STRUCTURAL SPECIFICITY IN PHOSPHOLIPID BIOSYNTHESIS. FORMATION OF 2-ACYL-1,2-ALKANEDIOLPHOSPHORYLCHOLINE IN A CELL-FREE SYSTEM

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SUMMARY: Microsomal preparations from rat liver catalyze the formation of 2-acyl-1,2-alkanediolphosphorylcholine from a long-chain 2-acyl-1,2-alkanediol and CDP-choline in the presence of Mg^{++} or Mn^{++}. The reaction is inhibited by Ca^{++}. The results indicate that 2-acyl-1,2-alkanediol can substitute for 1,2-diglyceride in phospholipid biosynthesis.

Long-chain 1,2-alkanediols occur as constituents of diester waxes (1) in the skin surface lipids of animals (2). We have shown that long-chain 1,2-alkanediols can also be incorporated into mammalian phospholipids by the formation of a glycerol ether bond leading to 1-O-2'-hydroxyalkyl-2-acyl glycerophosphatides (3-5) and by the formation of phospholipids having a long-chain 1,2-alkanediol backbone (6). It is reasonable to assume that a long-chain 2-acyl-1,2-alkanediol may substitute for 1,2-diacylglycerol in phospholipid biosynthesis as established by Kennedy et al. (7,8). In this case, a requirement for CDP-choline or CDP-ethanolamine would be expected. Alternatively, a transfer of phosphorylcholine from lecithin to 2-acyl-1,2-alkanediol could be postulated in analogy to the phosphorylcholine transfer from lecithin to ceramide in the course of sphingomyelin biosynthesis (9,10).

In the present communication, we report the biosynthesis of 2-tetradecanoyl-1,2-hexadecanediolphosphorylcholine by subcellular preparations from rat liver and demonstrate the requirement for

CDP-choline and other factors characteristic of CDP-choline: diacylglycerol cholinephosphotransferase (7,8) in this reaction.

EXPERIMENTAL

Fatty acids and glycerides were obtained from Nu-Chek-Prep, Inc., Elysian, Minn., α-hydroxypalmitic acid and [1-^{14}C]α-hydroxypalmitic acid from Applied Science Laboratories, Inc., State College, Penn. The sodium salts of cytidine 5'-diphosphate choline (CDP-choline) and cytidine 5'-diphosphate ethanolamine (CDP-ethanolamine) were from Sigma Chemical Co., St. Louis, Mo. Tetradecanal (11) was synthesized from myristic acid *via* the methyl ester, alcohol and mesylate (12). 1,2-Hexadecanediol was prepared by $LiAlH_4$-reduction of α-hydroxy-palmitic acid, and 2-tetradecanoyl-1,2-hexadecanediol was synthesized according to the method of Baumann and Madson (13) as described previously (6).

Adsorption chromatography was carried out on layers of Silica Gel H (Merck), 0.5 mm thick, in tanks lined with filter paper. Proportions of developing solvents are given by volume. Radioactivities were determined in a Packard Tri-Carb liquid scintillation spectrometer using Aquasol (New England Nuclear, Boston, Mass.) counting solution (68-70% counting efficiency). Radioactivity of TLC fractions was determined after scraping bands of adsorbent into counting vials which contained 1 ml of water and adding 10 ml of Aquasol.

[1-^{14}C]α-Hydroxypalmitic acid (55.7 Ci/mole), 50 μCi, was converted to its methyl ester which was subjected to $LiAlH_4$-reduction to obtain [1-^{14}C]1,2-hexadecanediol. The latter (9.8×10^7 dpm) was checked for purity by TLC of a small aliquot and was reacted with 2.8 mg of tetradecanal in 2 ml of dry benzene containing 40 mg/ml of *p*-toluenesulfonic

acid at 80°C for 3 hr. The cyclic acetal (3.1×10^7 dpm) was purified by TLC using hexane-diethyl ether, 95:5, and was subjected to ozonolysis in 25 ml of dichloromethane-ethyl acetate, 1:1, at -20°C for 45 min. The 2-acyl-1,2-diol was separated from the 1-isomer by TLC using hexane-diethyl ether, 7:3 (developed twice). Repeated purification by TLC yielded 2-tetradecanoyl[1-^{14}C]hexadecanediol (6.1×10^6 dpm) and 1-tetradecanoyl[1-^{14}C]hexadecanediol (2.6×10^6 dpm) in a radiopurity of better than 98%.

Livers taken from six adult male rats (Sprague-Dawley) were washed with ice-cold 0.25 M sucrose, transferred to 2 volumes of fresh 0.25 M sucrose, and homogenized in a Sorvall omnimixer. The homogenate was filtered through cheesecloth and the cells were ruptured by rapid decompression from 900 psi using a pressure bomb. Subcellular fractions were obtained by differential centrifugation with a Beckman type-30 fixed angle rotor under the following conditions: 600 x g for 10 min (nuclei and cell debris), 15,000 x g for 15 min (mitochondria), 100,000 x g for 120 min (microsomes, supernatant). The fractions were stored at -40°C for not more than one month. The pellets were resuspended as needed. Protein was determined according to Lowry et al. (14). All incubations were carried out in 5 ml screw-cap vials. Each flask was subjected to a 1 min vibration and then held at 37°C in an Eberbach metabolic shaker at 150 strokes per min. The reactions were terminated by adding 2 ml of chloroform-methanol, 2:1, and the lipids were extracted.

RESULTS AND DISCUSSION

When 2-tetradecanoyl[1-^{14}C]1,2-hexadecanediol was incubated with subcellular fractions from rat liver in the

TABLE I

Incorporation of Radioactivity from 2-Tetradecanoyl-[1-^{14}C]1,2-Hexadecanediol into Choline Phospholipid

Complete system[a]	Radioactivity[b]
Total homogenate	384
Mitochondria	170
Microsomes	609
Supernatant	13
Boiled microsomes	7
Deletions and additions[c]	
-CDP-choline	37
-CDP-choline, +choline glycerophosphatide[d] (1 mg)	3
$-Mg^{++}$	24
$-Mg^{++}$, $+Mn^{++}$ (5 mM)	541
+1,2-Dipalmitin (0.5 mg)	47
$+Ca^{++}$ (0.1 mM)	188
$+Ca^{++}$ (0.6 mM)	65

[a]Each vial contained: 2-Tetradecanoyl[1-^{14}C]1,2-hexadecanediol, 2.67 x 10^4 dpm; Triton-20, 1 mg/ml; cysteine, 20 mM; $MgCl_2$, 20 mM; CDP-choline, 0.5 mg; and the subcellular preparation, 2 mg of protein; in a total of 0.2 ml 0.1 M Tris-HCl buffer (pH 7.4 at 37°C). Incubation time: 2 hours.

[b]Total dpm in PC fraction (excluding background).

[c]Complete microsomal system.

[d]Prepared from bovine heart muscle.

presence of CDP-choline, as listed in Table I, incorporation of radioactivity into a polar lipid was observed. As demonstrated in Figure 1, radioactivity was also present in 1,2-hexadecanediol. The radioactive phospholipid was isolated

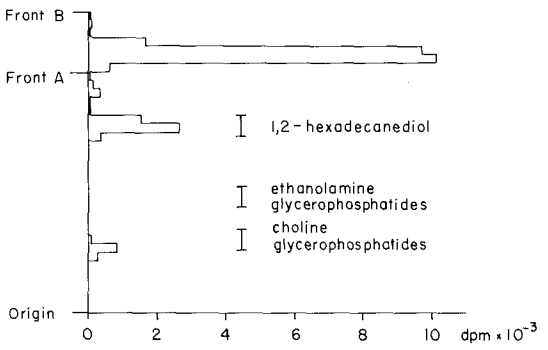

Figure 1. Radioactivity in lipid fractions after a 2-hour incubation of 2-tetradecanoyl[1-^{14}C]1,2-hexadecanediol (3.66×10^4 dpm) with the complete microsomal system (Table I). TLC on Silica Gel H using chloroform-methanol-water, 65:25:4 (A) and hexane-diethyl ether, 1:1 (B) consecutively. Migration rates of standards are indicated (the major radioactive fraction is unreacted substrate).

by TLC and characterized: a) In TLC using three different solvent systems (chloroform-methanol-water, 65:25:4; chloroform-methanol-acetic acid-water, 50:25:4:2; and chloroform-methanol-conc. ammonia, 65:35:5) it migrated with 1,2-diacyl glycerophosphorylcholine; b) Hydrolysis of the purified product (1.40×10^3 dpm) by phospholipase C (*Cl. welchii*) as described (6) yielded almost all radioactivity (1.25×10^3 dpm) in a fraction whose migration rate in TLC corresponded to a synthetic 2-tetradecanoyl-1,2-hexadecanediol.

Of the four subcellular preparations examined, the microsomal fractions exhibited the highest activity (Table I) and were used throughout. At low protein concentration, the amount of product formed increased linearly within a 3-hour period. The effect of protein concentration on product formation was approximately linear up to 5 mg/ml (Figure 2).

The requirement for CDP-choline and Mg^{++} or Mn^{++} for the formation of the diol phospholipid and its inhibition by Ca^{++} is evident from Table I. As these are the requirements for the CDP-choline: diacylglycerol cholinephosphotransferase

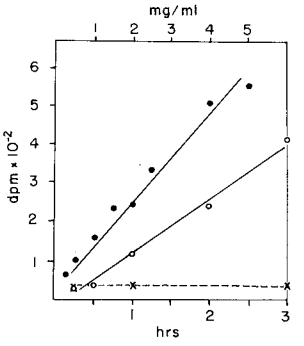

Figure 2. Effect of protein concentration [•] (3.66 x 10^4 dpm of substrate, 1 hr of incubation) and time [o] (1.3 mg/ml of protein) on product formation in the complete microsomal system; boiled microsomes [x].

described by Kennedy et al. (7,8), it appears that a long-chain 2-acyl-1,2-alkanediol can successfully substitute for 1,2-diacylglycerol in this reaction. This assumption is further supported by the finding that an addition of 1,2-diacylglycerol inhibits the formation of the diol phospholipid and that lecithin cannot substitute for CDP-choline. However, our data do not rule out the possibility that CDP-choline merely stimulates this reaction rather than serving as the donor of phosphorylcholine. Formation of 2-acyl-1,2-alkanediolphosphorylethanolamine from 2-acyl-1,2-diol and CDP-ethanolamine under the same conditions was not observed.

Incubation of 1-tetradecanoyl-1,2-hexadecanediol (7.7 x 10^4 dpm) with the complete microsomal system (Table I) yielded radioactivity (4.6 x 10^3 dpm) in an unidentified polar lipid whose migration rate in TLC using chloroform-methanol-water, 65:25:4, was between ethanolamine and choline glycerophosphatides; using chloroform-methanol-acetic acid, 65:25:8, it migrated above ethanolamine glycerophosphatide.

When 1,2-hexadecanediol (7.3 x 10^4 dpm) was incubated with the complete microsomal system (Table I), only small amounts

of polar lipid were formed (7.1×10^2 dpm); larger amounts of radioactivity were present in monoacyldiols, i.e., mainly in 1-acyl-1,2-alkanediol (4.0×10^3 dpm) and also in 2-acyl-1,2-alkanediol (6.7×10^2 dpm). 1,2-Diacylalkanediol was not formed.

Using a particulate fraction from rat liver, Kiyasu and Kennedy (15) have demonstrated plasmalogen biosynthesis from alk-1-enylacylglycerol and CDP-choline and CDP-ethanolamine. Snyder et al. (16) showed the requirement for CDP-choline and CDP-ethanolamine in the biosynthesis of the corresponding alkylacyl glycerophosphatides from alkylacylglycerol with microsomes from preputial gland tumors. It has been suggested (15,16) that the same enzyme, CDP-choline: diacylglycerol cholinephosphotransferase, may catalyze these reactions.

If this enzyme can also utilize a long-chain 2-acyl-1,2-alkanediol, it would seem to require only the presence of an acyl group vicinal to the primary hydroxy group of the lipid substrate (7,8,15). Apparently, phosphorylcholine transfer occurs if either an acyl, alkyl or alk-1-enyl group, or no functional group at all, occupies the position adjacent to this acyl moiety. Studies on the stereospecificity of the phosphorylcholine transfer to 2-acyl-1,2-diols are in progress in our laboratory.

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