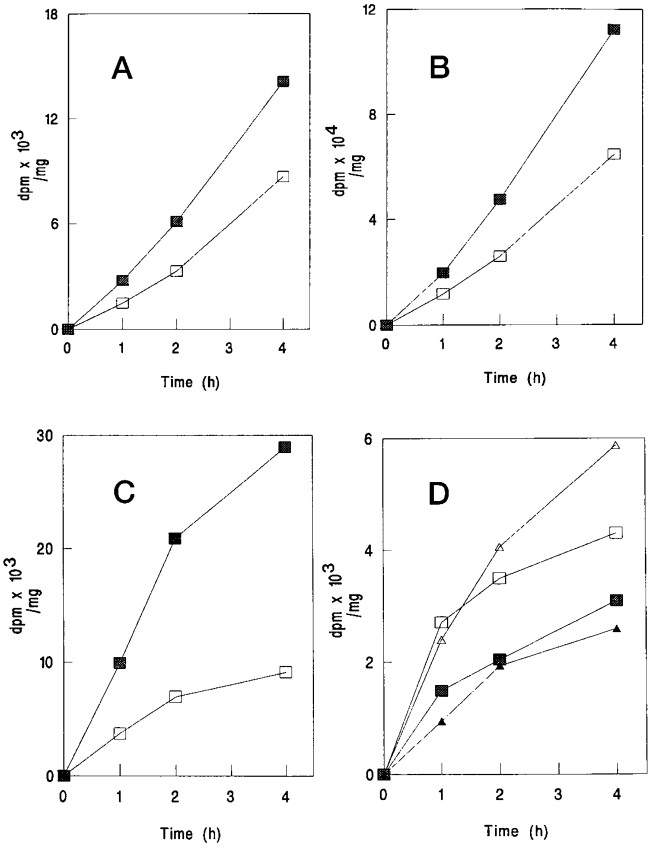

FIG. 2. **The biosynthesis of PC, PE, PI, DG, and TG from [1,3-^{3}H]glycerol in H9c2 cardiac myoblasts treated with CPEC.** H9c2 cardiac myoblasts were incubated for 12 h in the absence or presence of 5 μM CPEC and subsequently incubated with [1,3-^{3}H]glycerol for up to 4 h, and the radioactivity incorporated into PE (*A*), PC (*B*), PI (*C*), and DG (*squares*) and TG (*triangles*) (*D*) was determined. *Shaded symbols*, control; *open symbols*, CPEC-treated. Results represent the mean of two separate experiments.

incorporated into phospholipids and neutral lipids was restored to control values. In addition, the presence of cytidine alone did not affect the incorporation of radioactivity into phospholipids or neutral lipids. The increase in radioactivity incorporated into DG and TG might be expected if there were a re-direction of PA radioactivity toward DG and TG at the expense of acidic phospholipid biosynthesis. To confirm this we performed a time course experiment in which cells were incubated in the absence or presence of CPEC and then incubated with [1,3-^{3}H]glycerol for up to 4 h. The total amount of radioactivity in these cells was 1.37×10^5 dpm/mg protein at 1 h and by 4 h increased to 4.67×10^5 dpm/mg protein, and the presence of CPEC in the incubation medium did not affect this. As seen in Figs. 1 and 2 radioactivity incorporated into phospholipids and neutral lipids increased with time of incubation in both control and CPEC-treated cells. However, radioactivity incorporated into CDP-DG, CL, PG, PI, PE, and PC were all inhibited by the presence of CPEC in the incubation. In contrast, radioactivity incorporated into PA, DG, and TG were increased and accumulated with time in CPEC-treated cells compared with controls indicating a re-direction away from phospholipid toward neutral lipid biosynthesis.

The glycerol labeling studies indicated that PC and PE biosynthesis were affected by CPEC. We investigated if reduction in the CTP level would affect PC biosynthesis from the CDP-choline pathway and PE biosynthesis from the CDP-ethanolamine pathway since these pathways require CTP. Cells were incubated for 12 h in the absence or presence of CPEC or CPEC plus cytidine and subsequently incubated for 4 h with either [*methyl*-^{3}H]choline (2 μCi/dish) or [1-^{3}H]ethanolamine (2 μCi/dish), and the radioactivity incorporated into PC and PE was determined. Total radioactivity incorporated into [*methyl*-^{3}H]choline-incubated cells was $3.87 \pm 0.34 \times 10^5$ dpm/mg protein but was reduced to $2.52 \pm 0.29 \times 10^5$ dpm/mg protein when CPEC was present. Total radioactivity incorporated into [1-^{3}H]ethanolamine-incubated cells was $7.71 \pm 0.47 \times 10^5$ dpm/mg protein but was reduced to $5.65 \pm 0.22 \times 10^5$ dpm/mg protein when CPEC was present. The biosynthesis of PC from [*methyl*-^{3}H]choline and PE from [1-^{3}H]ethanolamine was inhibited by about 50% when the cells were incubated with CPEC (Fig. 3, *A* and *B*). Supplementation of CPEC-treated cells with cytidine restored PC and PE biosynthesis. Thus, the reduction in PE and PC biosynthesis observed in CPEC-treated cells was likely due to a reduction in CTP level.

It was possible that the observed reduction in radioactive glycerol incorporated into CL was due to an inhibition of PA:CTP cytidylyltransferase or a reduced conversion of glycerol 3-phosphate and CDP-DG into lipids (CL, PG, PI) or a reduced CL synthase activity. The activity of PA:CTP cytidylyltransferase and the conversion of [U-^{14}C]glycerol 3-phosphate and CDP-DG to organic products were determined in homogenates prepared from these cells. PA:CTP cytidylyltransferase activity in H9c2 cells was 2.49 ± 0.54 pmol/min·mg protein (average of four determinations) and was unaltered when cells had been treated with CPEC or CPEC plus cytidine. In addition, the conversion of [U-^{14}C]glycerol 3-phosphate and CDP-DG to or-

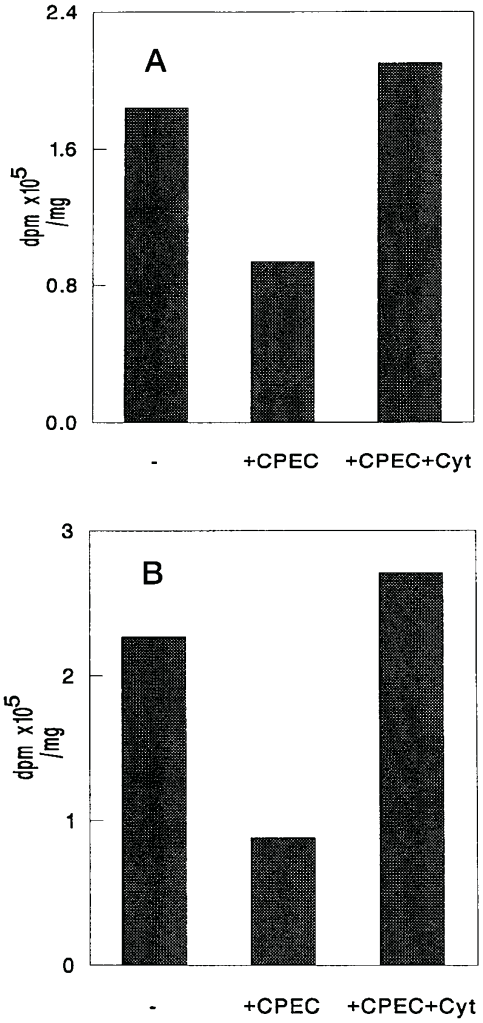

FIG. 3. **The biosynthesis of PC from [*methyl*-^{3}H]choline and PE from [1-^{3}H]ethanolamine in H9c2 cardiac myoblast cells incubated with CPEC.** H9c2 cardiac myoblasts were incubated for 12 h in the absence or presence of 5 μM CPEC or 5 μM CPEC plus 0.1 mM cytidine and subsequently incubated with [*methyl*-^{3}H]choline or [1-^{3}H]ethanolamine for 4 h, and the radioactivity incorporated into PC from [*methyl*-^{3}H]choline (*A*) and PE (from [1-^{3}H]ethanolamine) (*B*) was determined. Control, −; plus CPEC, +*CPEC*; plus CPEC plus cytidine, +*CPEC* + *Cyt.* Results represent the mean of two separate experiments.

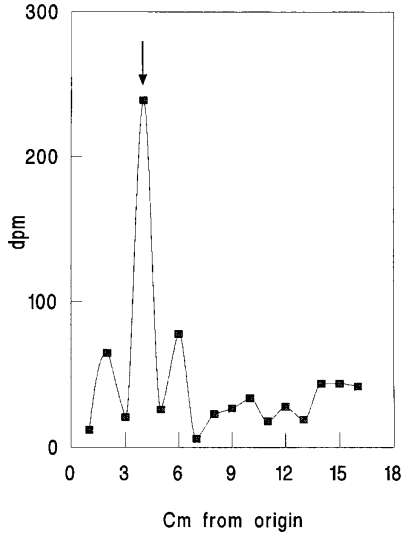

FIG. 4. **Thin layer chromatograph of organic fractions from [^{3}H]CPEC-treated cells.** H9c2 cells were incubated for 12 h with 5 μM [^{3}H]CPEC, and the organic fraction was isolated. The organic fraction was separated on a thin layer plate and radioactivity determined in 1-cm sections removed from the plate. The location corresponding to the migration of authentic CDP-DG standard is indicated by an *arrow*. Background radioactivity was 20–40 dpm. A representative chromatograph is shown.

ganic products (product identification revealed mainly PG, PGP and PI) was 7.9 ± 1.8 pmol/min·mg protein (average of four determinations) and was unaltered when cells had been treated with CPEC or CPEC plus cytidine. CL synthase activity was also measured in mitochondria prepared from these cells and was determined to be 1.2 ± 0.3 pmol/min·mg protein (average of four determinations) and was unaltered in cells treated with CPEC or CPEC plus cytidine compared with controls. One other possibility was that CPEC may have stimulated PA phosphohydrolase activity which would re-direct radioactivity toward DG and TG. However, this clearly could not be the case since [1,3-^{3}H]glycerol accumulated in PA in the CPEC-treated cells. Thus, since the maximum *in vitro* activities of the enzymes involved in CL biosynthesis were unaffected, the decrease in radioactivity incorporated into CL in CPEC-treated cells was due to a decrease in CTP levels. Taken together, the results clearly indicate that CL biosynthesis in H9c2 cells is regulated by the intracellular CTP concentration. In addition, the results suggest that this regulatory mechanism may apply for all phospholipids.

It was surprising that such a dramatic reduction in CTP levels (less than 10%) in the CPEC-treated cells did not reduce CL biosynthesis greater than 50%. One possibility for this is that the CPEC-triphosphate formed in these cells could serve as a high energy nucleotide precursor for charging PA to its corresponding nucleotide intermediate. Indeed, when CPEC was added to cells it was rapidly phosphorylated to CPEC-triphosphate (23). To test this hypothesis, cells were incubated for 12 h in the presence of 5 μM [^{3}H]CPEC, and the organic fraction from these cells was analyzed for radioactivity incorporated into lipids via thin-layer chromatography. A significant amount of radioactivity was observed 4–5 cm from the origin and co-migrated with authentic CDP-DG standard (see *arrow* Fig. 4). Thus, it was possible that CPEC-triphosphate might serve to a limited extent as a high energy nucleotide source for the charging of PA. This might explain why in the presence of CPEC CL biosynthesis was not completely reduced.

Reduction in CTP in H9c2 Cells Does Not Affect the Resynthesis of Cardiolipin from Linoleic Acid—To determine if the above observed reduction in CTP level affected phospholipid resynthesis, H9c2 cells were incubated for 12 h in the absence or presence of CPEC, CPEC plus cytidine, or cytidine and subsequently labeled for 4 h with [1-^{14}C]linoleic acid. Linoleic acid was used since it would be significantly incorporated into cardiolipin which contains a high proportion of this molecular species (15). Treatment with CPEC did not affect the radioactivity incorporated into CL and most other phospholipids (Table IV). In contrast, radioactivity incorporated into PI and PG was reduced by approximately 50% and corresponded with the level of reduction observed when cells were incubated in the presence of [1,3-^{3}H]- or [U-^{14}C]glycerol (Fig. 1, Table III). Significant radioactivity in PA was not observed (but see note Table IV). These data suggest that linoleic acid may be preferentially incorporated into only newly synthesized PG and PI. Interestingly, linoleic acid incorporation into DG and especially TG was dramatically elevated. This might be expected if there was elevated *de novo* synthesis of DG and TG. The increase in DG and TG radioactivity in the glycerol labeling studies support this conclusion.

TABLE IV
The resynthesis of lipids from [1-^{14}C]linoleic acid in H9c2 cardiac myoblasts treated with CPEC

H9c2 cardiac myoblasts were incubated for 12 h in the absence or presence of 5 μM CPEC, 5 μM CPEC plus 0.1 mM, cytidine or 0.1 mM cytidine and subsequently incubated with [1-^{14}C]linoleic acid for 4 h, and the radioactivity incorporated into neutral lipids and phospholipids was determined. Results represent the mean of two separate experiments. (Note, radioactivity observed in PA was four to five times the background in CPEC-treated cells compared with controls which were in the background (20–40 dpm) range.)

	Treatment			
	Control	CPEC	CPEC + cytidine	Cytidine
	dpm $\times$ 10^4 */mg protein*			
Phospholipids				
Cardiolipin	1.12	1.13	1.10	1.17
Phosphatidylglycerol	0.11	0.05	0.10	0.11
Phosphatidylethanolamine	5.48	4.73	5.40	5.91
Phosphatidylcholine	41.8	42.0	39.1	45.4
Phosphatidylserine	0.41	0.40	0.45	0.43
Phosphatidylinositol	1.02	0.43	0.99	1.09
Neutral lipids				
Diacylglycerol	1.12	3.74	1.12	1.21
Triacylglycerol	1.60	7.43	1.48	1.46

DISCUSSION

The objective of this study was to determine if a reduction in the intracellular CTP level could regulate CL biosynthesis in H9c2 cardiac myoblast cells. We took advantage of the ability of the specific and potent inhibitor of CTP synthetase, CPEC-triphosphate, to reduce intracellular CTP levels in these cells. The major findings are as follows. 1) When the intracellular CTP level was reduced to less than 10% of normal levels all phospholipid biosynthesis including CL was attenuated, and this resulted in a re-direction of lipid synthesis toward DG and TG. 2) When the CPEC-treated cells were supplemented with cytidine, which restored CTP levels, biosynthesis of all phospholipids including CL was restored to control levels as was the synthesis of DG and TG. 3) Reduction in the CTP level in H9c2 cells did not affect the activities of PA:CTP cytidylyltransferase, CL synthase, or the *in vitro* synthesis of phospholipids from glycerol 3-phosphate and CDP-DG. 4) Reduction in the CTP level of these cells did not affect the resynthesis of CL from linoleic acid. 5) The data strongly indicate that the intracellular CTP level is a universal signal/switch for all phospholipid biosynthesis in eukaryotic cells.

Upon entrance into cells the anti-tumor agent CPEC is rapidly phosphorylated to CPEC mono-, di, and triphosphate, a specific inhibitor of CTP synthetase (23, 31–36). In the present study, treatment of H9c2 cardiac myoblast cells with CPEC for 12 h caused a reduction in cellular CTP concentration but did not significantly affect the concentration of the other high energy nucleotides ATP, UTP, and GTP. Incubation of cells with CPEC did not significantly affect cellular protein content and phospholipid phosphorus content of individual phospholipids. In addition, incorporation of [1,3-^{3}H]glycerol into most phospholipids in CPEC-treated cells was linear, and incorporation of [1-^{14}C]linoleic acid into CL and most phospholipids was not affected by CPEC. Thus, cell viability over the time course of these experiments was not compromised. Prolonged exposure (24 h) of Molt-4 T-cell leukemia cells to CPEC resulted in considerable cytotoxicity (37). When control or CPEC-treated H9c2 cells were incubated with cytidine, the CTP pool increased 2.5-fold that of controls but again did not significantly affect the level of ATP, UTP, or GTP. We were not able to increase the CTP levels beyond this level when control or CPEC-treated cells were supplemented with more cytidine (data not shown). This was likely due to the feedback mechanism of CTP on the CTP synthetase and the uridine/cytidine kinase (31, 38). In any case, incubation of H9c2 cells with CPEC proved to be a useful tool for dramatically and specifically decreasing the cellular CTP concentrations to less than 10% of control cells.

In 1979 Vance and Choy (39) postulated that since all phospholipid backbones in all mammalian cells are produced by a reaction that requires CTP, this nucleotide might serve as a universal signal for the biosynthesis of all phospholipids. These authors suggested that control of phospholipid biosynthesis through the concentration of CTP would provide a facile mechanism for relating the rate of membrane synthesis to the energy state of the cell. Previous studies have set a precedent for elevated synthesis of PC as a result of increased cellular CTP level. In cytidine-supplemented PC12 cells PC biosynthesis via the CDP-choline pathway was stimulated, and this was correlated with an increase in cellular CTP levels (40). In more direct studies, HeLa cells infected with poliovirus demonstrated a 3-fold increase in cellular CTP level, and this was accompanied by a 2-fold stimulation of PC biosynthesis (41, 42). Overexpression of CTP synthetase in *Saccharomyces cerevisiae* resulted in a 2.4-fold elevation of cellular CTP, and this was associated with a 2-fold stimulation of the utilization of the CDP-choline pathway for PC biosynthesis (43). Conversely, starvation for 24 h reduced rat and hamster liver PC biosynthesis, and this coincided with a decrease in liver CTP levels (44–46). In BHK-21 cells infected with Semliki Forest virus a 70% reduction in cellular CTP was observed compared with mock-infected cells, and this was accompanied by a decreased biosynthesis of PC from [*methyl*-^{3}H]choline (47). To our knowledge in no instance had it ever been directly demonstrated that the biosynthesis of CL was modulated specifically by reduced cellular levels of CTP. Since biosynthesis of CDP-DG from PA:CTP cytidylyltransferase was directly CTP-dependent (48) and eukaryotic *de novo* CL biosynthesis requires CDP-DG (11), regulation of CL biosynthesis by the level of the cellular CTP pool might be expected.

Treatment of cells with CPEC resulted in a dramatic elevation of DG and TG biosynthesis from both radioactive glycerol and linoleic acid concomitant with a decrease in PI, PG, and CL biosynthesis. This would be expected if there were a decreased utilization of CTP by the PA:CTP cytidylyltransferase. Since the activity of this enzyme, CL synthase and the synthesis of phospholipids from glycerol 3-phosphate and CDP-DG was unaltered in homogenates derived from CPEC-treated cells compared with controls, the reduction of CTP level in these cells likely caused a re-routing of lipid biosynthesis away from PI, PG, and CL biosynthesis toward DG and TG biosynthesis. PA lies at a branch point in phospholipid and neutral lipid biosynthesis (for review see Ref. 49). Regulation of this branch step by elevated ATP levels favored phospholipid biosynthesis at the expense of TG biosynthesis and vice versa in yeast (50). We found that in CPEC-treated cells the ATP, UTP, and GTP levels remained unchanged, yet radioactivity incorporated into DG and TG was dramatically increased. These results suggest that, in addition to ATP, the cellular CTP concentration may also be involved in the regulation of DG and TG biosynthesis. Furthermore, the reduction in CDP-DG formation in cardiac H9c2 cells treated with CPEC concomitant with the reduction of CTP level provides additional evidence for the rate-limiting role of the PA:CTP cytidylyltransferase for CL biosynthesis.

Fatty acids require ATP to be charged to their acyl-CoA derivatives prior to the addition to lysophospholipid substrates by reacylation (for review see Ref. 51). It was not surprising that [1-^{14}C]linoleic acid incorporated into CL and most other phospholipids was unaffected by incubation of cells with CPEC

since the ATP level was essentially unaltered. These data suggest that CL and other phospholipids may be readily deacylated and reacylated with linoleic acid in H9c2 cells. In rat liver mitochondrial preparations, resynthesis of CL from monolyso-CL and linoleoyl-CoA had been documented (52). Interestingly, radioactivity incorporated into PI and PG from [1-^{14}C] linoleic acid was reduced to a similar extent as that observed in [1,3-^{3}H]- or [U-^{14}C]glycerol-labeled cells. These data might imply that in H9c2 cells newly synthesized PI and PG may be preferentially utilized for deacylation and reacylation with linoleic acid since the pool size of these phospholipids did not change.

The question might be asked why does reduction in CTP to such low levels not completely inhibit CL biosynthesis? In the time course study with [1,3-^{3}H]glycerol most phospholipid biosynthesis proceeded linearly, albeit at a reduced rate, as did controls (Figs. 1 and 2). The rate of formation of phospholipids appeared to be about 50% of the control, yet CTP levels were less than 10% of controls. Possible explanations for this are as follows: 1) the compartmentation of CTP into pools at or near the lipid biosynthetic enzymes (53), 2) a preferential utilization of the salvage-derived pool of CTP for synthesis of liponucleotides including CDP-choline and CDP-ethanolamine (54), or 3) that the high energy CPEC-triphosphate, synthesized in cells treated with CPEC, could itself serve as a substrate for the charging of PA to the CPEC-liponucleotide intermediate. In support of the latter, when cells were incubated with [^{3}H]CPEC, radioactivity was observed in the organic fraction and in a location on the thin-layer plates that co-migrated with authentic CDP-DG. The liponucleotides of dideoxycytidine, 3′-deoxythymidine, and 3′-azido-3′-deoxythymidine were shown to substitute to varying extents for CDP-DG in the biosynthesis of PI, PG, and CL in rat liver subcellular fractions, although each of these was not as efficient as CDP-DG itself (55).

Another intriguing question is why did supplementation of the CPEC-treated cells with cytidine, in which a 2.5-fold stimulation of CTP level occurred, not stimulate CL biosynthesis beyond that of control values? The reported true K_m for yeast PA:CTP cytidylyltransferase and the apparent K_m for the guinea pig liver enzyme was 1.0 mM (19, 25). The pool size of CTP in the control cells was 333 μM. The elevated CTP levels seen in the CPEC plus cytidine (835 μM) or cytidine (758 μM) supplemented cells was still below the K_m of the PA:CTP cytidylyltransferase; therefore, an increase in CL biosynthesis in these cells might have been expected. This was clearly not the case. A likely explanation is that the size of the PA pool may also be limiting the PA:CTP cytidylyltransferase reaction. In summary, this study in which reduced cellular CTP levels reduced all phospholipid biosynthesis combined with the many previous studies in which alterations in cellular CTP levels accompany corresponding alterations in PC biosynthesis strongly support the hypothesis that CTP is a universal signal/switch for all phospholipid biosynthesis in eukaryotic cells.

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