INCORPORATION OF FATTY ACIDS INTO THE OUTER AND INNER MEMBRANES OF ISOLATED RAT LIVER MITOCHONDRIA

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Received 5 March 1969

Acyl-CoA: phospholipid acyl-transferase activity as well as phospholipase A activity were detected in inner and outer membrane preparations from rat liver mitochondria. Both enzyme systems have an optimum pH around 8 and act preferentially on phosphatidylethanolamine. While phospholipase A activity is much lower in the inner membrane than in the outer membrane of mitochondria the reverse is true for the incorporation of (^{14}C)-oleic acid into endogenous phosphatidylethanolamine. These results bring an indirect evidence that the inner membrane *per se* possesses a phospholipase A activity.

1. Introduction

The study of the intracellular distribution of enzymes able to modify membrane structures has been undertaken as a way of approach to the understanding of the functional activities and possible renewal of cellular membranes.

Phospholipids which are the main lipid constituents of membranes differ essentially by the fatty acids attached to the glycerol moiety of glycerolphosphate. The long chain fatty acids constitute the apolar moiety of membranes and may be involved in the permeability barrier to hydrophilic solutes. Hydrolytic enzymes such as phospholipase A may create discontinuities in a membrane; if its activity is low enough, these discontinuities may only amount to localized increases in permeability which may only be temporary if a reacylating enzyme, acting *in situ* on membrane lysophosphatides, is also present.

The following experiments were designed to estimate, in submitochondrial membranes, the presence and assess the possible physiological importance of such a couple of systems as phospholipase A and lysophosphatide acylating system functioning in a cyclic manner.

Abbreviations:
Phosphatidylcholine : PC ; Phosphatidylethanolamine : PE ; Phosphatidylinositol : PI ; Cardiolipin : CL ; Lysophosphatidylethanolamine : Lyso-PE.

In rat liver, phospholipase A activities have been detected in mitochondria [1–6] as well as in microsomes [4,7] and in lysosomes [5,6,8]. Acylation of lysophosphatides may be catalyzed by the acyl-CoA phospholipid acyltransferase discovered by Lands [9,10]; among rat tissues, liver is the most active [11]. Besides microsomes [10,12] mitochondria can exhibit acyl-CoA transferase activity [6,12,13]. This acyl-CoA transferase activity has been found in the mitochondrial soluble material [14] and in the outer membrane [15]. The soluble fraction of liver cell was also shown to have high reacylating capacity [16] depending upon the presence of acyl-CoA derivatives.

2. Methods

2.1. *Isolation of mitochondrial membranes*

Rat liver mitochondria were fractionated [17] into inner membrane + matrix and other membranes after phosphate treatment as described by Parsons et al. [18]. After purification by centrifugation on a three-layer sucrose density gradient the "inner membrane + matrix" fraction which is collected at the bottom of the tube is disrupted by sonication (in a Branson sonifier at 10 A for 2 min). The sonicated inner membrane was separated from the soluble matrix protein by centrifugation at 30,000 rpm for one hour (Spinco rotor 30), then purified by centrifugation on a

three-layer sucrose gradient. By spinning at 24,000 rpm for 2 hours in a SW 25-2 Spinco rotor (8×10^6 g/min) the sonicated inner membrane gathers at the second interface of the gradient, i.e. between the 51.3% sucrose (w/v) and 37.7% sucrose (w/v) solution layers. The "inner membrane + matrix" fraction can also be disrupted by repeated passages through three-layer sucrose density gradient, a procedure which is milder than sonication to obtain inner membrane.

2.2. *Enzymic determinations*

The degree of purification of the submitochondrial membrane preparations was monitored by enzyme markers: acid phosphatase for lysosomes, glucose-6-phosphatase for microsomes, monoamine oxidase for the outer mitochondrial membrane, and cytochrome oxidase for the inner membrane fractions (for details cf. ref. 17).

The phospholipase activity was estimated by the amount of free fatty acids and lysophosphatides formed when using either exogenous substrates : egg phosphatidylethanolamine (PE) or rat liver (^{32}P)-PE, or endogenous phospholipids.

The reacylation of membrane phospholipids was measured after incubation of mitochondrial fractions with (^{14}C)-oleate in the presence of ATP, CoA, Ca^{++}, Mg^{++} as indicated in the tables, extraction of the lipids by chloroform/methanol (2/1) and isolation of phospholipids by thin layer chromatography [4]. The phosphorus content was determined by the method of Bartlett [19].

3. Results and discussion

It was shown earlier that (^{14}C)-oleic acid can be incorporated into mitochondria phospholipids [6]. The distribution of radioactivity among the different subfractions of rat liver mitochondria after their incubation with (^{14}C)-oleic acid is given in table 1. Incorporation of (^{14}C)-oleic acid into endogenous phospholipids is ATP-dependent. GTP is also active. In mitochondria, phosphatidylethanolamine becomes more rapidly labelled than lecithin. The specificity of the reacylating system for phosphatidylethanolamine is more marked in the inner than in the outer membrane of mitochondria. Phosphatidylethanolamine is also a better substrate for phospholipase A. The pH-curve (fig. 1) shows

Table 1
Incorporation of ^{14}C-oleic acid into membrane phospholipids.

Membrane preparation	Incubation mixture	Phospholipid	Radioactivity *
		PC	PE
Inner membrane + matrix	Ca^{++}	1	20
	Complete	163	1,460
Sonicated inner membrane	Ca^{++}	1	17
	Complete	304	1,450
Outer membrane	Ca^{++}	2	7
	Complete	365	787

* cpm/μg of lipid phosphorus.

The Ca^{++} incubation medium was made of 0.0025 M $CaCl_2$ and 0.04 M triethanolamine buffer, pH 8.0, and contained 0.2 μmole (^{14}C)-oleic acid (250,000 cpm/μmole) emulsified by sonication.
The complete incubation mixture was made of 0.2 μmole (^{14}C)-oleic acid (250,000 cpm/μmole) emulsified by sonication, 0.020 M ATP, 0.025 M $MgCl_2$, 0.0025 M $CaCl_2$, 0.0005 M CoA, 0.2 M NaF, in 0.04 M Triethanolamine buffer, pH 8.0.
5 to 11 mg of protein. Total 2.0 ml. Incubation for 30 min at 37°.

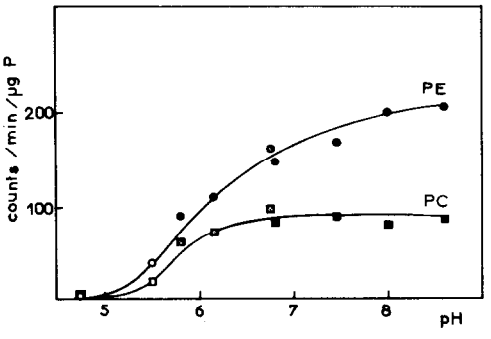

Fig. 1. Effect of pH on the incorporation of (^{14}C)-oleic acid into mitochondrial inner membrane phospholipids.
The incubation system was the same as in table 1 and contained 0.2 µmole of (^{14}C)-oleic acid (120,000 cpm) and 6 mg of protein of sonicated inner membrane.
○, □ : acetate buffer;
◎, ▨ : Tris, maleate buffer;
●, ■ : Tris, HCl buffer.

a better activity at alkaline pH in the range of 8.0–8.5.

The degree of purity of the submitochondrial fractions used can be roughly estimated from the phospholipid analysis of these fractions (table 2) and from the specific activities of the marker enzymes (table 3). It was also controlled by electron microscopy.

The distribution of phospholipase activity in the submitochondrial fractions has been evaluated with (^{32}P)phosphatidylethanolamine as exogenous substrate (table 4). The outer membrane exhibited the higher

Table 2
Phospholipid content of submitochondrial fractions.

	Lipid phosphorus *	Percent of lipid phosphorus PC	PI	PE	CL
Mitochondria					
Total	190	45	3	38	14
Inner membrane + matrix	150	41	3	38	18
Sonicated inner membrane	350	40	–	37	21
Outer membrane	500	50	12	34	3

* nmole/mg of protein.

phospholipase activity. The phospholipase activity present in the other fractions : inner membrane + matrix and sonicated inner membrane is of the order of magnitude of the reacylating activity found in these fractions.

Incubation of mitochondria or of mitochondrial membranes in the reacylating medium (table 1) does not alter the content of endogenous phospholipids. In contrast, in the absence of ATP and in the presence of Ca^{++}, endogenous phospholipids found at the end of the incubation period are notably lowered. This suggests that either the activity of the reacylating system compensates the phospholipase activity or that

Table 3
Activities of marker enzymes in submitochondrial fractions.

Fraction	Ac. * phosphatase	Cytochrome ** oxidase	MAO **	Glucose-6 * phosphatase
Mitochondria	0.032	1.8	0.006	0.010
Inner membrane + matrix	0.004	2.8	0.004	0.004
Sonicated inner membrane	0.006	2.7	0.006	–
Outer membrane	0.100	0.3	0.060	0.110

* µmoles of phosphate released at 37°/min/mg protein (*p*-nitrophenylphosphate substrate for acid phosphatase).
** µmoles/min/mg protein at 25°.

Table 4
Phospholipase activity in submitochondrial fractions.

Fraction	(^{32}P)-lyso PE formed *
Mitochondria	19.5
Inner membrane + matrix	19.1
Sonicated inner membrane	11.8
Outer membrane	52.5

* nmoles/mg protein/hr.

The incubation medium contained 2 μmoles of $CaCl_2$, 50 μmoles of Triethanolamine buffer, pH 8.0, 0.3 μmole of (^{32}P)-phosphatidylethanolamine (6,000 cpm) emulsified by sonication and submitochondrial preparations (0.5 mg to 1.8 mg of protein) in a final volume of 1 ml. Prior to incubation the fractions had been submitted to 10 cycles of freezing and thawing.

phospholipase is partially inhibited in the presence of ATP.

Acknowledgements

We are indebted to Miss Alix of the "Service de Microscopie Electronique" for checking our submitochondrial fractions at the electron microscope. The skilful technical assistance of Miss J.Baranne and Mr. R.Césarini is gratefully acknowledged.

This work was supported by the "Centre National de la Recherche Scientifique", the "Délégation Générale à la Recherche Scientifique et Technique" and the "Fondation pour la Recherche Médicale".

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