D.E. Vance and J.E. Vance (Eds.) *Biochemistry of Lipids, Lipoproteins and Membranes (4th Edn.)*

CHAPTER 8

Phospholipid biosynthesis in eukaryotes

Dennis E. Vance

CIHR Group on Molecular and Cell Biology of Lipids and Department of Biochemistry, University of Alberta, Edmonton, AB T6G 2S2, Canada, Tel.: +1 (780) 492-8286; Fax: +1 (780) 492-3383; E-mail: dennis.vance@ualberta.ca

1. Introduction

The objective of this chapter is to provide an overview of eukaryotic phospholipid biosynthesis at an advanced level. Phospholipids make up the essential milieu of cellular membranes and act as a barrier for entry of compounds into cells. Phospholipids also function as precursors of second messengers such as diacylglycerol (DG) and inositol-1,4,5-P_3 which is covered in Chapter 12. A third, and usually overlooked function of phospholipids, is storage of energy in the form of fatty acyl components. This function is probably quantitatively important only under extreme conditions such as starvation.

2. Phosphatidic acid biosynthesis and conversion to diacylglycerol

Phosphatidic acid (PA) is an intermediate that occurs at a branch point in glycerolipid biosynthesis as shown in Fig. 1. Significant developments in elucidation of the biosynthetic pathway occurred in the 1950s when Kornberg and Pricer demonstrated that fatty acids are activated to acyl-CoA prior to reaction with glycerol-3-P. Subsequent studies from the laboratories of Kennedy, Shapiro, Hübscher and others delineated the biosynthetic pathway for PA.

An important step in PA biosynthesis is the activation of fatty acids by acyl-CoA synthetases to yield acyl-CoA (Fig. 2). Five different forms of rat acyl-CoA synthetase have been cloned, each encoded by a separate gene (R.A. Coleman, 2001). Different forms of the enzyme have been found on endoplasmic reticulum (ER), mitochondria and mitochondrial associated membrane (MAM), a subfraction of the ER (J.E. Vance, 1990). Hence, these synthetases may function differently in providing substrate for phospholipid and triacylglycerol (TG) biosynthesis.

2.1. Glycerol-3-P acyltransferase

This enzyme catalyzes the first committed reaction in the biosynthesis of PA. The relative importance of this acyltransferase in regulation of phospholipid biosynthesis has not been clearly established. In mammals, two glycerol-3-P acyltransferases have been identified, one associated with mitochondria and the other on the ER (Fig. 2). The ER acyltransferase is inhibited by *N*-ethylmaleimide whereas the mitochondrial

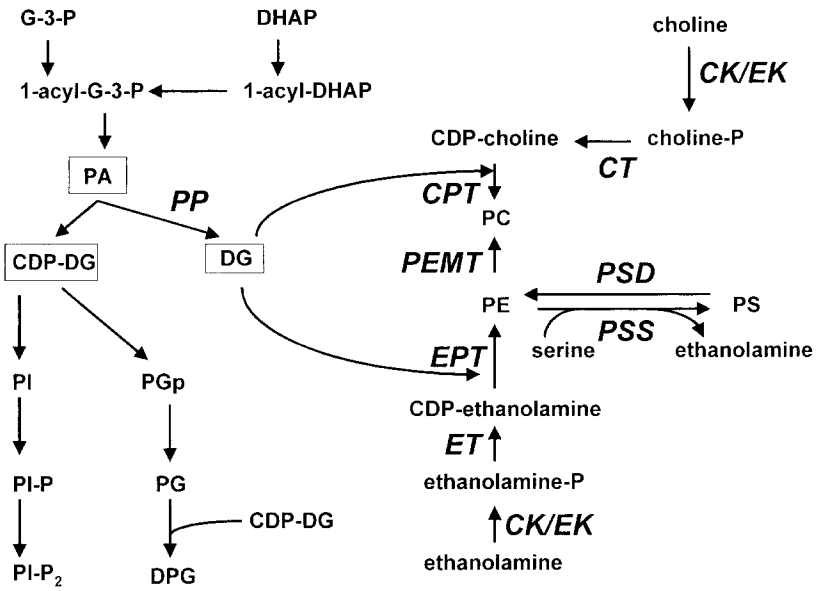


Fig. 1. Phospholipid biosynthetic pathways in animal cells. The abbreviations are: DHAP, dihydroxyacetone phosphate; G-3-P, glycerol-3-phosphate; PA, phosphatidic acid; DG, diacylglycerol; CDP-DG, cytidine diphosphodiacylglycerol; PI, phosphatidylinositol; PG, phosphatidylglycerol; PGp, phosphatidylglycerol phosphate; DPG, diphosphatidylglycerol; PP, phosphatidic acid phosphatase; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PEMT, phosphatidylethanolamine *N*-methyltransferase; CT, CTP : phosphocholine cytidylyltransferase; PS, phosphatidylserine; CK/EK, choline/ethanolamine kinase; CPT, CDP-choline : 1,2-diacylglycerol cholinephosphotransferase; EPT, CDP-ethanolamine : 1,2-diacylglycerol ethanolaminephosphotransferase; ET, CTP : phosphoethanolamine cytidylyltransferase; PSD, phosphatidylserine decarboxylase; PSS, phosphatidylserine synthase.

enzyme is not. The mitochondrial acyltransferase prefers palmitoyl-CoA as an acyl donor compared to oleoyl-CoA, whereas the ER enzyme does not show a preference for saturated versus unsaturated acyl-CoAs. For this and other reasons the mitochondrial enzyme is thought to be primarily responsible for the abundance of saturated fatty acids in the SN-1 position of glycerophospholipids (D. Halder, 1994). Mitochondrial glycerol-3-P acyltransferase has been localized to the outer mitochondrial membrane with the active site facing the cytosol (R.A. Coleman, 2001). Mitochondrial glycerol-3-P acyltransferase has been purified and the cDNA cloned.

Transcription of the mitochondrial acyltransferase gene is decreased by starvation and glucagon, and increased by a high carbohydrate diet [1]. These responses make physiological sense since animals do not need to make triacylglycerols and to a lesser extent phospholipids when energy supply is limited. The microsomal activity is not significantly altered by these treatments. The 5′ flanking region of the murine gene for the mitochondrial acyltransferase was linked to a luciferase reporter plasmid and expressed in 3T3-L1 pre-adipocytes. Deletion analysis on the promoter indicated that sequences between −86 and −55 bp were important for the expression of luciferase activity. Subsequent studies identified −78 to −55 bp as sites that bind sterol response

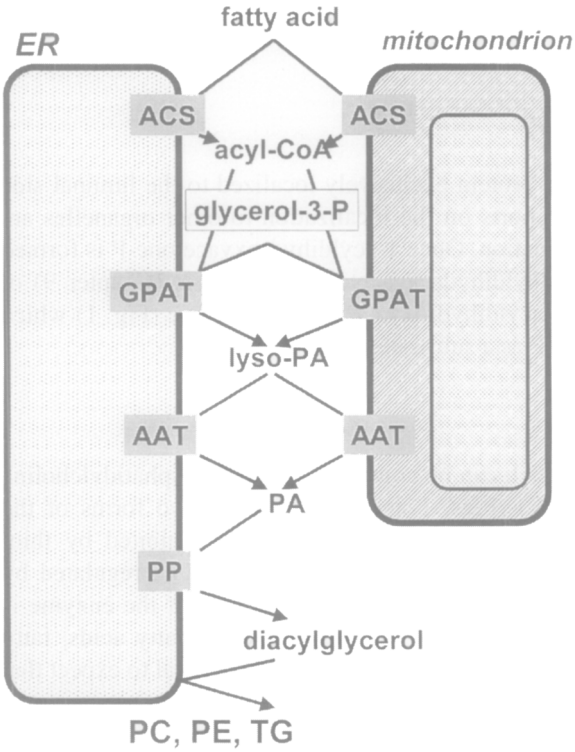


Fig. 2. Biosynthesis of phosphatidic acid (PA) can occur on both the endoplasmic reticulum and the outer membrane of mitochondria. The abbreviations are: ACS, acyl-CoA synthetase; GPAT, glycerol-3-P acyltransferase; AAT, 1-acylglycerol-3-P acyltransferase; PP, phosphatidic acid phosphatase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TG, triacylglycerol.

element binding protein (SREBP) and NF-Y transcription factors (P.A. Edwards, 1997). As indicated in Chapters 6, 7 and 15, SREBP is a key regulator of cholesterol and fatty acid synthesis, and fatty acid desaturation. Thus, it makes physiological sense that SREBP would also enhance glycerolipid, particularly TG, biosynthesis by increasing the expression of glycerol-3-P acyltransferase. Disruption of the mitochondrial glycerol-3-P acyltransferase gene in mice caused a decrease in the TG and cholesterol content in liver and striking changes in glycerolipid fatty acid composition (R.A. Coleman, 2002).

2.2. 1-Acylglycerol-3-P acyltransferase

Much less is known about the second step in the PA biosynthetic pathway (Fig. 2). The activity of this acyltransferase is much lower in mitochondria than in ER. It is presumed that much of the lyso-PA formed in mitochondria is transferred to ER for the second acylation. In vitro studies indicate that a carrier protein is not required (A.K. Hajra, 1992). The esterification at position 2 is specific for unsaturated fatty acids. However, the types of fatty acyl-CoAs available also influence the acyl-CoA selected for transfer

to lyso-PA. Two human isoforms of 1-acylglycerol-3-P acyltransferase have been cloned and expressed [2].

2.3. Dihydroxyacetone-P acyltransferase

This enzyme is an integral membrane protein exclusively localized to the luminal side of peroxisomes (A. Poulos, 1993). Reports on the localization to other organelles are likely a result of peroxisomal contamination. Once 1-acyldihydroxyacetone-P is formed it can be used as a substrate for 1-alkyldihydroxyacetone-P synthesis (Chapter 9) or reduced to lyso-PA by a peroxisomal acyldihydroxyacetone-P reductase (Fig. 1) which also utilizes 1-alkyldihydroxyacetone-P as a substrate.

2.4. Phosphatidic acid phosphatase

This enzyme hydrolyses PA to DG which can be converted to TG, phosphatidylcholine (PC) or phosphatidylethanolamine (PE) (Figs. 1 and 2). There are two forms of the phosphatase. The cytosolic ER form is dependent on Mg^{2+} and inhibited by thiol reagents such as *N*-ethylmaleimide. The activity of this enzyme can be regulated by reversible translocation between cytosol and ER. The cytosolic form of the enzyme is inactive and is translocated to the ER membrane in the presence of fatty acids, fatty acyl-CoAs and PA. Since the substrate, PA, is found on the ER it is logical to expect that the ER is where the enzyme functions in the cell.

The second PA phosphatase is neither inhibited by *N*-ethylmaleimide nor stimulated by Mg^{2+}. The cDNA for this phosphatase was cloned and expressed [3]. It appears to be a glycosylated protein on the plasma membrane with 6 putative transmembrane regions. This lipid phosphatase also hydrolyzes ceramide-1-phosphate, sphingosine-1-phosphate as well as lyso-PA. The active site is predicted to face outside the cell and has activity on lyso-PA but low activity on PA (H. Kanoh, 2000). Thus, the plasma membrane phosphatase probably has no role in intracellular phospholipid biosynthesis. Consistent with a signaling role, the plasma membrane phosphatase shares 34% sequence identity with Wunen protein (also has phosphatase activity) that functions in germ cell migration in *Drosophila* embryos. Furthermore, both of these proteins are very similar to Dri42, a protein that is upregulated in expression during epithelial differentiation in rat intestinal mucosa.

Yeast also has two PA phosphatases, a 104-kDa, microsomal form and a 45-kDa, mitochondrial form [4]. Addition of inositol induces the expression of the 45-kDa enzyme but not the 104-kDa enzyme (see Section 10 for more on regulation of phospholipid biosynthesis in yeast).

A novel DG pyrophosphate phosphatase has also been identified in yeast [4]. The enzyme has PA phosphatase activity but prefers DG pyrophosphate as substrate. This phosphatase has broad specificity but only DG pyrophosphate and PA have been shown to be substrates in vivo. A yeast mutant defective in the pyrophosphatase gene was viable and accumulated DG pyrophosphate (G.M. Carman, 1998). The enzyme expression is induced by inositol and zinc deprivation (G.M. Carman, 2001). The enzyme is similar to the mammalian plasma membrane PA phosphatase which also hydrolyzes DG

pyrophosphate. The function of DG pyrophosphate is unknown. Since it occurs at a very low level in yeast (0.18 mol% of total phospholipids), DG pyrophosphatase may have a signaling function [4].

3. *Phosphatidylcholine biosynthesis*

3.1. Historical background

PC was first described by Gobley in 1847 as a component of egg yolk and named 'lecithin' after the Greek equivalent for egg yolk (*lekithos*). In the 1860s Diakonow and Strecker demonstrated that lecithin contained two fatty acids linked to glycerol and that choline was attached to the third hydroxyl by a phosphodiester linkage. The first significant advance in understanding PC biosynthesis occurred in 1932 with the discovery by Charles Best that animals had a dietary requirement for choline. In the 1950s the CDP-choline pathway for PC biosynthesis (Fig. 3) was described by Eugene Kennedy and coworkers. A key observation was that CTP, rather than ATP, was the activating nucleotide for PC biosynthesis [5]. CTP is required not only for PC biosynthesis but also for the de novo synthesis of all phospholipids (prokaryotic and

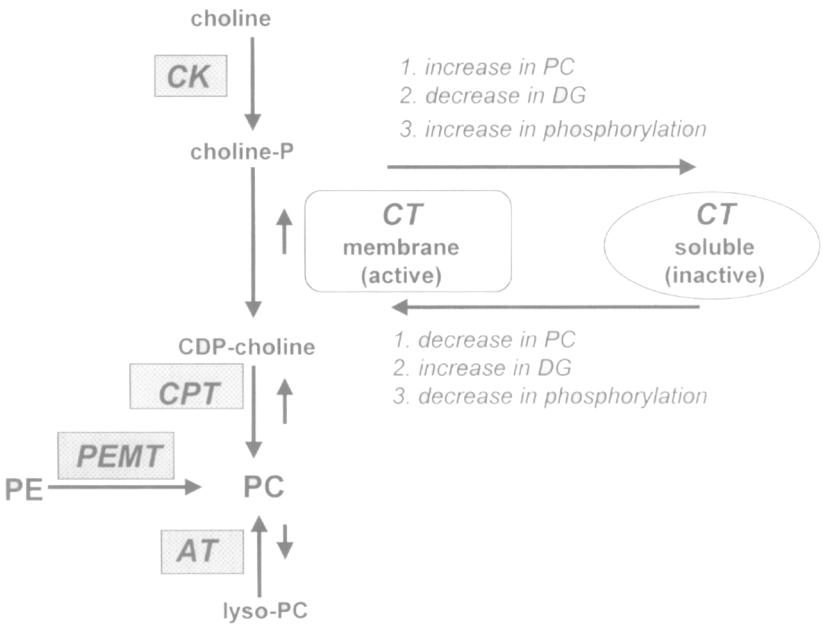


Fig. 3. Regulation of PC biosynthesis via the CDP-choline pathway by modulation of the binding of CTP : phosphocholine cytidylyltransferase (CT) to membranes. Three different modes of regulation of CT activity are indicated. The abbreviations are: CK, choline kinase; CPT, CDP-choline : 1,2-diacylglycerol cholinephosphotransferase; PEMT, phosphatidylethanolamine *N*-methyltransferase; AT, lyso-PC acyltransferase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; DG, diacylglycerol.

eukaryotic, excluding PA which can be considered to be an intermediate in glycerolipid biosynthesis).

An alternative pathway for PC biosynthesis, of quantitative significance only in liver, is the conversion of PE to PC via PE methylation (Fig. 3). The first observation of this pathway was in 1941 when Stetten fed [^{15}N]ethanolamine to rats and isolated [^{15}N]choline. Two decades later Bremer and Greenberg detected a microsomal enzyme that converted PE to PC via transfer of methyl groups from *S*-adenosylmethionine.

3.2. Choline transport and oxidation

Choline is not made de novo in animal cells except by methylation of PE to PC and subsequent hydrolysis of the choline moiety. Therefore, choline must be imported from extracellular sources. There are two distinct transport mechanisms for choline [6]; a high affinity (K_m or $K_t < 5$ μM), Na-dependent transporter and a lower affinity ($K_t > 30$ μM), Na-independent transporter. Several cDNAs encoding proteins that show high affinity transport of choline have been reported. A human cDNA is predicted to have 13 transmembrane spanning domains (R.D. Blakely, 2000).

Once choline is inside the cell, its normal fate is rapid phosphorylation by choline kinase (Fig. 3). In neurons choline is also converted to the neurotransmitter, acetylcholine. Choline is also oxidized to betaine [$^{-}$OOC–CH$_2$–N^{+}(CH$_3$)$_3$] in the liver and kidney. In liver betaine is an important donor of methyl groups for methionine biosynthesis and the one carbon pool. Betaine is produced in mitochondria into which choline is transported by a specific transporter on the inner membrane. Next, choline is oxidized to betaine aldehyde by choline dehydrogenase on the inner leaflet of the inner mitochondrial membrane. The conversion to betaine is catalyzed by betaine-aldehyde dehydrogenase located in the mitochondrial matrix.

Betaine can be transported into kidney medulla by a betaine transporter. In renal medulla, eubacteria, halotolerant plants, marine invertebrates and cartilaginous fish, betaine accumulates as an osmolyte (a small organic solute that accumulates in response to hypertonicity without adverse affect to the cell or organism) (J.S. Handler, 1992). Hypertonicity of the renal medulla is important for the kidney's ability to concentrate urine.

3.3. Choline kinase

The enzyme was first demonstrated in yeast extracts by J. Wittenberg and A. Kornberg (more famous for his contributions to DNA replication) in 1953. The enzyme was purified by K. Ishidate (1984) from rat kidney and shown also to phosphorylate ethanolamine [6]. This kinase is now referred to as choline/ethanolamine kinase β. The cDNA for a rat liver choline/ethanolamine kinase encoded an enzyme that is now referred to as choline/ethanolamine kinase α1. Northern analyses indicate that the mRNA for choline/ethanolamine kinase α1 is most abundant in testis. Choline/ethanolamine kinase α2 appears to be a splice variant of choline/ethanolamine kinase α1. The choline/ethanolamine kinase α and β genes have been characterized. The length of the gene was 40 kb for the choline/ethanolamine kinase α gene whereas the β gene was only 3.5 kb in length (K. Ishidate, 2000).

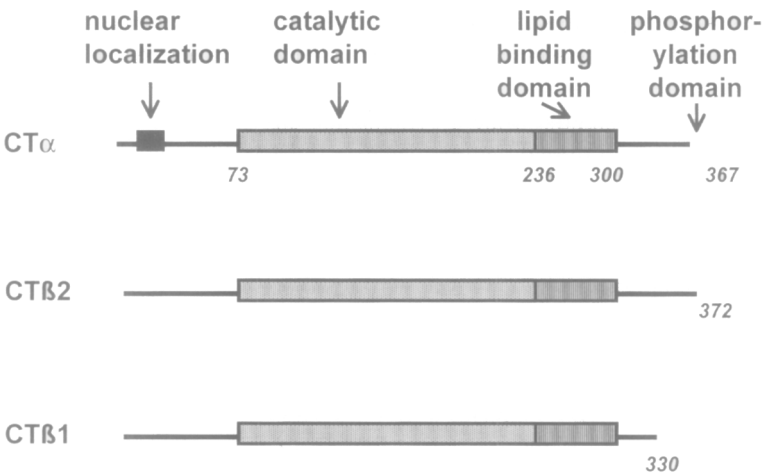


Fig. 4. Domain structures of CTP: phosphocholine cytidylyltransferase (CT) α, β1 and β2. CTα contains a nuclear localization signal, a N-terminal catalytic domain, an amphipathic helical (lipid binding) domain and a C-terminal phosphorylation domain. The CTβ forms lack the nuclear localization signal but contain catalytic and amphipathic helical domains. CTβ1 is missing the phosphorylation domain whereas CTβ2 has a phosphorylation domain that is different from that of CTα.

Choline is not only required in the diet of animals but also in the medium of animal cells in culture (H. Eagle, 1955). Choline is essential because of the cell's requirement for PC to grow and divide.

There is evidence that the activity of choline kinase might be regulatory for cell division in some cases [6].

3.4. CTP : phosphocholine cytidylyltransferase

This enzyme activity was first described by Kennedy and Weiss in 1955 [5]. Over three decades later CT was finally purified to homogeneity (P.A. Weinhold, 1987). The CT gene was cloned from *S. cerevisiae* (S. Yamashita, 1987) by complementation of a yeast mutant defective in CT activity. The cDNA of rat liver CT was subsequently cloned (R.B. Cornell, 1990). CT is a homodimer in soluble extracts of rat liver and is also found on membranes. In most cells CT is thought to exist in an inactive reservoir in its soluble form and to be active when associated with membranes (Fig. 3).

Two genes encode different forms of CT, α and β. The CTα gene spans approximately 26 kb. Exon 1 is untranslated, exon 2 encodes the translation start site and a nuclear localization signal, exons 4–7 encode the catalytic domain, exon 8 codes for the alpha helical membrane binding domain and exon 9 encodes a C-terminal phosphorylation domain (I. Tabas, 1997) (Fig. 4).

The CTβ gene is located on the X chromosome and encodes two isoforms, CTβ1 and CTβ2 (Fig. 4), presumably derived by mRNA splicing. Both isoforms differ from CTα at the amino terminus, lack the nuclear localization signal and are found in the cytoplasm of animal cells [7]. The primary sequences of CTβ1 and CTβ2 are identical except at

the carboxyl terminal. CTβ1 lacks most of the phosphorylation domain that is present in CTβ2. There are significant differences between the sequences of the phosphorylation domains of CTα and CTβ.

CT has classically been considered to be a cytoplasmic enzyme since its activity is found in the cytosol and on microsomal membranes in cellular homogenates. However, Kent and coworkers demonstrated that CT was found in the nuclear matrix and associated with the nuclear membrane [8]. The role of the nuclear localization signal in CTα was explored by mutagenesis. Mutated CTα was expressed in a CHO mutant (MT-58) that was temperature-sensitive for CT activity (C. Raetz, 1980). In MT-58 cells, CT activity is present at low levels and the cells grow at 33°C. At the restrictive temperature of 40°C, there was no CT activity and the cells died via apoptosis (F. Tercé, 1996). Expression of CTα in which residues 8–28 (the nuclear localization signal) was deleted resulted in expression of CT largely, but not exclusively, in the cytoplasm [8]. These cells were able to survive at the restrictive temperature. Since some CTα was expressed in the nucleus, the experiment does not yet prove that cells can grow and divide when CTα is present only in the cytoplasm. There is intriguing evidence that CTα migrates into the cytoplasm during the G1 phase of the cell cycle, a time when PC biosynthesis is activated (R.B. Cornell, 1999). Thus, the role the nuclear localization signal of CT plays in cellular PC biosynthesis remains an intriguing question.

The lipid binding domain and the phosphorylated domains are involved in the regulation of CT activity. These domains of CTα have been deleted by either proteolysis with chymotrypsin or by construction of CTα truncation mutants [9]. CTα cDNAs that were truncated in the region of residue 314 (Fig. 4) lacked the phosphorylation segment, and CT truncated at residues 236, 231 or 228 lacked both the phosphorylation and lipid binding domains. When the lipid binding and phosphorylation domains were deleted, CT was a soluble, active enzyme that did not bind to membranes. Thus, the lipid binding domain is regulatory for the binding to membranes and the activation of CT. The binding of phospholipids to CT appears to activate the enzyme by decreasing the apparent K_m value for CTP (S.L. Pelech, 1982; S. Jackowski, 1995).

CT activity is modulated by phosphorylation. Experiments with CT truncation mutants have demonstrated that the phosphorylation domain is not required for lipid binding or CT activity. In vitro, CT is phosphorylated by casein kinase II, cdc2 kinase, cAMP kinase, protein kinase C and glycogen synthase kinase-3 but not by MAP kinase. However, the stoichiometry of phosphorylation is less than 0.2 mol P/mol CT with any of the kinases and in vitro phosphorylation does not affect enzyme activity. Exactly what role phosphorylation of CT plays in a physiologically relevant system remains to be demonstrated (see Section 4.4).

3.5. CDP-choline : 1,2-diacylglycerol cholinephosphotransferase

This enzyme was also discovered by Kennedy and coworkers [5] and is considered to be located on the ER but is also found on the Golgi, MAM and nuclear membranes [10]. Even though the enzyme has been known for more than four decades and despite intense efforts in many laboratories, the cholinephosphotransferase has never been purified. The difficulty is that the enzyme is an intrinsic membrane-bound protein that

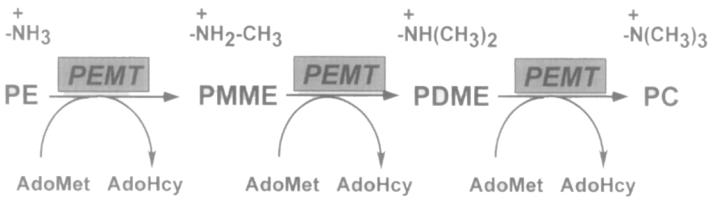


Fig. 5. Reactions catalyzed by phosphatidylethanolamine *N*-methyltransferase (PEMT). AdoMet, *S*-adenosylmethionine; AdoHcy, *S*-adenosylhomocysteine; PMME, phosphatidylmonomethylethanolamine; PDME, phosphatidyldimethylethanolamine; PC, phosphatidylcholine.

requires detergents for solubilization. Moreover, the detergents complicate purification procedures commonly used such as gel filtration because the protein binds to micelles that are hard to separate on the basis of molecular size. The purification of membrane-bound enzymes has been described as 'masochistic enzymology' (D.E. Vance, 1990).

Yeast genetics and molecular biology have, however, allowed for the cholinephosphotransferase to be cloned. Two genes, *CPT1* and *EPT1*, each account for 50% of the cholinephosphotransferase activity in yeast extracts [10]. By the use of null mutations in these two genes, it has been established that *CPT1* is responsible for 95% of the PC made and *EPT1* gene product accounts for 5%. The *EPT1* gene product utilizes both CDP-choline and CDP-ethanolamine whereas *CPT1* catalyzes only reactions with CDP-choline.

More recently a human choline/ethanolaminephosphotransferase cDNA (hCEPT1) was cloned and expressed (C.R. McMaster, 1999). The open reading frame predicts a protein with 7 membrane-spanning domains. Subsequently, the same lab cloned a human cDNA that encoded for a CDP-choline-specific enzyme (hCPT1) with 60% sequence identity to hCEPT1. hCEPT1 mRNA was detected in all tissues tested whereas the expression of hCPT1 was highest in heart, testis, intestine and colon.

Cholinephosphotransferase acts at a branch point in the metabolism of DG that can also be converted to PE, TG or PA (Fig. 1). Most studies indicate that there is an excess of cholinephosphotransferase in cells, hence, the amount of active enzyme does not limit PC biosynthesis. However, it is clear that the in vivo activity of cholinephosphotransferase is regulated by substrate supply. The supply of CDP-choline is regulated by the activity of CT (Section 3.4). The supply of DG in liver seems to be controlled by the supply of fatty acids. Excess DG not utilized for PC or PE biosynthesis is stored in liver as TG.

3.6. Phosphatidylethanolamine N-methyltransferase

All nucleated cells contain PC and the CDP-choline pathway. Thus, it was not obvious why the pathway for PE methylation (Fig. 5) survived during evolution. Nor was it obvious why PE methyltransferase (PEMT) activity is mostly found in liver whereas 2% or less of the hepatic PEMT activity is found in other tissues of the body.

PEMT was purified from rat liver microsomes although it is an intrinsic membrane protein (N.R. Ridgway, 1987). Sequence of the amino terminal enabled the cloning of

the cDNA for PEMT (Z. Cui, 1993). Preparation of an antibody to the deduced sequence of the carboxyl terminal peptide permitted subcellular localization of the enzyme. The major activity for PEMT is found on the ER but the antibody only recognized a protein that was exclusively localized to MAM (J.E. Vance, 1990). This isoenzyme of PEMT is referred to as PEMT2 and the activity on the ER is called PEMT1. Both PEMTs catalyze all three transmethylation reactions that convert PE to PC (Fig. 5).

A mouse was generated in which the *Pemt* gene was disrupted and there was no PEMT activity [11]. The $Pemt^{-/-}$ mice lived and bred normally and there was a 50% increase in CT activity in their livers. Since the mice retained the CDP-choline pathway, the lack of an obvious phenotype was not surprising. However, when the mice were fed a choline-deficient diet for 3 days, which attenuates PC synthesis via the CDP-choline pathway, the $Pemt^{-/-}$ mice exhibited severe liver failure [12]. $Pemt^{+/+}$ mice fed a choline-deficient diet were normal with no obvious liver pathology. Thus, it seems that the PEMT pathway has survived in evolution to provide PC at times when the CDP-choline pathway is less active such as might occur during starvation. Moreover, pregnant rats and suckling mothers can also have choline reserves depleted (S.H. Zeisel, 2000), hence, the PEMT pathway might provide an evolutionary advantage in this respect. The structurally related compound, dimethylethanolamine [$HOCH_2$–CH_2–$N^+(CH_3)_2$] would not substitute for choline in the $Pemt^{-/-}$ mice even though it was converted to phosphatidyldimethylethanolamine (K.A. Waite, 2002). Thus, it seems that the third methyl group on the phospholipid has a critical function in mice.

Further studies on $Pemt^{-/-}$ mice showed that male, but not female, $Pemt^{-/-}$ mice fed a high fat/high cholesterol diet have a defect in secretion of very low density lipoproteins that contain apolipoprotein B100 (A. Noga, 2002) (Chapter 19). The mechanism(s) for this sexual dimorphism is not clear.

The human gene encoding PEMT has been cloned and characterized. Whereas only one mRNA transcript has been identified in mice, human liver has three separate mRNAs that differ only at the 5′ end, in a non-coding region of the transcript (D.J. Shields, 2001). Thus, the three transcripts encode the same protein. The function of separate PEMT mRNAs is going to be difficult to study in humans.

Yeast also has both the PE methylation pathway and the CDP-choline pathway. In yeast two enzymes are used for the conversion of PE to PC [13]. The methylation of PE to phosphatidylmonomethylethanolamine is catalyzed by the *PEM1/CHO2* gene product whereas the subsequent two methylations are catalyzed by the *PEM2/OPI3* gene product. Deletion of both *PEM1* and *PEM2* genes is lethal unless the yeast is supplied with choline. Yeast normally grows in the absence of choline and depend on the PEMT pathway. Thus, the CDP-choline pathway and the PE methylation pathway can compensate for each other in yeast.

Bacteria generally do not contain PC but *Rhodobacter sphaeroides* make PC by methylation of PE. Interestingly, this enzyme is soluble and has virtually no homology to PEMT or the yeast enzymes (V. Arondel, 1993). Also in one bacterium, a novel choline-dependent pathway was recently discovered in *Sinorhizobium meliloti* in which choline reacts with CDP-DC to form PC (O. Geiger, 2000).

4. Regulation of phosphatidylcholine biosynthesis

4.1. The rate-limiting reaction

The CT reaction usually limits the rate of PC biosynthesis. The first evidence in favor of this conclusion was measurement of pool sizes of the aqueous precursors (in rat liver, choline = 0.23 mM, phosphocholine = 1.3 mM, CDP-choline = 0.03 mM). These values assume that 1 g wet tissue is 1 ml and there is no compartmentation of the pools. The second assumption may not be valid as there is evidence for compartmentation of PC precursors (M. Spence, 1989). Nevertheless, the relative amounts of these compounds might be correct in the biosynthetic compartment(s). The concentration of phosphocholine is 40-fold higher than CDP-choline which is consistent with a 'bottleneck' in the pathway at the reaction catalyzed by CT.

Pulse-chase experiments demonstrate this bottleneck more vividly. After a 0.5 h pulse of hepatocytes with [*methyl*-^{3}H]choline, more than 95% of the radioactivity in the precursors of PC was in phosphocholine, the remainder in choline and CDP-choline. When the cells were chased with unlabeled choline in the medium, labeled phosphocholine was quantitatively converted to PC (Fig. 6). The radioactivity in CDP-choline remained low during the chase and CDP-choline was rapidly converted to PC. There was minimal radioactivity in choline which suggests that choline is immediately phosphorylated after it enters the cell.

One additional point should be made. If a cell or tissue is in a steady state, pool sizes and reaction rates are not changing. Thus, although the rate of PC synthesis is determined by the CT reaction, the rates of the choline kinase and cholinephosphotransferase reactions will be the same as that catalyzed by CT. Otherwise, changes in the pool sizes of precursors would occur. For example, if the choline kinase reaction were faster than

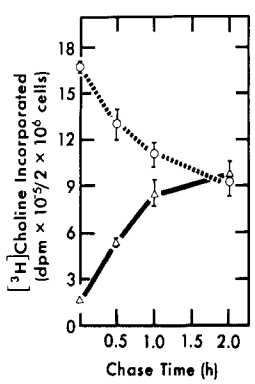


Fig. 6. Incorporation of [^{3}H-*methyl*]choline into phosphocholine and PC as a function of time. Hepatocytes from rat liver were incubated with labeled choline for 30 min. Subsequently, the cells were washed and incubated (chased) for various times with unlabeled choline. The disappearance of radioactivity from phosphocholine (dashed line) and its appearance in PC (solid line) are shown. Adapted from fig. 1 of Pelech et al. (1983), J. Biol. Chem. 258, 6783, with permission.

the CT reaction, there would be an increase in the amount of phosphocholine. Thus, CT sets the pace, but the other reactions proceed at the same rate.

4.2. The translocation hypothesis

CT is recovered from cells and tissues in both cytosol and microsomal fractions. However, in the early 1980s evidence from several laboratories suggested a close correlation between CT activity on the microsomal membranes and the rate of PC biosynthesis. The hypothesis was that the active form of the enzyme was on cellular membranes and CT in the cytosol acted as a reservoir (Fig. 3). In agreement with this proposal, cytosolic fractions contain essentially no phospholipid and CT requires phospholipids for activity. Thus, cells have a facile mechanism for altering the rate of PC biosynthesis by a reversible translocation of CT between a soluble, inactive reservoir and cellular membranes. This mechanism for activation applies to CTα and both CTβ1 and CTβ2. This hypothesis remains basically valid except much of the 'cytosolic' CT may originate from the nucleus and activated CT may be associated with the nuclear membrane as well as the endoplasmic reticulum.

Binding of CT to membranes begins by electrostatic adsorption followed by hydrophobic interactions that involve intercalation of the protein into the nonpolar core of the membrane [9] (Fig. 7). When insertion of CT into the membrane lipids is blocked by using viscous gel phase lipids, CT binds electrostatically to the membrane but is not activated. Four properties of membranes promote CT insertion [9]: (1) interfacial packing defects as might occur when lipids with small head groups such as DG are in the membrane; (2) low lateral surface pressure (loose packing) as observed in highly curved compared to planar bilayers; (3) acyl chain disorder that can be caused by oxidation of the fatty acyl chains; (4) curvature strain that would occur when membranes are enriched in hexagonal phase-preferring lipids such as PE and DG. Synthesis of PC would reverse these properties of membranes and form a more stable bilayer.

4.3. Regulation of phosphatidylcholine biosynthesis by lipids

As indicated in Fig. 3, the association of CT with membranes and CT activation can be modulated by lipids. Both feed-forward and feed-back mechanisms for regulation of CT activity have been identified. DG may alter the rate of PC biosynthesis both as a substrate and as a modulator of CT binding to membranes. In vitro an increase in the content of DG in membranes enhanced the binding of CT.

Feedback regulation of CT and PC biosynthesis by PC has also been described (H. Jamil, 1990). Regulation of a metabolic pathway by product inhibition is commonly observed. In hepatocytes derived from choline-deficient rats, the rate of PC biosynthesis was inhibited by approximately 70% compared to choline-supplemented rats, the amount of PC declined and there was a corresponding increased binding of CT to membranes. CT appeared to sense a need for increased PC biosynthesis and was poised on the membrane prepared for catalysis. However, in choline-deficient cells there is less substrate, phosphocholine, so increased PC biosynthesis could not occur. When choline-deficient hepatocytes were supplied with choline, there was a positive

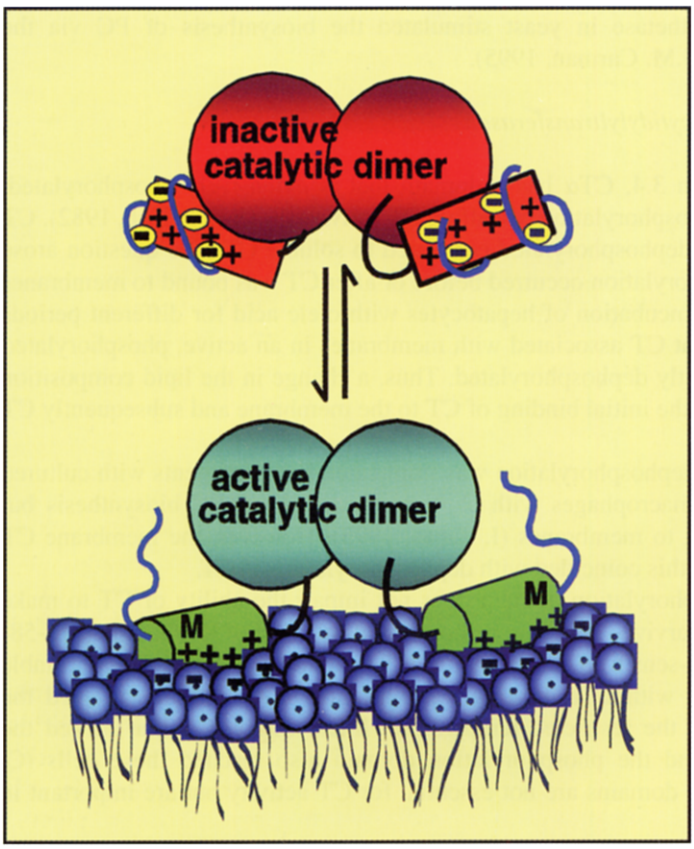


Fig. 7. Translocation of CTP : phosphocholine cytidylyltransferase (CT) from an inactive soluble form to a membrane-associated activated form. The reversible interaction of CT with membranes involves the amphipathic helical region lying on the surface of the membrane with the hydrophilic side interacting with the negatively charged lipid head-groups and the hydrophobic side intercalating into the membrane core. From Cornell and Northwood [9] with permission.

correlation between the increased level of PC and the release of soluble CT. Similar correlations were observed when the level of PC was increased, either by providing methionine for enhanced conversion of PE to PC, or by providing lyso-PC which is imported into hepatocytes and acylated to PC.

An elegant feedback regulation of CT has been shown in the yeast *Saccharomyces cerevisiae* (V.A. Bankaitis, 1995). SEC14p is a phospholipid transfer protein that when assayed in vitro prefers phosphatidylinositol (PI) and PC and is an essential gene product (Chapter 17). SEC14p inhibited the CDP-choline pathway when PC was bound to SEC14p. In contrast, when PI was bound to SEC14p, there was minimal inhibition of CT. Thus, in yeast under conditions where PC is abundant, there is a feedback inhibition of CT and the CDP-choline pathway.

CTP, has also been implicated as regulatory in animal systems and yeast. Over-

expression of CTP synthetase in yeast stimulated the biosynthesis of PC via the CDP-choline pathway (G.M. Carman, 1995).

4.4. Phosphorylation of cytidylyltransferase

As mentioned in Section 3.4, CTα has a domain that is extensively phosphorylated. Moreover, the state of phosphorylation can affect CT activity [8] (S.L. Pelech, 1982). CT bound to membranes is dephosphorylated compared to soluble CT. The question arose whether or not dephosphorylation occurred before or after CT was bound to membranes (M. Houweling, 1994). Incubation of hepatocytes with oleic acid for different periods of time demonstrated that CT associated with membranes in an active, phosphorylated form and was subsequently dephosphorylated. Thus, a change in the lipid composition of membranes mediated the initial binding of CT to the membrane and subsequently CT was dephosphorylated.

Activation of CT by dephosphorylation was implicated in experiments with cultured macrophages. Loading macrophages with cholesterol stimulated PC biosynthesis but did not alter CT binding to membranes (I. Tabas, 1995). However, the membrane CT increased in activity and this coincided with dephosphorylation of CT.

Deletion of the phosphorylation domain does not impair the ability of CT to make enough PC for cells to survive. This was demonstrated in a line of CHO cells (MT-58) that have a temperature-sensitive defect in the CT reaction (C. Kent, 1995). Stable transfection of the cells with CTα lacking the phosphorylation domain allowed the MT-58 cells to grow at the restricted temperature. Interestingly, CT that lacked the lipid binding domain and the phosphorylation domain also rescued these cells (C. Kent, 1999). Thus, these domains are not essential for CT activity but are important in regulating CT activity.

4.5. Transcriptional and post-transcriptional regulation of CTα

Most studies on CT activity and PC biosynthesis have not indicated regulation at the level of gene expression. The ability of a cell to activate the soluble form of CT would normally satisfy the cell's requirement for PC. Nevertheless, some control over the expression of the genes encoding CT must occur. The proximal promoter of the CTα gene has numerous potential regulatory elements (I. Tabas, 1997). Subsequent studies showed that Sp1, the first mammalian transcription factor purified and cloned (R. Tjian, 1986, 1987), had an important role in regulating the expression of the CTα gene [14]. The related nuclear factor, Sp3, could also activate CTα transcription (M. Bakovic, 2000). DNase protection assays indicated several elements in the proximal promoter bound unidentified nuclear factors. The yeast one hybrid system was utilized to clone the cDNA for one of these factors and transcription enhancer factor 4 (TEF4) was identified as a regulator of CTα transcription (H. Sugimoto, 2001). These initial studies were done in experiments involving transfections with various cDNA constructs. The first experiments to search for transcriptional regulation in a cell physiology-relevant system were on CTα expression in a murine fibroblast cell line as a function of the cell cycle (L. Golfman, 2001). During the G0 to G1 phase of the cell cycle there is an

increase in PC biosynthesis (S. Jackowski, 1996; L. Golfman, 2001) but there was no enhanced transcription of the CTα gene. Instead, increased transcription occurred during the S phase of the cell cycle, possibly to increase the amount of CTα in preparation for mitosis. Obviously, there is much to be done to elucidate the factors and DNA elements involved in transcriptional regulation of the CTα gene. There are no reports on transcriptional regulation of the CTβ gene.

SREBPs play a critical role in regulating the expression of genes involved in fatty acid (Chapters 6 and 7) and cholesterol (Chapter 15) metabolism. Thus, several labs explored whether or not SREBPs might alter the expression of the CTα gene. Interestingly, one report indicated there was no direct modulation of CTα transcription (N. Ridgway, 2000) whereas research from another lab implicated sterols and SREBPs in the regulation of CTα transcription (P.A. Edwards, 2001). Further work is required on the relationship between SREBPs and PC biosynthesis.

The level of CTα mRNA can also be regulated by alterations in mRNA stability. When a macrophage cell line was depleted of colony stimulating factor and then repleted there was a 4-fold induction of mRNA for CTα (S. Jackowski, 1991). The stability of CTα mRNA increased after the addition of colony stimulating factor. An increase in CTα mRNA in fetal lung type II cells has also been ascribed to enhanced mRNA stability (M. Post, 1996).

Finally, enhanced turnover of CTα via the ubiquitin–proteosome pathway appears to be the mechanism by which tumor necrosis factor decreases the level of CTα in alveolar type II cells (R. Mallampalli, 2000).

4.6. Transgenic and gene-disrupted murine models of CTα

To determine if enhanced PC biosynthesis would protect macrophages from excess cholesterol-induced toxicity, genetically modified mice have been generated. A truncated version of CTα lacking the phosphorylation domain was expressed specifically in macrophages of mice under control of the scavenger receptor (Chapter 21) promoter (I. Tabas, 1999). These cells were protected from cholesterol-induced toxicity. In another approach, CTα expression was eliminated in macrophages using the Cre-lox method for selective disruption of a gene in specific cells (I. Tabas, 2000). The lack of the CTα gene and hence decreased PC biosynthesis caused enhanced sensitivity to cholesterol loading. In the absence of cholesterol loading, the macrophages without CTα appeared normal, possibly due to increased expression of CTβ2.

5. Phosphatidylethanolamine biosynthesis

5.1. Historical background and biosynthetic pathways

PE was first alluded to in a book published by Thudichum in 1884. He described 'kephalin' as a nitrogen- and phosphorus-containing lipid that was different from lecithin. In 1913, Renall and Baumann independently isolated ethanolamine from

kephalin. In 1930, Rudy and Page isolated the first pure preparation of PE. The structure of PE was established in 1952 by Baer and colleagues.

The biosynthesis of PE in eukaryotes can occur via four pathways (Fig. 8). The route via CDP-ethanolamine constitutes de novo synthesis of PE. The other pathways arise as a result of the modification of a pre-existing phospholipid. The CDP-ethanolamine pathway was first described by Kennedy and Weiss in 1956. The decarboxylation of phosphatidylserine (PS) to yield PE (Fig. 8) was shown in 1960 to occur in animal cells. PS decarboxylation is the only route for PE biosynthesis in *E. coli* (Chapter 3). The PE generated by this pathway can react with serine to generate PS and ethanolamine (Fig. 8). This appears to be one mechanism by which ethanolamine is made in cells. The other involves degradation of sphingosine (Chapter 14). The ethanolamine generated by either pathway can be utilized for PE biosynthesis via the CDP-ethanolamine pathway. No one has ever been able to show the decarboxylation of serine to ethanolamine in animal cells. Such a reaction was shown to occur in a plant, *Arabidopsis thaliana* (A.D. Hanson, 2001). PE can also be formed by reacylation of lyso-PE or reaction of ethanolamine with PS (Fig. 8).

5.2. Enzymes of the CDP-ethanolamine pathway

As mentioned in Section 3.3, the phosphorylation of ethanolamine in liver can be catalyzed by choline/ethanolamine kinase (Figs. 1 and 8). The cDNA encoding an ethanolamine kinase was cloned from *Drosophila* (P. Pavlidis, 1994). These scientists did not plan on cloning this cDNA since their approach was to determine the gene responsible for the easily shocked (*eas*) phenotype in this insect. These mutant flies display transient paralysis following a brief mechanical shock. In the *eas* mutant, a 2 base pair deletion caused formation of a premature stop codon in the ethanolamine kinase gene. Analysis of the phospholipids showed a decrease in PE from 59% of the total phospholipid in wild type to 56% in *eas*. How this minor change mediates the paralysis is not known. The difference may reflect a major change in PE content in a particular tissue or subcellular membrane. More recently, the gene that encodes a yeast ethanolamine kinase (G.M. Carman, 1999) and a human cDNA for ethanolamine-specific kinase (S. Jackowski, 2001) were cloned and expressed.

The second step in the CDP-ethanolamine pathway is catalyzed by CTP : phosphoethanolamine cytidylyltransferase [15]. The enzyme is distinct from CT and is not activated by lipids. Although the phosphoethanolamine cytidylyltransferase is recovered in cytosol from cell extracts, much of the enzyme has been localized to rough ER of rat liver by immunoelectronmicroscopy. Unlike CTα, there is no report of the phosphoethanolamine cytidylyltransferase in the nucleus.

CDP-ethanolamine : 1,2-diacylglycerol ethanolaminephosphotransferase is an integral membrane protein found on the ER, Golgi and MAM. The enzyme shows a distinct preference for DG species that contain 1-palmitoyl-2-docosahexaenoyl (22 : 6) fatty acids. In hepatocytes in culture, nearly 50% of PE made via the ethanolaminephosphotransferase reaction is this species. The purpose of this extraordinary selectivity is unknown. The bovine hepatic enzyme was purified and exhibited both ethanolamine- and choline-phosphotransferase activity (L. Binaglia, 1999).

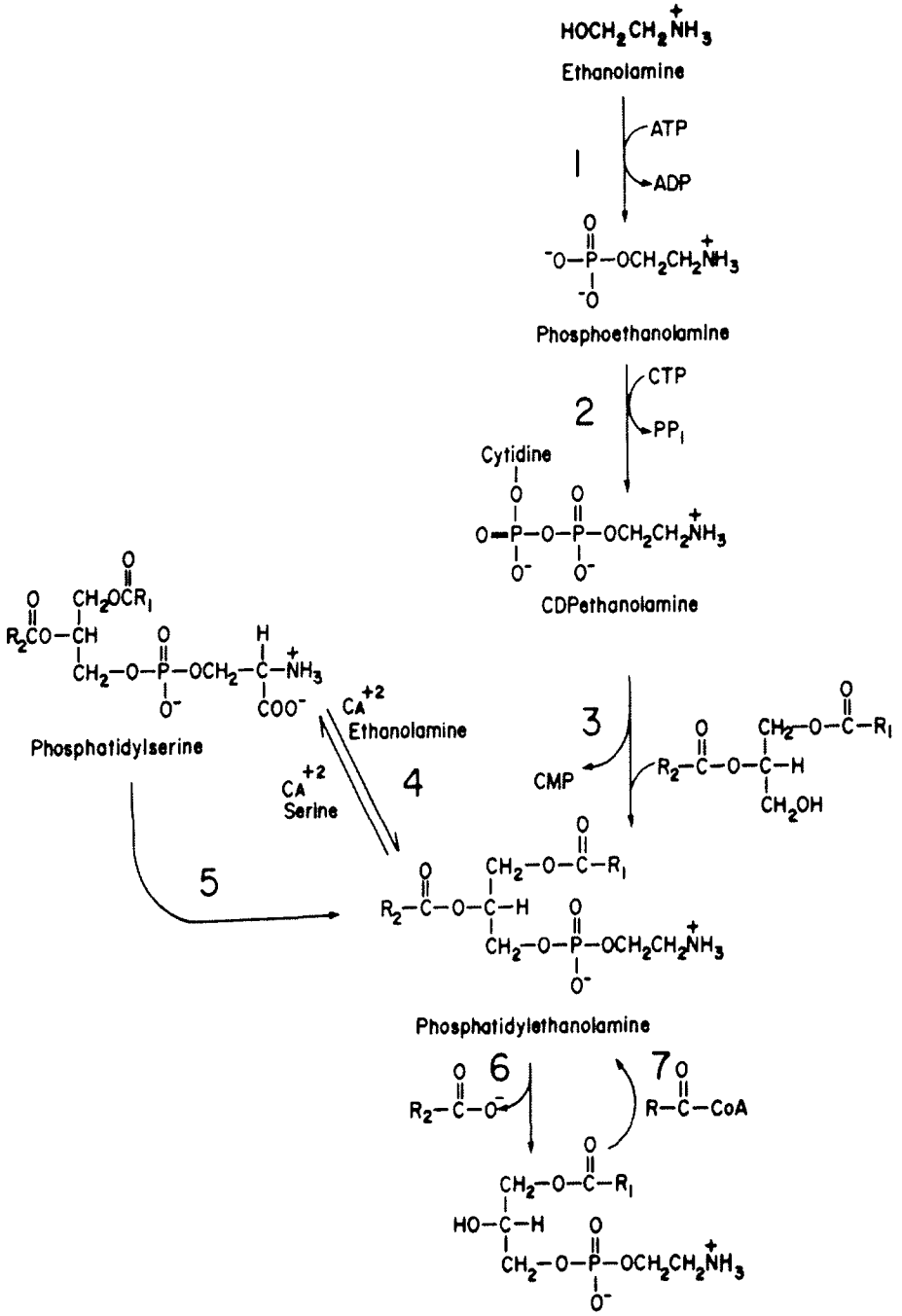


Fig. 8. Pathways for the biosynthesis of PE and PS. The numbers indicate the enzymes involved. 1, ethanolamine(choline) kinase; 2, CTP : phosphoethanolamine cytidylyltransferase; 3, CDP-ethanolamine : 1,2-diacylglycerol ethanolaminephosphotransferase; 4, PS synthase; 5, PS decarboxylase; 6, phospholipase A2; 7, acyl-CoA : lyso-PE acyltransferase.

A yeast gene (*EPT1*) that encodes an ethanolaminephosphotransferase and a human cDNA that encodes a choline/ethanolaminephosphotransferase have been cloned as discussed in Section 3.5.

5.3. Regulation of the CDP-ethanolamine pathway

Unlike CT, there is minimal literature on the mechanisms that control the activity of phosphoethanolamine cytidylyltransferase. Åkesson and Sundler in the 1970s found that phosphoethanolamine cytidylyltransferase was rate-limiting for PE biosynthesis. However, the supply of DG as a substrate can also limit the rate of PE biosynthesis (L.B.M. Tijburg, 1989). Thus, both the supply of CDP-ethanolamine from the cytidylyltransferase reaction and the supply of DG can regulate PE biosynthesis. Two studies have implicated channeling of intermediates in the biosynthesis of PE in mammalian cells [15].

5.4. Phosphatidylserine decarboxylase

PS decarboxylase is found in both prokaryotes (Chapter 3) and the mitochondria of eukaryotes. The enzyme activity was first described by Kanfer and Kennedy in 1964. The enzyme has not been purified from a eukaryotic source but the gene has been cloned and expressed from CHO cells (M. Nishijima, 1991) and yeast [16]. The yeast gene (*PSD1*) encodes a protein that is localized to mitochondria. However, when *PSD1* was disrupted in yeast, 5% PS decarboxylase activity remained and the yeast continued to grow. Subsequently, a second gene, *PSD2*, was isolated. When both *PSD1* and *PSD2* were disrupted, the yeast became ethanolamine auxotrophs. The PSD2 protein has been localized to the vacuolar and Golgi compartments. The function of *PSD2* is not known other than it can supply enough PS decarboxylase to allow growth of yeast in the absence of *PSD1*. The rate of PS decarboxylation is determined by the rate of PS transport into mitochondria (Chapter 17).

6. Phosphatidylserine biosynthesis

6.1. Historical developments and biosynthesis

PS accounts for 5–15% of the phospholipids in eukaryotic cells. The lower concentration of PS compared to PC and PE is probably the reason PS was not discovered as a separate component of 'kephalin' (originally identified to be only PE in 1930) until 1941 by Folch. The correct structure was proposed by Folch in 1948 and confirmed by chemical synthesis in 1955 by Baer and Maurukas. PS is a required cofactor for protein kinase C and is required for initiation of the blood clotting cascade. In the plasma membrane of cells PS is normally located on the inner monolayer. During apoptosis, exposure of PS on the cell surface (outer monolayer) leads to recognition and removal of these cells by macrophages (V. Fadok, 1992).

PS is made in prokaryotes (Chapter 3), in some plants and yeast (S. Yamashita, 1997) via the CDP-diacylglycerol pathway. This route does not exist in animals. Instead, PS is

made by a base-exchange reaction catalyzed by PS synthase first described by Hübscher in 1959 (reaction 4 in Fig. 8) in which the head group of a PC or PE is exchanged for serine.

6.2. Chinese hamster ovary cell mutants and regulation

CHO mutants were generated that were auxotrophic for PS and demonstrated that these cells have two PS synthases [17]. PS synthase 1 utilizes PC and serine as substrates whereas PS synthase 2 utilizes only serine and PE. The two PS synthases, when coupled with PS decarboxylase, yield PS at the expense of PC and generate both choline and ethanolamine which could be recycled into the biosynthesis of PC and PE. As a result, PS and PE can both be generated without a decline in the amount of PC.

$$\text{PC} + \text{serine} \xrightarrow{\text{PS synthase 1}} \text{PS} + \text{choline}$$

$$\text{PS} \xrightarrow{\text{PS decarboxylase}} \text{PE} + \text{CO}_2$$

$$\text{PE} + \text{serine} \xrightarrow{\text{PS synthase 2}} \text{PS} + \text{ethanolamine}$$

The sum of the reactions is:

$$\text{PC} + \text{two serines} \longrightarrow \text{PS} + \text{choline} + \text{ethanolamine} + \text{CO}_2$$

A CHO mutant defective in PS synthase 1 was used to clone by complementation the cDNA for this enzyme [17]. The deduced amino acid sequence for murine PS synthase 1 was >90% identical to the CHO enzyme (S. Stone, 1998). The cDNA for PS synthase 2 from CHO cells was cloned and shown to be 32% identical in amino acid sequence to PS synthase 1 [17]. Immunoblot analysis indicated that both of the murine PS synthases are mainly localized to MAM (S. Stone, 2000). The source of the substantial PS synthase activity in the rough and smooth ER remains unknown, possibly a third PS synthase activity. The mRNAs encoding PS synthases 1 and 2 were found in all murine tissues examined but PS synthase 2 was enriched in testis and kidney (J.E. Vance, 2001).

Our understanding of regulation of PS biosynthesis is in its infancy. Addition of exogenous PS to the medium of CHO cells feedback inhibited the biosynthesis of PS [17]. CHO mutants in which Arg-95 of PS synthase 1, or Arg-97 in PS synthase 2, were replaced by lysine were no longer sensitive to inhibition by PS (M. Nishijima, 1998, 1999). There also appears to be 'cross-talk' between PE biosynthesis via the CDP-ethanolamine pathway and PS synthase 1/PS decarboxylation pathway since over-expression of PS synthase 1 increased production of PE from decarboxylation of PS and decreased PE biosynthesis via the CDP-ethanolamine pathway (S. Stone, 1999). Interestingly, over-expression of PS synthase 2 did not alter the activity of the CDP-ethanolamine pathway.

The murine gene for PS synthase 1 has been cloned and characterized (J.E. Vance, 2001). This is an important step toward the generation of mice with a disrupted gene for PS synthase 1. The gene for PS synthase 2 has been disrupted and the mice are viable (S. Young, 2002).

7. *Inositol phospholipids*

7.1. Historical developments

A major fate of PA is conversion to DG that is metabolized to PC, PE and TG (Fig. 1). Alternatively, PA can react with CTP to form CDP-DG that is utilized for the biosynthesis of the inositol phospholipids, phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG) (Fig. 1).

Inositol is a cyclohexane derivative in which all 6 carbons are substituted with hydroxyl groups. The most common isoform is *myo*-inositol but other less abundant inositols with different structures also occur. The first report of an inositol-containing lipid was in 1930 from *Mycobacteria* [18] which is ironic since inositol lipids are rarely found in bacteria. Brain is the richest source of these lipids, as first discovered by Folch and Wooley in 1942. In 1949, Folch described PI phosphate (PI-P) which was later found to include PI and PI bisphosphate (PI-P_2). The chemical structures of PI, PI-P and PI-P_2 were determined by Ballou and coworkers between 1959 and 1961. PI (1.7 μmol/g liver) constitutes around 10% of the phospholipids in a cell or tissue. PI-P and PI-P_2 are present at much lower concentrations (1–3% of PI). Agranoff et al. published the first experiments in 1958 on the incorporation of [^{3}H]inositol into PI. Subsequently, Paulus and Kennedy showed that CTP was the preferred nucleotide donor.

7.2. CDP-diacylglycerol synthase

Regulation of the conversion of PA to CDP-DG is not well understood. The enzyme, CDP-DG synthase, is largely microsomal but is also found in the mitochondrial inner membrane.

A cDNA encoding CDP-DG synthase 1 was cloned from *Drosophila* (C.S. Zuker, 1995). This isoform is specifically located in photoreceptor cells of *Drosophila*. Mutations in this isoform lead to a defect in PI-P_2 biosynthesis. As a result mutant photoreceptor cells show severe defects in their phospholipase C-mediated signal transduction that can be rescued by re-introduction of the CDP-DG synthase cDNA.

CDNAs encoding human and murine CDP-DG synthases 1 and 2 were more recently cloned (S. Jackowski, 1997; B. Franco, 1999). CDP-DG synthase 2 is expressed during embryogenesis in the central nervous system whereas CDP-DG synthase 1 had a high level of expression in adult retina.

Curiously, in *Saccharomyces cerevisiae*, CDP-DG synthase activity is found in microsomes and the mitochondrial inner membrane even though only one gene encodes this activity [19]. Since only a single mRNA species was found, there may not be alternative splicing of the yeast gene. The yeast CDP-DG synthase gene is essential for cell viability as well as germination of spores.

7.3. Phosphatidylinositol synthase

Three potential sources for cellular inositol are: diet, de novo biosynthesis and recycling of inositol. Biosynthesis of inositol from glucose occurs in the brain and testes, and

other tissues to a lesser extent. The rate-limiting step appears to be the synthesis of inositol-3-phosphate from glucose-6-phosphate [20]. Inositol-3-phosphate is hydrolyzed to inositol by a phosphatase.

PI synthase was purified from human placenta [21]. When the cDNAs encoding either CDP-DG synthase 1 or phosphatidylinositol synthase, or both, were over-expressed in COS 7 cells, there was no change in the rate of PI biosynthesis indicating that the level of these enzymes was not limiting for PI biosynthesis (S. Jackowski, 1997).

Disruption of the PI synthase gene in yeast is lethal indicating that PI is essential [22]. Further information on the inositol phospholipids and their functions is covered in Chapter 12.

8. Polyglycerophospholipids

8.1. Historical developments and biosynthetic pathways

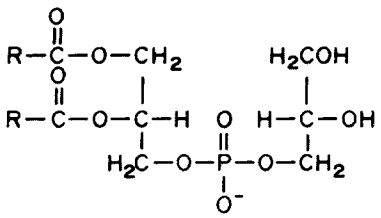
Diphosphatidylglycerol (DPG), commonly known as cardiolipin, was discovered in 1942 in beef heart by Pangborn. The correct structure (Fig. 9) was proposed in 1956–1957 and confirmed by chemical synthesis in 1965–1966 by de Haas and van Deenen. Phosphatidylglycerol (PG) was first isolated in 1958 from algae by Benson and Mauro. The structure was confirmed by Haverkate and van Deenen in 1964–1965. The third lipid in this class, bis(monoacylglycerol)phosphate was recovered from pig lung by Body and Gray in 1967. The stereochemistry differs from PG and DPG since bis(monoacylglycerol)phosphate contains *sn*-(monoacyl)glycerol-1-phospho-*sn*-1′-(monoacyl)-glycerol rather than a *sn*-glycerol-3-phospho linkage.

These three lipids (Fig. 9) are widely distributed in animals, plants, and microorganisms. In animals, DPG is found in highest concentration in cardiac muscle (9–15% of phospholipid), hence the name cardiolipin, and is exclusively found in the mitochondria. PG is generally present at a concentration of less than 1% of total cellular phospholipids, except in lung, where it comprises 2–5% of the phospholipid. In pulmonary surfactant and alveolar type II cells, PG is 7–11% of the total lipid phosphorous. Bis(monoacylglycerol)phosphate comprises less than 1% of total phospholipids in animal tissues, except in alveolar (lung) macrophages where it is 14–18% of total phospholipid.

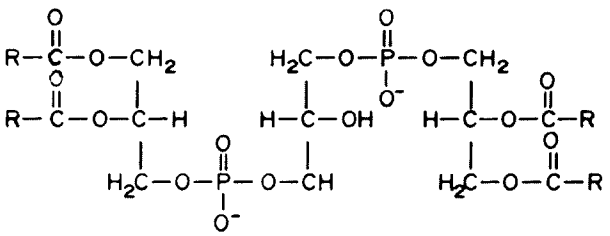
The biosynthesis of PG was elucidated by Kennedy and coworkers in 1963 (Fig. 1). For DPG biosynthesis PA is transferred from CDP-DG to PG to yield DPG. DPG synthesis in *E. coli* differs and involves the condensation of two molecules of PG (Chapter 3).

Understanding the biosynthesis of bis(monoacylglycerol)phosphate has been a particular challenge because the carbon linked to the phosphate residue is the *sn*-1 rather than *sn*-3 configuration. The likely biosynthetic pathway is depicted in Fig. 10 [23].

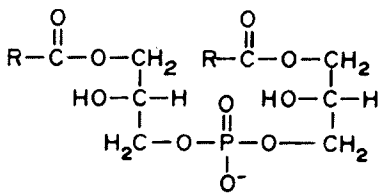
An intermediate in the biosynthesis of bis(monoacylglycerol)phosphate is 1-acyl-lyso-PG (Fig. 10), also known as lysobis-PA. Recent studies have shown that the inner membranes of late endosomes are enriched in lysobis-PA and that these membranes play an important role in the sorting of insulin growth factor receptor 2 and the mannose-



Phosphatidylglycerol



Diphosphatidylglycerol



Bis(monoacylglycero)phosphate

Fig. 9. Structures of polyglycerophospholipids.

6-phosphate receptor [24]. Moreover, lysobis-PA cross-reacts with antibodies produced in patients with antiphospholipid syndrome. Possibly, some of the pathological defects in this disease could arise from disruption of endosomal traffic. Moreover, the defect in cholesterol trafficking in Niemann-Pick C disease (Chapter 17) may also involve lysobis-PA (J. Gruenberg, 1999).

8.2. Enzymes and subcellular location

PG can be made in mitochondria and microsomes from various animal cells and, except for lung, appears to be primarily converted to DPG. DPG is biosynthesized exclusively on the matrix side of the mitochondrial inner membrane and is found only in this organelle. DPG synthase requires Co^{2+} for activity (K.Y. Hostetler, 1991). There is evidence that the rate-limiting step in DPG biosynthesis is the conversion of PA into CDP-DG (G.M. Hatch, 1994). Consistent with this idea, the levels of CTP have been shown to regulate DPG biosynthesis in cardiac myoblasts (G.M. Hatch, 1996).

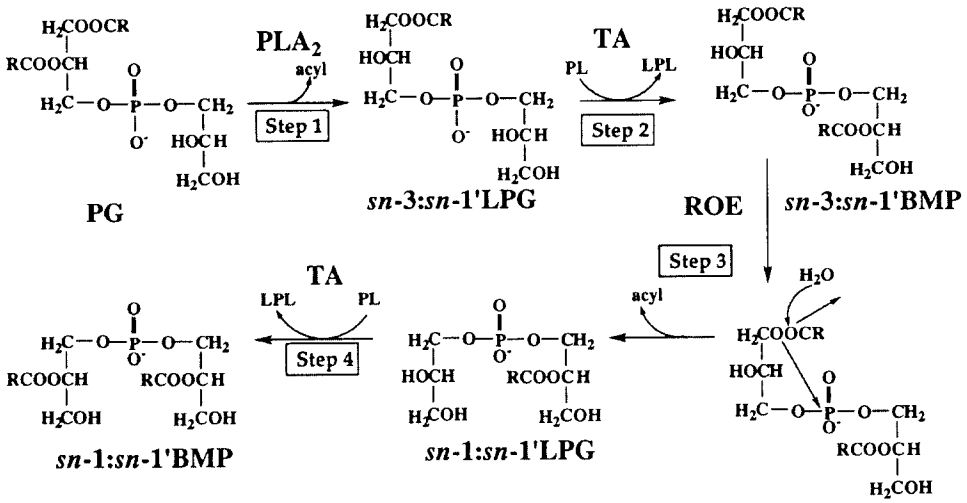


Fig. 10. Proposed pathway for the biosynthesis of bis(monoacylglycero)phosphate. Phospholipase A_2 (PLA_2) hydrolyzes PG to 1-acyl-lyso-PG (LPG). LPG is then acylated by a transacylase (TA), using a phospholipid (PL) as the acyl donor, to form bis(monoacylglycero)phosphate (BMP) that still retains the *sn*-3 : *sn*-1′ stereoconfiguration of the original PG and a lysophospholipid (LPL). The glycerol backbone of the *sn*-3 : *sn*-1′-BMP is reoriented by an enzymatic activity (ROE) to yield *sn*-1 : *sn*-1′-LPG (step 3). The final product, *sn*-1 : *sn*-1′-BMP, is formed upon acylation of *sn*-1 : *sn*-1′-LPG (step 4). The assignment of the acyl residues to the *sn*-2 positions of both glycerol moieties is based on their being primarily unsaturated and from degradation studies. It is believed that spontaneous rearrangement can occur so that the acyl residues end up on the *sn*-3 carbons as shown in Fig. 9. Figure from Amidon et al. [23] with permission.

Using techniques developed by Raetz and coworkers [25] M. Nishijima (1993) and coworkers isolated a temperature-sensitive mutant in PG-P synthase of CHO cells. The mutant had 1% of wild type CHO PG-P synthase activity at 40°C and a temperature-sensitive defect in PG and DPG biosynthesis. This mutant was used to show that DPG is required for the NADH-ubiquinone reductase (complex I) activity of the respiratory chain.

In yeast DPG synthesis has been genetically interrupted [26]. The yeast grows at temperatures between 16 and 30°C without DPG but fails to grow at 37°C on fermentable carbon sources such as glucose even though intact mitochondria are, therefore, not required for ATP synthesis. Thus, mitochondria must have some necessary function in yeast survival other than generating energy [26].

The fatty acyl content of phospholipids can also impact on mitochondrial function. Incubation of cardiomyocytes with palmitic acid increased the palmitic acid content of PA and PG and decreased DPG levels in mitochondria with a concomitant release of cytochrome *c* leading to apoptosis (W. Dowhan, 2001).

9. *Remodeling of the acyl substituents of phospholipids*

Phospholipids are made de novo with the fatty acid compositions present in the precursors DG and CDP-DG. Once the phospholipid is made, the fatty acid substituents

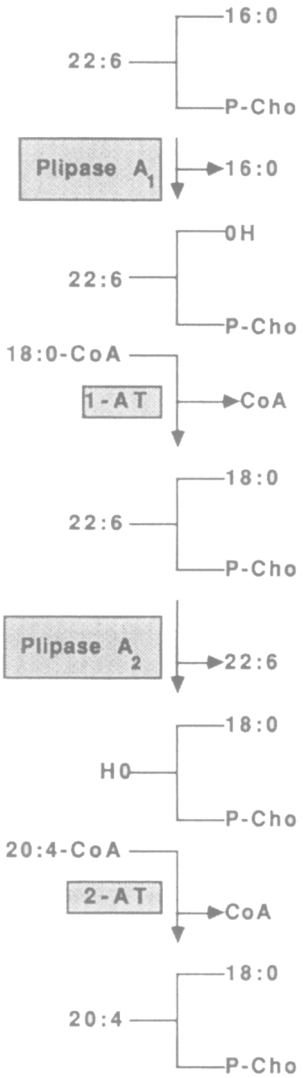


Fig. 11. Fatty acids at both the *sn*-1 and *sn*-2 positions of PC can be deacylated by phospholipases and reacylated by acyltransferases. Palmitic acid (16:0) can be removed from the *sn*-1 position and replaced with stearic acid (18:0). The fatty acid at the *sn*-2 position is depicted as docosahexaenoic acid (22:6) which can be replaced with 20:4 or 18:2. If the fatty acid at the *sn*-2 position were oleic acid, it could also be deacylated and reacylated. Alternatively, deacylation/reacylation could occur initially at the *sn*-2 position. Plipase, phospholipase; 1-AT, acyl-CoA : lyso-PC 1-acyltransferase; 2-AT, acyl-CoA : lyso-PC 2-acyltransferase; cho, choline.

can be remodeled via deacylation–reacylation reactions (Fig. 11). Remodeling can occur on either the *sn*-1 or *sn*-2 positions of the glycerolipid. For example, a major molecular species formed from the conversion of PE to PC is 16:0–22:6-PC (R.W. Samborski, 1990). This species of PC has a half-life of less than 6 h and appears not to be

significantly degraded but rather converted to other molecular species, particularly those with 18 : 0 on the *sn*-1 position and 20 : 4, 18 : 2 or 22 : 6 on the *sn*-2 position. Other studies have suggested that the main products of de novo PC and PE biosynthesis are 16 : 0–18 : 2, 16 : 0–18 : 1, 16 : 0–22 : 6 and 18 : 1–18 : 2. The major remodeled product is 18 : 0–20 : 4 for both PC and PE (H.H.O. Schmid, 1995). Why 18 : 0–20 : 4-PC and -PE are made by this circuitous route, rather than directly, is not known.

10. Regulation of gene expression in yeast

The pathways for the biosynthesis of phospholipids in yeast were largely elucidated by Lester and coworkers in the late 1960s (Fig. 12). These pathways are similar to those found in other eukaryotes except PS in yeast is made via a pathway similar to that found in *E. coli* where CDP-DG reacts with serine to yield PS and CMP.

Considerable interest in yeast as a model system has developed over the past two

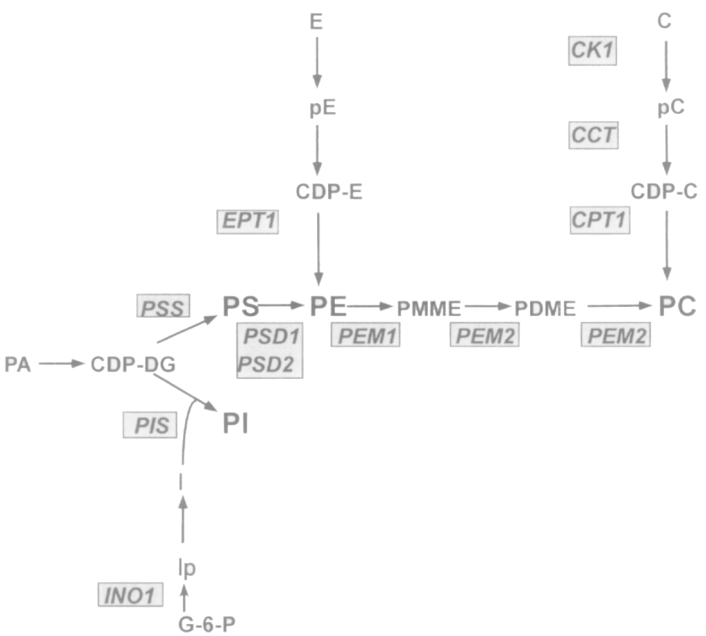


Fig. 12. The pathway for phospholipid biosynthesis in yeast and designation of the genes (italics in boxes) encoding the enzymes that catalyze the reactions. The abbreviations are: E, ethanolamine; pE, phosphoethanolamine; CDP-E, CDP-ethanolamine; C, choline; pC, phosphocholine; CDP-C, CDP-choline; PE, phosphatidylethanolamine; PMME, phosphatidylmonomethylethanolamine; PDME, phosphatidyldimethylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; PA, phosphatidic acid; CDP-DG, CDP-diacylglycerol; PI, phosphatidylinositol; I, inositol; Ip, inositol phosphate; G-6-P, glucose-6-phosphate. The genes encode the following enzymes: *INO1*, I-1-P synthase; *PIS*, PI synthase; *PSS* (also known as *CHO1*), PS synthase; *EPT1*, CDP-E : 1,2-diacylglycerol ethanolaminephosphotransferase; *PEM1* (*CHO2*), PE methyltransferase; *PEM2* (*OPI3*), phospholipid methyltransferase; *CK1*, choline kinase; *CCT*, CTP : phosphocholine cytidylyltransferase (abbreviated as CT elsewhere in this chapter); *CPT1*, CDP-C : 1,2-diacylglycerol cholinephosphotransferase; *PSD1* and *PSD2*, PS decarboxylase.

decades. Reasons for choosing *Saccharomyces cerevisiae* include a large knowledge base in classical genetics, the ease of making mutant strains and the ability to grow large amounts of yeast. Whereas understanding the regulation of expression of phospholipid biosynthetic enzymes in animal cells is still in its infancy, considerable progress has been made in the yeast system [26–28]. When yeast cells are grown in the presence of choline and inositol, the expression of the enzymes involved in the conversion of PA and glucose-6-P to PI, PC and PE is depressed (Fig. 12).

Both positive and negative regulatory factors are involved in the regulation of expression of phospholipid biosynthetic enzymes in yeast. The *INO2* and *INO4* genes encode transcription factors that are required for the expression of inositol-1-P synthase (*INO1*). In vitro transcribed and translated proteins derived from *INO2* and *INO4* form a heterodimer that binds a specific DNA fragment of the *INO1* gene referred to as UAS_{INO} (S.A. Henry, 1994). Ino4p (the protein encoded by *INO4*) and Ino2p exhibit basic helix–loop–helix domains. The Ino2p–Ino4p heterodimer binds to UAS_{INO} of the *INO1* promoter that contains two copies of a binding site (CANNTG) for basic helix–loop–helix-containing proteins.

The *OPI1* gene encodes a protein that is a negative regulatory factor for phospholipid biosynthesis [27]. Opi1p contains a leucine zipper, a motif implicated in protein–DNA interactions and transcriptional control. *Opi1* mutants exhibit a two-fold increase in the constitutive expression of inositol-1-P synthase and other enzymes involved in PI, PC and PE biosynthesis. The mechanism by which Opi1p mediates its negative regulatory role is unknown. Opi1p does not interact directly with UAS_{INO} or with Ino2p or Ino4p. Phosphorylation of Opi1 by protein kinase C may be involved (G.M. Carman, 2001).

Recent experiments have identified other proteins that interact with Ino4p (J.M. Lopez, 2000) indicating that there is still much to learn about transcriptional regulation of phospholipid biosynthetic genes in yeast. How the regulatory genes (*INO2*, *INO4*, *OPI1*) are themselves regulated is just beginning to be studied (J.M. Lopes, 2001).

11. Future directions

Since the first edition of this book was published in 1985 there have been astonishing developments in phospholipid metabolism. Some of these advances have dictated that a separate chapter be devoted to the role of glycerophospholipids in signal transduction (Chapter 12). The purification of some enzymes involved and the use of genetic screens has allowed molecular biological techniques to be used to clone and express cDNAs and genes for eukaryotic phospholipid biosynthetic enzymes. In addition, genetically modified mouse models are being developed.

(1) We can expect that crystal structures of some of the soluble proteins will be reported.
(2) More genes that encode phospholipid biosynthetic enzymes will be cloned and characterized. Elements of the genes involved in regulation of transcription will be mapped and positive and negative transcription factors should be identified.
(3) We can expect that more transgenic mice that over-express some of these enzymes,

as well as mice in which phospholipid biosynthetic genes have been disrupted, will be produced. Such studies should provide valuable insight into the role of these enzymes in whole animal physiology.

(4) The yeast system will continue to be exploited for studies on gene function and expression as well as regulation of phospholipid biosynthesis.

(5) There should be progress in understanding the regulation of PE, PI, PS and DPG biosynthesis.

(6) In the process of testing hypotheses and asking fundamental questions about phospholipid biosynthesis, we can continue to expect the unexpected.

Abbreviations

CDP-DG	CDP-diacylglycerol
CHO	Chinese hamster ovary
CT	CTP : phosphocholine cytidylyltransferase
DPG	diphosphatidylglycerol (cardiolipin)
DG	diacylglycerol
ER	endoplasmic reticulum
MAM	mitochondria associated membrane
PA	phosphatidic acid
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PEMT	phosphatidylethanolamine *N*-methyltransferase
PG	phosphatidylglycerol
PI	phosphatidylinositol
PS	phosphatidylserine
SREBP	sterol response element binding protein
TG	triacylglycerol

References

1. Dircks, L.K. and Sul, H.S. (1997) Mammalian mitochondrial glycerol-3-phosphate acyltransferase. Biochim. Biophys. Acta 1348, 10–16.
2. Coleman, R.A., Lewin, T.M. and Muoio, D.M. (2000) Physiological and nutritional regulation of enzymes of triacylglycerol synthesis. Annu. Rev. Nutr. 20, 77–103.
3. Kanoh, H., Kai, M. and Wada, I. (1997) Phosphatidic acid phosphatase from mammalian tissues: discovery of channel-like proteins with unexpected functions. Biochim. Biophys. Acta 1348, 56–62.
4. Carman, G.M. (1997) Phosphatidate phosphatases and diacylglycerol pyrophosphate phosphatases in *Saccharomyces cerevisiae* and *Escherichia coli*. Biochim. Biophys. Acta 1348, 45–55.
5. Kennedy, E.P. (1989) Discovery of the pathways for the biosynthesis of phosphatidylcholine. In: D.E. Vance (Ed.), Phosphatidylcholine Metabolism. CRC Press, Boca Raton, FL, pp. 1–9.
6. Ishidate, K. (1997) Choline/ethanolamine kinase from mammalian tissues. Biochim. Biophys. Acta 1348, 70–78.
7. Lykidis, A., Baburina, I. and Jackowski, S. (1999) Distribution of CTP : phosphocholine cytidylyl-

transferase (CCT) isoforms: identification of a new CCTβ splice variant. J. Biol. Chem. 274, 26992–27001.
8. Kent, C. (1997) CTP : phosphocholine cytidylyltransferase. Biochim. Biophys. Acta 1348, 79–90.
9. Cornell, R.B. and Northwood, I.C. (2000) Regulation of CTP : phosphocholine cytidylyltransferase by amphitropism and relocalization. Trends Biochem. Sci. 25, 441–447.
10. McMaster, C.R. and Bell, R.M. (1997) CDP–choline: 1,2-diacylglycerol cholinephosphotransferase. Biochim. Biophys. Acta 1348, 100–110.
11. Walkey, C.J., Donohue, R., Agellon, L.B. and Vance, D.E. (1997) Disruption of the murine gene encoding phosphatidylethanolamine *N*-methyltransferase. Proc. Natl. Acad. Sci. USA 94, 12880–12885.
12. Walkey, C.J., Yu, L., Agellon, L.B. and Vance, D.E. (1998) Biochemical and evolutionary significance of phospholipid methylation. J. Biol. Chem. 273, 27043–27046.
13. Kanipes, M.I. and Henry, S.A. (1997) The phospholipid methyltransferases in yeast. Biochim. Biophys. Acta 1348, 134–141.
14. Bakovic, M., Waite, K., Tang, W., Tabas, I. and Vance, D.E. (1999) Transcriptional activation of the murine CTP : phosphocholine cytidylyltransferase gene *(Ctpct)*: combined action of upstream stimulatory and inhibitory *cis*-acting elements. Biochim. Biophys. Acta 1438, 147–165.
15. Bladergroen, B.A. and van Golde, L.M.G. (1997) CTP : phosphoethanolamine cytidylyltransferase. Biochim. Biophys. Acta 1348, 91–99.
16. Voelker, D.R. (1997) Phosphatidylserine decarboxylase. Biochim. Biophys. Acta 1348, 236–244.
17. Kuge, O. and Nishijima, M. (1997) Phosphatidylserine synthases I and II of mammalian cells. Biochim. Biophys. Acta 1348, 151–156.
18. Hawthorne, J.N. (1982) Inositol phospholipids. In: J.N. Hawthorne and G.B. Ansell (Eds.), Phospholipids. Elsevier, Amsterdam, pp. 263–278.
19. Dowhan, W. (1997) CDP–diacylglycerol synthase of microorganisms. Biochim. Biophys. Acta 1348, 157–165.
20. Downes, C.P. and MacPhee, C.H. (1990) Myo-inositol metabolites as cellular signals. Eur. J. Biochem. 193, 1–18.
21. Antonsson, B. (1997) Phosphatidylinositol synthases from mammalian tissues. Biochim. Biophys. Acta 1348, 179–186.
22. Nikawa, J.-I. and Yamashita, S. (1997) Phosphatidylinositol synthase from yeast. Biochim. Biophys. Acta 1348, 173–178.
23. Amidon, B., Schmitt, J.D., Thuren, T., King, L. and Waite, M. (1995) Biosynthetic conversion of phosphatidylglycerol to *sn*-1 : *sn*-1′ bis(monoacylglycerol)phosphate in a macrophage-like cell line. Biochemistry 34, 5554–5560.
24. Kobayashi, T., Stang, E., Fang, K.S., de Moerloose, P., Parton, R.G. and Gruenberg, J. (1998) A lipid associated with the antiphospholipid syndrome regulates endosome structure and function. Nature 392, 193–197.
25. Zoeller, R.A. and Raetz, C.R.H. (1992) Strategies for isolating somatic cell mutants defective in lipid biosynthesis. Methods Enzymol. 209, 34–51.
26. Schlame, M., Rua, D. and Greenberg, M.L. (2000) The biosynthesis and functional role of cardiolipin. Prog. Lipid Res. 39, 257–288.
27. Nikoloff, D.M. and Henry, S.A. (1991) Genetic analysis of yeast phospholipid biosynthesis. Annu. Rev. Genet. 25, 559–583.
28. Swede, M.J., Hudak, K.A., Lopes, J.M. and Henry, S.A. (1992) Strategies for generating phospholipid synthesis mutants in yeast. Methods Enzymol. 209, 21–34.