PHOSPHATIDYL GLYCEROL PHOSPHATE PHOSPHATASE IN BHK-21 CELLS A COMPARISON WITH RAT LIVER MITOCHONDRIA

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Received September 2,1976

SUMMARY: Unlike mitochondria from rat liver, BHK-21 mitochondria are not autonomous for polyglycerolphosphatide biosynthesis. The phosphatase which converts phosphatidyl glycerol phosphate to phosphatidyl glycerol is located in the post-microsomal supernatant. It is somewhat heat stable, non-dialyzable, sulfhydryl-containing and its activity is similar to the phosphatase that can be found in the supernatant from sonicated rat liver mitochondria.

INTRODUCTION

Mitochondria have been shown to possess the capacity to catalyze the synthesis of several phospholipids, such as phosphatidic acid, CDP-diglyceride, phosphatidyl glycerol and cardiolipin (for review, see 1). The biosynthesis of the polyglycerolphosphatides, ie. phosphatidyl glycerol and cardiolipin, from sn-glycerol-3-phosphate and CDP-diglyceride is a process intrinsic to the inner membrane of mitochondria (2-5). This pathway involves the conversion of an obligatory intermediate, phosphatidyl glycerol phosphate, to phosphatidyl glycerol, a process that has been demonstrated in mitochondria from a number of tissues, such as chicken liver (2), sheep brain (3), rat liver (4-7), guinea pig heart (7), rat brain (8), rat heart (9), and guinea pig liver (10). Phosphatidyl glycerol is subsequently converted to cardiolipin (4,6,7,10-13).

While investigating the biosynthesis of polyglycerolphosphatides in mitochondria isolated from chloramphenicol-treated BHK-21 (baby hamster kidney) cells, it was observed that the dephosphorylation of phosphatidyl glycerol phosphate was rate limiting and that the activity of the phosphatase was very low (14). Partial characterization of the phosphatase and its probably soluble nature are described here.

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MATERIALS AND METHODS

Spinner adapted BHK-21 cells were maintained in culture in Alpha-MEM (Flow), containing 5% fetal bovine serum (Flow). Following centrifugation of exponentially growing cells (5-10 x 10^8 cells), they were washed and homogenized in 1 ml 0.25M sucrose-0.1mM EDTA using a Polytron® (15). Male Sprague-Dawley rats (100-250g) were used in the liver experiments. Liver homogenates were prepared in 0.25M sucrose-0.1mM EDTA, and subcellular fractions were isolated as described previously (16). Subcellular fractions from BHK-21 cells were similarly prepared except that the supernatant fraction was prepared on the first centrifugation in order to maintain its concentration. The pellet was resuspended and the remaining subcellular fractions isolated as before (16).

The assay of polyglycerolphosphatide synthesis and/or phosphatidyl glycerol phosphate hydrolysis was based on the *in vitro* system described by Stanacev (7,17), using *sn*-glycerol-3-phosphate [^{14}C(U)] (130.5 mCi mmole^{-1}) (New England Nuclear) and CDP-diolein (Serdary Research, London, Canada). Modifications of this assay are described in the legends of each figure or table. Following incubation, the samples were extracted with 8 volumes chloroform-methanol 1:1 (v/v) and washed with theoretical upper phase as described (16). Lipids were chromatographed (18), localized by spraying with 1% iodine in methanol, scraped and counted (19). Protein was determined by the method of Lowry *et al* (20).

RESULTS

Table I indicates the relative distribution of label in mitochondria

TABLE 1 Distribution of Labelled Glycerol-3-Phosphate Incorporated by Mitochondria from BHK-21 Cells and Rat Liver

The incubation mixture consisted of 50 µmoles tris-HCl, pH 7.4, 25 µmoles sucrose, 10 nmoles EDTA, 0.5 µmoles CDP-diolein, and 0.1 µmoles *sn*-glycerol-3-phosphate [^{14}C(U)] (11.1 x 10^6 DPM µmole^{-1}). Samples containing BHK-21 mitochondria (0.1 mg) and normal rat liver mitochondria (0.25 mg) were added to 5 µmoles 2-mercaptoethanol and treated rat liver mitochondria to 0.4 µmoles *p*-chloromercuribenzoic acid, potassium salt pH 8.5 to final volumes of 0.35 ml. Following 1 hour incubation at 37°C, the lipids were extracted as described in the Methods.

	Percent of Label Incorporated[a]		
Phospholipid	BHK-21 cells	Normal rat liver	PCMB-treated rat liver
PG	4.5	90.6	1.8
PGP	92.2	6.7	94.7
Total Incorporation[b]	16.5	14.0	6.2

[a] average of two experiments
[b] nmoles mg protein^{-1}

Abbreviations: DPG, cardiolipin, PG, phosphatidyl glycerol; PGP, phosphatidyl glycerol phosphate; PCMB, *p*-chloromercuribenzoic acid.

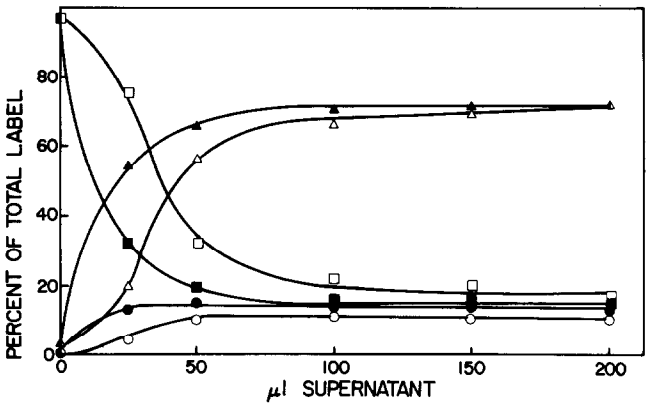

Figure 1. The Effects of Post-Microsomal Supernatant and Supernatant from Sonicated Rat Liver Mitochondria on the Incorporation of [^{14}C]-Glycerol-3-Phosphate by BHK-21 Cell Mitochondria. The assay mixture was the same as the untreated system described in Table I. To each tube was added 0.1 mg BHK-21 mitochondrial protein. Post-microsomal supernatant (7.5 mg protein ml^{-1}) was added to one set of tubes at the protein concentrations described. Frozen rat liver mitochondria were sonicated for 3 min with cooling using a microprobe equipped Branson Sonifier. The sonicate was centrifuged as described for the post-microsomal supernatant for BHK-21 cells and this supernatant (10.0 mg ml^{-1}) added to a second set of tubes. Following 1 hour incubation at 37^{o}C, 5 μmoles $CoCl_2$ was added to a final volume of 0.1 ml and incubated for a further 2 hours. Lipids were extracted as previously described. The lipid extracts were chromatographed (18) and the spots identified by spraying with 1% iodine in methanol, scraped and counted (19). o--o, DPG; Δ--Δ, PG; □--□ , PGP; BHK-21 post-microsomal supernatant. ●--●, DPG; ▲--▲, PG; ■--■ , PGP; sonicated rat liver mitochondrial supernatant.

from BHK-21 cells, and rat liver mitochondria with or without PCMB. In rat brain or liver, the conversion of phosphatidyl glycerol phosphate to phosphatidyl glycerol has been shown to be sensitive to sulfhydryl inhibitors (2,8). The labelling profile of BHK-21 mitochondria is identical with that of PCMB-treated rat liver mitochondria. This would seem to indicate that the activity of the PGP phosphatase in the BHK-21 mitochondria is very low, a feature not observed in rat liver mitochondria under normal conditions.

Since the PGP to PG conversion occurs in homogenates of BHK-21 cells (14), the effect of the addition of post-microsomal supernatant from BHK-21 cells or the supernatant from sonicated rat liver mitochondria on the labelling profile in BHK-21 mitochondria was examined (Figure 1). Examination of the individual polyglycerolphosphatides revealed that although total incorporation was stimulated to a greater extent by the sonicated rat liver mitochondrial supernatant (unpublished results) at optimal concentrations of both types of supernatant the production of PG and PGP occur at the same relative rates. In both cases, conversion of PGP to PG is stimulated many fold.

TABLE II Effects of Various Treatments on the Supernatant Stimulation of Phosphatidyl Glycerol Phosphate Conversion to Phosphatidyl Glycerol

The assay mixture was identical to that described in Fig. 1. Each tube contained 150 µg BHK-21 mitochondrial protein and the various supernatant fractions described below. Following 1 hour at 37°C, 5 µmoles $CoCl_2$ was added to a final volume of 0.5 ml and the samples incubated for an additional 2 hours. Lipids were extracted as before.

Sample	Lipid Synthesized[a] (Percent of Total) Total	DPG	PG	PGP
Control - no supernatant	1.51	0.056(3.7)	0.087(5.8)	1.37(90.5)
+ 750 µg BHK-21 post-microsomal supernatant	3.49	1.39(39.8)	1.42(40.7)	0.68(19.5)
+ 750 µg dialyzed[b] BHK-21 post-microsomal supernatant	3.42	1.44(42.1)	1.44(42.1)	0.54(15.8)
+ 250 µg heat treated[c] BHK-21 post-microsomal supernatant	2.18	0.98(45.0)	0.79(36.2)	0.41(18.8)
+ 1.0 mg sonicated[d] rat liver mitochondria supernatant	4.65	2.06(44.3)	1.99(42.8)	0.60(12.9)
+ 1.0 mg dialzyed[b] sonicated rat liver mitochondrial supernatant	4.76	2.03(42.6)	1.97(41.4)	0.76(16.0)
+ 0.5 mg heat treated[c] sonicated rat liver mitochondrial supernatant	1.80	0.23(12.8)	0.34(18.9)	1.23(68.3)
+ 2.0 mg rat liver post-microsomal supernatant	1.55	0.64(41.3)	0.57(36.8)	0.34(21.9)

[a] nmoles per samples - average of two experiments

[b] dialyzed against 1000 volumes 0.25M sucrose-0.1mM EDTA - 0.2% 2-mercaptoethanol for 20 hours at 4°C

[c] 10 min. at 100°C, centrifuged at 10,000g x 10 min.

[d] prepared as described in Figure 1

A partial characterization of the nature of this stimulation was undertaken (Table II). The total incorporation of labelled sn-glycerol-3-phosphate was stimulated by both BHK-21 post-microsomal supernatant and sonicated rat liver mitochondrial supernatant, as is the percent of the label converted to PG and DPG. Dialysis has no effect on either stimulation. When an equivalent original volume of either heat-treated supernatant is utilized, total incorporation is reduced to close to the control values. It appears that the PGP to PG conversion by BHK-21 post-microsomal supernatant is not as heat labile as for the rat liver extract, and that the enhancement of total incorporation of label is an effect not related to conversion of PGP to PG and DPG. In

TABLE III Conversion of PGP to PG by BHK 21 Post-Microsomal Supernatant

The assay mixture consisted of 1.21 nmoles [^{14}C]-PGP (6.6×10^3 DPM nmole^{-1}), 25 µmoles tris-HCl, pH 7.4 ± 2.5 µmoles 2-mercaptoethanol sonicated together for 30 seconds in a bath-equipped Branson Sonifier. BHK-21 post-microsomal supernatant (250 µg protein, 12.5 µmoles sucrose, 5 nmoles EDTA) was added to a final volume of 0.25 ml. Following up to 90 min. incubation at 37°C, the lipids were extracted as before.

Sample	Total PG Produced (pmoles)
Control - 0 min.	12.3
- 30 min.	66.4
- 60 min.	121
- 90 min.	182
- 90 min.-2-mercaptoethanol	105
- 90 min.-2-mercaptoethanol + PCMB	30.6

addition, a factor in the rat liver post-microsomal supernatant will stimulate conversion of PGP to PG, with no enhancement of total incorporation.

The hydrolysis of PGP by post-microsomal supernatant from BHK-21 cells was examined using [^{14}C]-PGP isolated from rat liver mitochondria treated with PCMB as in Table I. These results (Table III) indicate that the hydrolysis of PGP to PG by supernatant is probably due to a sulfhydryl-containing phosphatase rather than a factor which activates a mitochondrial phosphatase.

DISCUSSION

Unlike rat liver mitochondria, mitochondria from BHK-21 cells are not autonomous for the synthesis of polyglycerolphosphatides. Instead, it appears that the phosphatase involved in the conversion of PGP to PG is a cytoplasmic enzyme that has free access to the mitochondrially-synthesized PGP and rapidly stimulates PG formation. The rate of DPG synthesis is dependent on the synthesis of PG from PGP. In addition, a cytoplasmic factor stimulates the total synthesis of polyglycerolphosphatides in BHK-21 cells. This latter factor is heat-labile, while the phosphatase seems to be relatively stable. Both these activities can be replaced by adding a heat-labile soluble factor(s) isolated from rat liver mitochondria.

The reason for this interesting difference in compartmentalization of this biosynthetic pathway is uncertain. Rat and hamster kidney mitochondria, or mitochondria from HeLa, KB and L-cells isolated in the same manner, all possess PGP phosphatase activity, although post-microsomal supernatant from HeLa cells will stimulate the activity. In addition, BHK-21 cells grown in

monolayer show identical phosphatase distribution as spinner grown cells. Studies are presently in progress to elucidate further this variation.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Medical Research Council of Canada. J.H. Lipton is the recipient of an M.R.C. Studentship. The excellent technical assistance of E.C. Jarvis is gratefully appreciated.

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