EFFECT OF TEMPERATURE ON PHOSPHOLIPID SYNTHESIS IN EHRLICH ASCITES TUMOR CELLS

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Received 22 March 1976

1. Introduction

In view of the available experimental data it is generally believed that mitochondria are not able to synthesize their own complex phospholipids, with the exception of phosphatidic acid and cardiolipin. The pathways for the de novo synthesis of phosphatidylcholine and phosphatidylethanolamine with CDP-choline and CDP-ethanolamine as precursors are located entirely in the endoplasmic reticulum (cf. [1]). Phospholipids can exchange between intracellular structures being transported by specific exchange proteins present in the cytosol (for review see [2]). However, previous studies from this laboratory [3] showed that labelled phospholipids from microsomes were transported mainly into the outer, and not the inner, mitochondrial membrane of intact mitochondria. Thus, the origin of phospholipids of the inner membrane still remains unclear.

Intact cells seem to be a good material for studying the intracellular localization and control of phospholipid synthesis. The present investigation shows that the incorporation of inorganic [^{32}P]phosphate into phospholipids of Ehrlich ascites cells greatly depends on the temperature of incubation. In cells incubated at 37°C endoplasmic reticulum becomes highly labelled and the highest specific radioactivity among nitrogen-containing phospholipids is found in phosphatidylcholine. At lower temperatures (10°C–30°C) the specific labelling of mitochondrial phospholipids prevails, especially that of phosphatidylethanolamine.

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2. Materials and methods

2.1. *Biological material*

Ehrlich ascites tumor cells, grown in the peritoneal cavity of white mice, strain CFW, were harvested 7 to 9 days after transplantation. The isolation of cells, their disruption by osmotic shock and isolation of mitochondria were performed according to Borst [4]. The post-mitochondrial supernatant was centrifuged at 20 000 *g* for 20 min and the sediment was discarded. Microsomal fraction was obtained by centrifuging the supernatant at 100 000 *g* for 1 h.

2.2. *Enzymic assays*

Cytochrome oxidase (EC 1.9.3.1.) was determined by measuring oxygen uptake in a system containing ascorbate and cytochrome *c* [5]. The assayed material was solubilized by pre-treatment with Lubrol WX. NADPH-cytochrome *c* reductase was measured according to Sottocasa et al. [6], and glucose 6-phosphatase (EC 3.1.3.9.) according to Swanson [7]. Cholesterol esterase (EC 3.1.1.13.) was determined essentially according to Goodman [8], except that labelled cholesterol oleate was replaced by the non-labelled ester (25 μg/ml). After incubation, lipids were extracted by ethyl ether–ethanol (3:1, v/v) and separated by thin-layer chromatography on silicic acid G (E. Merck, Darmstadt, GFR) using petroleum ether–ethyl ether–acetic acid (60:40:1.6, v/v/v) as the solvent. The spot of free cholesterol was scraped out and cholesterol was determined without elution from the gel according to Searcy et al. [9].

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2.3. *Incubation*

Cells corresponding to about 500 mg protein were incubated in 100 ml of the incubation medium at 10°C, 20°C, 30°C, 37°C and 45°C under air containing 5% CO_2 as the gas phase, with constant shaking for 1 h. The medium was calcium-free Krebs-Ringer bicarbonate solution, pH 7.4, [10] containing in addition 5 mM glucose and 0.1 mM $KH_2{}^{32}PO_4$ (about 7 μCi/ml). After incubation, subcellular fractions were isolated as described above and precipitated with trichloroacetic acid (10% final concentration). The precipitate was washed 2 times with 10 mM KH_2PO_4 and extracted for lipid analysis.

2.4. *Lipid fractionation and determination*

Lipids were extracted from the precipitate using a mixture of methanol and chloroform (1:2, v/v) as described by Wojtczak et al. [11]. Phospholipids were separated and identified by thin-layer chromatography on silica gels G and H. With silica gel G, chromatograms were developed with a mixture of chloroform, methanol and water (60:25:4, v/v/v) according to Wagner et al. [12], and with silica gel H in two-step chromatography according to Rouser et al. [13]. For separation of phosphatidylglycerol from other phospholipids the solvent system of chloroform–methanol–acetic acid–water (65:25:8:4, by vol) according to Marshall and Kates [14] was also used. Spots on thin-layer chromatograms were visualized using iodine vapour or ninhydrin, identified by comparing their positions with those of known standards or known R_f values, and assayed for phospholipid phosphorus and radioactivity. The periodate–Schiff stain [15] was used to identify phosphatidylglicerol. Because plasmalogens also give a positive reaction with the Schiff reagent, they were detected by spraying a parallel chromatogram with dinitrophenylhydrazine (Reitsema test) [16]. The spot of phospholipid identified as phosphatidylglycerol was ninhydrin-negative, exhibited a positive colour reaction with the periodate–Schiff reagent, was negative in Reitsema test and had a similar R_f as described for phosphatidylglycerol by Marshall and Kates [14].

Phospholipids were also fractionated by column chromatography on silicic acid 'Mallincrodt' (100 mesh, Serva, Heidelberg, GFR) eluted with increasing concentrations of methanol in chloroform and on alumina oxide (neutral, grade I, M. Woelm, Eschwege, GFR) [17]. Fractions from columns were assayed for radioactivity. Phosphatidylethanolamine was also identified by mild alkaline hydrolysis [18] and paper chromatography of the hydrolysis products [19].

2.5. *Other procedures and chemicals*

Protein was determined by the biuret method [20]. Phospholipid phosphorus was determined according to Bartlett [21]. Radioactivity was measured with a scintillation spectrometer making use of the Čerenkov effect for ^{32}P [22]. [^{32}P]Phosphoric acid, carrier-free, was obtained from the Institute for Nuclear Research (Swierk, Poland). Standards of phosphatidic acid, cardiolipin, phosphatidylinositol and phosphatidylserine were obtained from Koch-Light Laboratories Ltd. (Colnbrook, England). As standards of phosphatidylcholine and phosphatidylethanolamine the mixture of phospholipids from hen egg yolk was used.

3. Results

The assay of cytochrome oxidase showed that the microsomal fraction from Ehrlich ascites cells as obtained in the present investigation was contaminated by mitochondrial fragments in less than 10%. A virtual absence in microsomes of the radioactive spot of phosphatidylglycerol (fig.1) after incubating the cells with [^{32}P]phosphate also indicates a negligible contamination by mitochondria. On the other hand, the mitochondrial fraction exhibited a high activity of both glucose 6-phosphatase and NADPH-cytochrome *c* reductase, enzymes usually considered to be characteristic for microsomes. Attempts to further purify the mitochondrial fraction were unsuccessful in diminishing the NADPH-cytochrome *c* reductase and glucose 6-phosphatase activities. On the contrary, the specific activity of these enzyme systems was occasionally increased and exceeded the value observed in the microsomal fraction. It was therefore concluded that NADPH-cytochrome *c* reductase and glucose 6-phosphatase may be intrinsic to Ehrlich ascites mitochondria. Similarly, Bergelson et al. [23] found glucose 6-phosphatase unsuitable as a marker for microsomal contamination in hepatoma mitochondria. In this situation we introduced cholesterol esterase to check microsomal contamination [23]

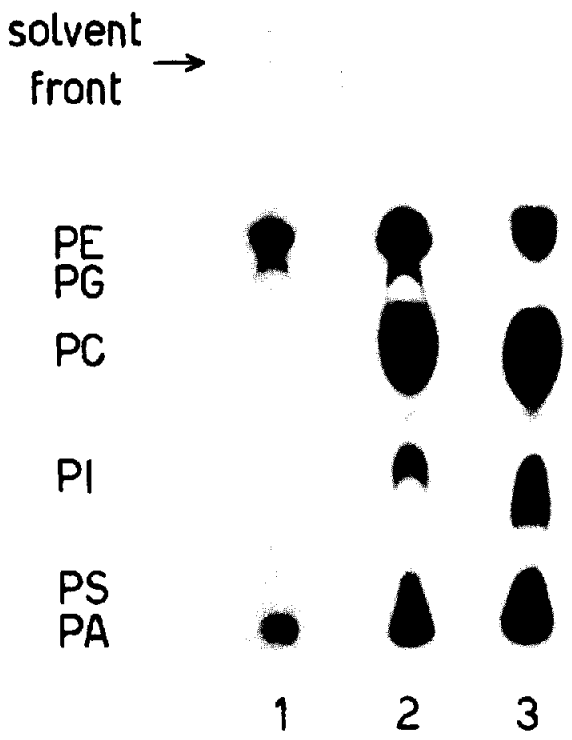

Fig.1. Thin-layer chromatography of mitochondrial and microsomal phospholipids from Ehrlich ascites tumor cells. The cells were incubated for 60 min with [^{32}P]phosphate and the lipids from mitochondria and microsomes were separated on silica gel G with chloroform–methanol–water (60:25:4, v/v/v). The figure represents an autoradiogram of the plate. (1) Mitochondria incubated at 10°C; (2) mitochondria incubated at 37°C; (3) microsomes incubated at 37°C; (PA) phosphatidic acid; (PS) phosphatidylserine; (PI) phosphatidylinositol; (PC) phosphatidylcholine; (PG) phosphatidylglycerol; (PE) phosphatidylethanolamine.

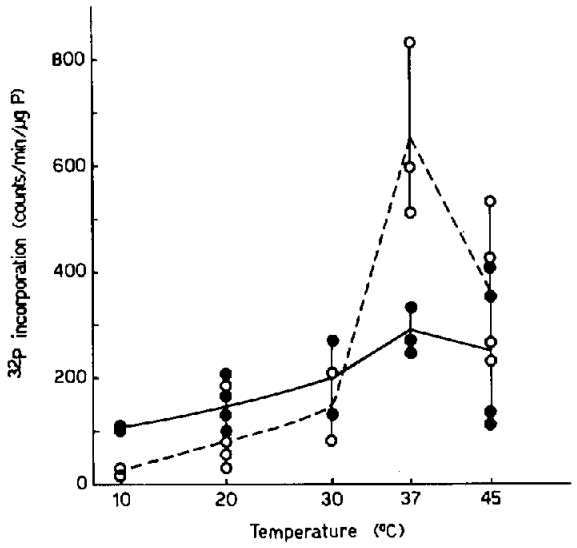

Fig.2. Incorporation of [^{32}P]phosphate into total phospholipids of mitochondria and microsomes of Ehrlich ascites tumor cells. Whole cells were incubated during 60 min at temperatures indicated. The symbols represent values obtained from individual experiments. (●——●) Mitochondria; (o- - -o), microsomes.

and found that, in the mitochondrial fraction, it did not exceed 7%.

The labelling of mitochondrial and microsomal phospholipids after incubation of intact cells in the presence of [^{32}P]phosphate is shown in fig.2. It can be seen that, at 37°C, the specific labelling of microsomal phospholipids is higher than that of mitochondrial phospholipids, what is in good agreement with earlier observations of David and Rossiter [24]. In contrast, at lower temperatures (10°C–30°C) these proportions are reversed. At 45°C the scatter of the individual measurements was very high (fig.2) because of varying viability of the cells. Therefore, the results for that temperature are unreliable and are not shown in fig.3 and table 1.

Table 1 shows the percentage distribution of the label between individual phospholipids after incubating the cells at various temperatures. In microsomes this distribution is fairly independent on the temperature and phosphatidylcholine remains the main labelled phospholipid at all temperatures tested. On the contrary, the percentage distribution of the label in mitochondrial phospholipids varies considerably with varying temperature of incubation. At 10°C and 20°C phosphatidylethanolamine is the main labelled nitrogen-containing phospholipid. The labelling of phosphatidylcholine is negligible at 10°C, but gradually increases with increasing temperature and attains the value of 49% of the total labelling at 37°C. It then becomes the main labelled phospholipid of mitochondria. It is also to be noted that phosphatidic acid contributes to about 30% of the total labelling of both mitochondrial and microsomal phospholipids at temperatures between 10°C and 30°C while it amounts to only 13% at 37°C.

Table 1
Incorporation of [^{32}P]phosphate into individual phospholipids of mitochondria and microsomes of whole Ehrlich ascites tumor cells

Temperature (number of experiments)	10°C (2)	20°C (4)	30°C (2)	37°C (3)
Microsomes				
Phosphatidic acid		29 ± 4	23 ± 4	13 ± 2
Phosphatidylserine		6 ± 2	8 ± 3	6 ± 3
Phosphatidylinositol		7 ± 1	10 ± 0.3	7 ± 2
Phosphatidylcholine		40 ± 1	45 ± 1	58 ± 1
Phosphatidylethanolamine		12 ± 3	12 ± 3	11 ± 2
Mitochondria				
Phosphatidic acid	30 ± 11	31 ± 1	25 ± 5	13 ± 1
Phosphatidylserine	7 ± 3	5 ± 2	4 ± 1	5 ± 2
Phosphatidylinositol	4 ± 1	4 ± 1	4 ± 3	10 ± 1
Phosphatidylcholine	4 ± 1	18 ± 2	38 ± 3	49 ± 3
Phosphatidylglycerol	19 ± 3	13 ± 2	7 ± 1	5 ± 1
Phosphatidylethanolamine	32 ± 10	25 ± 4	19 ± 4	17 ± 2

The phospholipids are listed in the order of increasing R_f in the chromatographic system of fig.1. The numbers represent radioactivity distribution expressed as percentage of the total radioactivity incorporated ± standard deviation (for 3 or 4 experiments) or mean deviation (for 2 experiments). Incorporation into microsomes at 10°C was too small to be evaluated precisely.

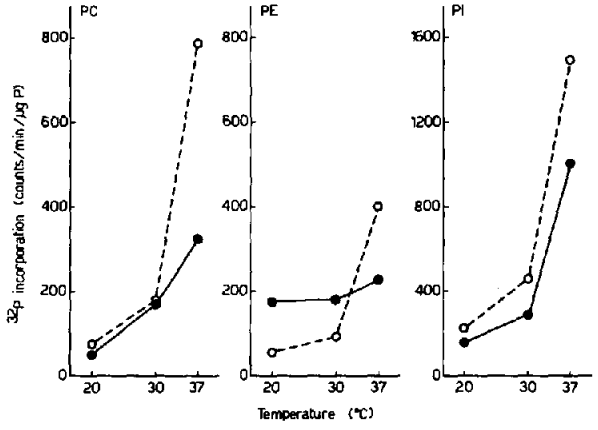

Fig.3. The effect of incubation temperature on the specific radioactivity of phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI) in Ehrlich ascites microsomes and mitochondria. Subcellular fragments after incubations of whole cells at temperatures indicated were isolated and phospholipids separated by thin-layer chromatography (see fig.1). Spots of phospholipids were scraped out from plates for counting and for phospholipid phosphorus determination. (●——●) Mitochondria; (o- - -o) microsomes.

A different picture is obtained when the specific labelling of various phospholipids is looked upon. It can be then observed (fig.3) that the specific radioactivity of phosphatidylcholine and phosphatidylinositol strongly increases with increasing temperature and more.so in microsomes than in mitochondria. A large increase at 37°C can also be observed in the specific labelling of microsomal phosphatidylethanolamine. In contrast, the specific labelling of mitochondrial phosphatidylethanolamine remains almost unchanged between 20°C and 37°C and at 20°C and 30°C it exceeds the specific labelling of this phospholipid in microsomes. This fact, together with a rather high contribution from mitochondrial phosphatidylglycerol, explains a higher overall specific labelling of mitochondrial phospholipids below 30°C (fig.2).

Probably the highest specific labelling among all phospholipids occurs in phosphatidic acid which, under conditions of fig.1, remained at the start line. However, because of minute amounts of this phospholipid its specific labelling was difficult to determine precisely.

4. Discussion

As can be seen from fig.3, the specific labelling of phosphatidylcholine and phosphatidylinositol runs parallel in mitochondria and in microsomes at various incubation temperatures. This is competible with the well known exchange of these phospholipids between intracellular membranes as mediated by specific exchange proteins [2]. Thus, these phospholipids synthesized in the endoplasmic reticulum can be incorporated into mitochondria (for the synthesis and exchange of phosphatidylinositol see also [25]). The most intriguing is the labelling of phosphatidylethanolamine which, at temperatures of 30°C and below, is much higher in mitochondria than in microsomes.

The explanation of this phenomenon is not clear. Two lines of speculation may be proposed. First, that phosphatidylethanolamine synthesized in the endoplasmic reticulum does not equilibrate with the bulk of phosphatidylethanolamine in these membranes but is rapidly transported to mitochondria where it accumulates. Since phosphatidylethanolamine is transported sluggishly (cf. [2]), it might be proposed that the newly synthesized small pool of phosphatidylethanolamine is first transformed into phosphatidylserine by the base exchange mechanism [26,27], then it is transported to mitochondria, and finally decarboxylated to phosphatidylethanolamine [28,29]. A second possibility is that mitochondria possess phospholipid synthesizing machinery which is, however, normally masked by prevailing synthetic processes proceeding in the endoplasmic reticulum and becomes only evident when the latter are decreased by lowering the temperature. If mitochondria are able, in fact, to synthesize phosphatidylethanolamine it would point to an additional similarity between mitochondria and bacteria, as it is well known that phosphatidylethanolamine is the main nitrogen-containing phospholipid formed in eubacteria [30].

Acknowledgements

The authors wish to thank Professor L. Wojtczak for his constant interest in this study and helpful discussions during preparations of the manuscript. Excellent technical assistance of Mrs Maria Bednarek is gratefully aknowledged.

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