AN ALTERNATE ENZYMIC ROUTE FOR THE SYNTHESIS OF THE ALKYL ANALOG OF PHOSPHATIDIC ACID INVOLVING ALKYLGLYCEROL

Kun Chae, Claude Piantadosi, and Fred Snyder*

From the Medical Division, Oak Ridge Associated Universities, Oak Ridge, Tennessee 37830 in collaboration with The Department of Medicinal Chemistry, School of Pharmacy, University of North Carolina, Chapel Hill, North Carolina 27514

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SUMMARY: A key intermediate in the biosynthesis of complex ether lipids is 1-alkyl-2-acyl-*sn*-glycero-3-phosphate. This lipid is known to be formed by enzymic reduction of alkyldihydroxyacetone phosphate and subsequent acylation. We have now obtained evidence for an alternate pathway for its formation from hexadecylglycerol. The reaction is catalyzed by a mitochondrial supernatant fraction prepared from mouse preputial gland tumors; CoA, ATP, and Mg^{++} are required as cofactors.

In 1968, Thompson (1) reported that after feeding [1-^{14}C]hexadecyl[2-^{3}H]-glycerol to terrestrial slugs, most of the radioactivity absorbed was incorporated into *O*-alkyl and *O*-alk-1-enyl phospholipids without cleavage of the ether linkage. Subsequent *in vivo* experiments demonstrated that similar results could be obtained in mammalian systems (2, 3). These data suggest that phosphorylation had occurred leading to the formation of 1-alkyl-*sn*-glycero-3-phosphate, which could then be converted to alkylacylglycerophosphate (4) and more complex ether-linked lipids (5). Such a pathway would provide for the biosynthesis of alkyl phospholipids without involving alkyldihydroxyacetone phosphate (6) which is formed from long-chain fatty alcohols (7) and acyldihydroxyacetone phosphate (8, 9).

Our earlier studies with homogenates from various tissues showed no phosphorylation of alkylglycerols (10); however, using slightly different conditions, we have been able to demonstrate the biosynthesis of alkylglycerophosphate and alkylacylglycerophosphate by a postmitochondrial fraction of

*Please address all correspondence to this author at Oak Ridge Associated Universities.

preputial gland tumors. This alternate metabolic route for the formation of the alkyl analog of phosphatidic acid is documented by the experimental data described in this report.

EXPERIMENTAL METHODS

rac-O-[1-^{14}C]Hexadecyl[2-^{3}H]glycerol (chimyl alcohol) which had a $^{3}H/^{14}C$ ratio of 2.65 was prepared by the method of Oswald *et al.* (11); its specific activity was 1.23 μCi per μmole for ^{14}C and 3.26 μCi per μmole for ^{3}H. Alkylglycerophosphate, which was used as a standard, was prepared by reduction of synthetic alkyldihydroxyacetone phosphate (12) with $NaBH_4$. Diacylglycerophosphate was used as a chromatographic marker for the isolation of alkylacylglycerophosphate, since both compounds have the same R_F values in the chromatographic systems used. The ATP and CoA were obtained from Nutritional Biochemicals, Cleveland, Ohio.

The enzyme source used in all experiments was a postmitochondrial fraction (15,000 *g* x 10 min supernatant) obtained from transplantable preputial gland tumors (ESR-586) grown subcutaneously in mice. Incubations were carried out essentially as before (13); all preparations were used fresh except where indicated. The components of the incubation systems are described in the tables. Incubations were terminated by extracting the lipids using the Bligh and Dyer procedure (14). Protein was determined by the method of Lowry *et al.* (15).

Lipid extracts were chromatographed for radioassay (16) on 250-micron layers of Silica Gel G developed in two solvent systems: System I, chloroform-methanol-glacial acetic acid (90:10:10, v/v); System II, chloroform-methanol-ammonium hydroxide (55:45:8, v/v). Identification of the products was based on cochromatography with the standards described above. In one experiment, the alkylacylglycerophosphate formed was isolated and reduced with Vitride (17) to yield alkylglycerol.

RESULTS AND DISCUSSION

The ^{14}C zonal profile scan of products isolated from an incubation

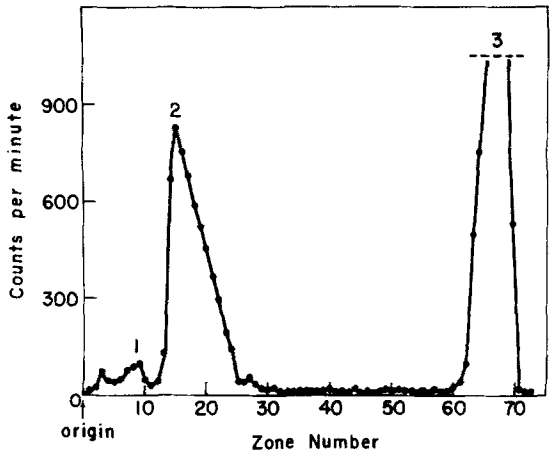

Fig. 1. Thin-layer chromatographic ^{14}C zonal profile scan (2 mm) of labeled products formed in an incubation system containing [1-^{14}C]hexadecyl[2-^{3}H]-glycerol, ATP, Mg^{++}, NaF, and a postmitochondrial fraction prepared from mouse preputial gland tumors (see Table I). The numbered peaks refer to (1) alkylglycerophosphate, (2) alkylacylglycerophosphate, and (3) neutral lipids (*rac*-hexadecylglycerol and *rac*-alkylacylglycerols). The chromatogram was developed on Silica Gel HR layers in a solvent system of chloroform-methanol-ammonium hydroxide (55:45:8, v/v).

mixture containing [1-^{14}C]hexadecyl[2-^{3}H]glycerol, ATP, Mg^{++}, NaF, and a postmitochondrial fraction from preputial gland tumors is shown in Fig. 1. Although alkylacylglycerophosphate was the main phosphorylated product formed at the end of a 1-hr incubation, we were also able to detect a small amount of alkylglycerophosphate that had been synthesized. The radioactivity associated with the neutral lipid fraction (Peak 3) consisted of unreacted hexadecylglycerol and *rac*-1-alkyl-3-acyl-*sn*-glycerol (or 3-alkyl-1-acyl-*sn*-glycerol), a product previously characterized by us (10); the latter was formed by acyltransferases.

As seen in Fig. 2, the time course for the biosynthesis of alkylacyl-glycerophosphate from alkylglycerol by the postmitochondrial fraction from tumors of mouse preputial glands is linear up to 30 min. Cofactor requirements for the phosphorylation of alkylglycerol were ATP and Mg^{++} (Table I). Figure 2 also shows the formation of alkylacylglycerophosphate from alkyl-glycerol as a function of ATP concentration. The reaction reaches its maximum rate at approximately 10 mM ATP. Almost all of the newly formed

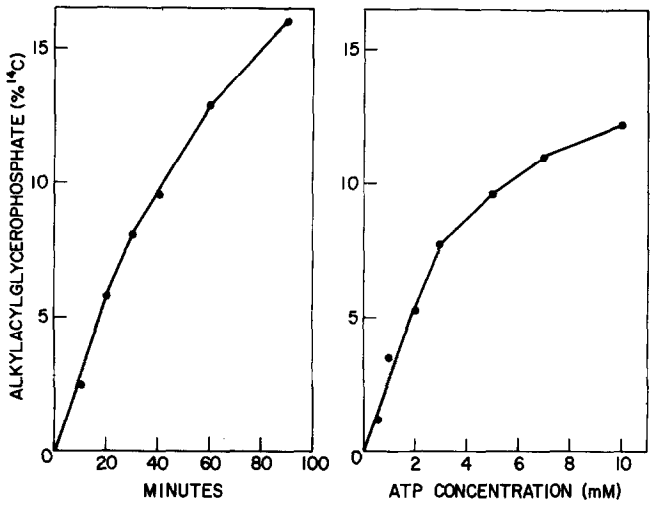

Fig. 2. The formation of alkylacylglycerophosphate from [1-^{14}C]hexadecyl[2-^{3}H]-glycerol as a function of time and ATP concentration. The complete system and conditions were the same as listed for Table I.

alkylglycerophosphate was acylated by the endogenous fatty acids when ATP and Mg^{++} were present; apparently there was sufficient endogenous CoA present. However, in the absence of CoA, an increase in the labeling of alkylglycerophosphate occurred (see values for samples 1 and 8 in Table I). Boiled or frozen preparations possessed no enzyme activity (Table I).

The ratio of $^{3}H/^{14}C$ in the alkylacylglycerophosphate formed was essentially the same as that of the doubly labeled hexadecylglycerol used as substrate. This indicates that the *O*-alkyl chain remained attached to the glycerol moiety during the reaction.

Addition of NaF was necessary to inhibit the phosphatase and thus increase the yield of phosphorylated products. We tested the effect of various concentrations of NaF (0-80 mM) on the reaction catalyzed by the postmitochondrial fraction of the tumor and found that maximal inhibition of the phosphatase activity required NaF levels greater than 20 mM.

Our data demonstrate that alkylacylglycerophosphate can be biosynthesized independently of the previously established pathway involving the reduction of alkyldihydroxyacetone phosphate and subsequent acylation (4, 6). This

TABLE I

Effect of Cofactors on the Phosphorylation of Labeled Hexadecylglycerol by a Mitochondrial Supernatant Fraction from Mouse Preputial Gland Tumors

System[b]	Products synthesized[a]		
	Alkylglycerophosphate	Alkylacylglycerophosphate	
	% ^{14}C	% ^{14}C	$^{3}H/^{14}C$
1. Complete[b]	1.3, 1.6	20.4, 18.1	2.53, 2.81
2. Complete minus ATP	0.1, 0.4	0.1, 0.8	2.65, 2.90
3. Complete minus Mg^{++}	0.8, 0.7	11.0, 11.8	2.54, 2.61
4. Complete minus ATP, Mg^{++}	0, 0.1	0.1, 0.1	-- --
5. Complete minus NaF	0.9, 1.2	9.6, 10.0	2.65, 2.58
6. Complete except enzyme preparation was boiled[c]	0.1, 0.1	0.3, 0.2	-- --
7. Complete except enzyme preparation was frozen[d]	0.1, 0.2	0.8, 0.8	-- --
8. Complete[e]	2.2, 2.0	17.4, 16.9	2.87, 2.70

[a]Calculations based on percent ^{14}C in the total recovered radioactivity after separation in solvent systems I and II; values are given for duplicate incubations.

[b]Each incubation vial contained [1-^{14}C]hexadecyl[2-^{3}H]glycerol (0.123 μCi based on ^{14}C, 33.3 μM), ATP (10 mM), Mg^{++} (4 mM), NaF (40 mM), 2-mercaptoethanol (20 mM), and mitochondrial supernatant fraction (9.5 mg protein) in a final volume of 3 ml of 0.1 M Tris-maleate buffer (pH 7.0). The samples were incubated for 1 hr at 37°. After the initial 1-hr incubation, CoA (0.1 mM) was added and incubated for another 30 min.

[c]Boiled in water bath for 15 min.

[d]Frozen overnight.

[e]Samples were incubated for 90 min without addition of CoA.

alternate route appears to be essential for incorporating alkylglycerols, derived from the diet and catabolic processes, into phospholipids. The phosphorylation step in this pathway apparently occurs via 1-alkyl-*sn*-glycerol kinase and not via 1-alkyl-2-acyl-*sn*-glycerol kinase. This reasoning is supported by the facts that (a) no 1-alkyl-2-acyl-*sn*-glycerols (or 3-alkyl-2-acyl-*sn*-glycerols) were detected in the neutral lipid fraction, which is in agreement with our previous acylation studies (10) using hexadecylglycerol as a substrate and (b) when CoA was not added to the otherwise complete incubation mixture, the quantity of alkylglycerophosphate was substantially increased. Further investigation of the enzyme system described in this communication should unequivocally elucidate the sequence of the phosphorylation

and acylation steps involved in the utilization of free alkylglycerols as precursors of ether-linked phospholipids.

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