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MEMBRANE LIPIDS COMPOSITION AND METABOLISM DURING EARLY EMBRYONIC DEVELOPMENT

PHOSPHOLIPID SUBCELLULAR DISTRIBUTION AND ^{32}P LABELING

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Phospholipid composition and ^{32}P metabolism were studied in oocytes and early developing embryos of the toad, *Bufo arenarum*, Hensel. The content and distribution of phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidic acid, sphingomyelin, phosphatidylserine,and diphosphatidylglycerol in embryos, whole oocytes, and the subcellular fractions of both were determined. Phosphatidylcholine and phosphatidylethanolamine were the major constituents of yolk platelet. Diphosphatidylglycerol was confined to the mitochondrial fraction, where it represented about 7% of the total phosphoacylglycerols. Relatively large amounts of sphingomyelin were found in microsomal and postmicrosomal supernatants. After in vivo labeling with ^{32}P, the early development of individual phospholipids in subcellular fractions and in whole eggs was followed. The greatest uptake was found in mitochondrial and yolk platelet fractions. A steady increase in the amount of ^{32}P present in phosphatidylethanolamine, phosphatidylcholine, and phosphatidylinositol was seen in the whole embryo from oocyte to late gastrula stage and in all subcellular fractions. Phosphatidic acid exhibited a slight decrease in specific activity, except in the yolk platelet fraction. This high ^{32}P incorporation would indicate a rapid and uneven polar headgroup turnover determined by phospholipid class and subcellular fraction. At the same time, the phospholipid content of the subcellular fractions studied remained unchanged during early embryogenesis. Moreover, ^{32}P was actively incorporated into the individual phospholipids in the absence of measurable net synthesis.

Introduction

Changes taking place in phospholipids during growth and development of embryos have been described previously [1–6]. However, no detailed study of individual phospholipids in the whole oocyte, embryo, and subcellular fractions has been done. During early cell cleavage, the embryo provides an excellent model for in vivo studies on membrane biogenesis; the synthesis and turnover of total phospholipids in this model have been reported [7–9]. The ^{32}P labeling of total phospholipids, phosphatidylcholine, and phosphatidylethanolamine in the whole embryo [2] shows an active increase as a function of early development. A similar pattern is also seen in the total phospholipids of embryo subcellular fractions [4]. To our knowledge, this report is the first time the content and ^{32}P metabolism of phosphatidic acid, phosphatidylserine, phosphatidylinositol, phos-

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phatidylcholine, phosphatidylethanolamine, sphingomyelin and diphosphatidylglycerol found in subcellular fractions of unfertilized oocytes and early developing vertebrate embryos have been described.

Material and Methods

Mature female toads, *Bufo arenarum* Hensel, were used. Ovulation was induced during hibernation by injection of homologous pituitary extract into the dorsal lymphatic sacs. Pituitary glands were stored and used as described by Pisáno [10]. When oviposition had begun, the animals were killed, ovisacs excised, and the oocytes obtained. In order to label oocytes endogenously with ^{32}P, 100 μCi of sterile $NaH^{32}PO_4$ (127.6 Ci/g P, Comision Nacional de Energia Atomica, Argentina) per 100 g of body weight were injected simultaneously with the pituitary extract. In vitro fertilization was performed by adding a homologous testicular homogenate. Development was carried out in a 1:10 amphibian Ringer solution at a temperature of 20–25°C. Embryological stages were characterized morphologically [11]; eggs showing surface irregularities or abnormalities were discarded. At a given stage, oocytes and embryos were released from the jelly coat by brief exposure to 2% thioglycollic acid neutralized with NaOH.

Cell fractions

Subcellular fractionation was performed using previously described procedures [4,12,13] with the following modifications: 10% homogenates were prepared in a 50 mM Tris-HCl buffer solution (pH 7.4) containing 10^{-3} M EDTA and 0.3 M sucrose. A Potter-Elvejhem homogenizer with a motor-driven Teflon pestle was used. All steps were carried out at temperatures between 0 and 4°C. The homogenate was centrifuged at $1500 \times g$ for 15 min. The pellet was transferred to a Potter-Elvejhem tube, rehomogenized by two strokes with a Teflon pestle, and sedimented at $1500 \times g$ for 15 min. The washing was repeated, after which the yolk platelet fraction was obtained. Supernatants and washings were combined and centrifuged at $20000 \times g$ for 15 min to obtain the mitochondrial fraction. The postmitochondrial supernatant was centrifuged (Beckman LS-50) at $133000 \times g$ for 60 min to produce a microsomal fraction pellet and postmicrosomal supernatant.

TABLE I

PHOSPHOLIPID AND TOTAL PROTEIN DISTRIBUTION IN SUBCELLULAR FRACTIONS FROM OOCYTE AND GASTRULA EMBRYOS

Phospholipid content is expressed as μmol phospholipid-P per 100 mg protein. Data represent mean ± standard deviation. *N*, number of samples. Numbers in parentheses indicate percent of total phospholipid in fraction. U, undetectable. Total protein content

Phospholipid	Yolk platelets fraction		Mitochondrial fraction	
	Unfertilized oocyte $N=5$	Late gastrula $N=3$	Unfertilized oocyte $N=8$	Late gastrula $N=3$
PC	6.35 ± 1.06 (63.6)	6.17 ± 1.08 (62.9)	26.23 ± 2.53 (51.3)	29.45 ± 3.68 (50.8)
PE	2.55 ± 0.27 (24.8)	2.15 ± 0.19 (22.7)	13.52 ± 1.13 (26.8)	16.14 ± 1.49 (26.9)
PI	1.33 ± 0.52 (9.4)	1.42 ± 1.05 (8.7)	3.93 ± 0.43 (7.4)	4.83 ± 0.60 (7.7)
PS	0.10 ± 0.02 (1.0)	0.10 ± 0.01 (1.1)	1.45 ± 0.38 (3.2)	1.59 ± 0.37 (2.9)
PA	0.02 ± 0.005 (0.2)	0.01 ± 0.00 (0.2)	0.08 ± 0.02 (0.2)	0.10 (2) (0.2)
Sph	0.43 ± 0.07 (4.6)	0.43 ± 0.07 (4.7)	2.30 ± 0.48 (3.1)	1.99 ± 0.61 (2.6)
DPG	U	U	3.12 ± 0.59 (6.9)	4.28 ± 0.68 (7.6)
Total	10.42 ± 1.52	9.70 ± 1.42	51.10 ± 4.31	58.79 ± 6.31
Total proteins	868.4 ± 240.8		18.0 ± 3.0	
% distribution total proteins	89.0 ± 1.5	92.2 ± 1.8	1.8 ± 0.1	1.4 ± 0.08

Extraction and separation of phospholipids

Lipid extracts were prepared from whole oocytes and embryos, yolk platelets, and mitochondrial and microsomal fractions using the method of Folch et al. [14]. Postmicrosomal supernatants were extracted according to Bligh and Dyer [15].

Phospholipids were isolated by two-dimensional thin-layer chromatography on silica gel H using chloroform/methanol/concentrated ammonia (65:25:5, by vol.) and chloroform/acetone/methanol/acetic acid/water (4 : 60 : 15:15:7.5, by vol.) as developing solvents [16]. Spots were visualized by iodine vapours. All extracts were stored under N_2 at −20°C.

Analytical procedure

Proteins were extracted with 1 M NaOH at 100°C for 20 min and identified by the method of Lowry et al. [17] using crystalline bovine serum albumin as a standard. Lipid phosphorus was measured after digestion with $HClO_4$ using the method of Rouser et al. [16]. Radioactivity was determined in a Beckman LS-250 liquid scintillation counter using 4% Omnifluor in toluene.

Results

Phospholipid patterns in subcellular fractions of early developing embryos

Cleavage, the first phase of embryonic development, involves nuclear division and the formation of cell membranes between daughter cells. The phospholipid distribution within subcellular fractions of oocyte and gastrula was characterized by the retention of about 80% of the whole embryo phospholipids found in the yolk platelet fraction. However, the phospholipid/protein ratio was low, due to the high protein concentration of this fraction (Table I). Phosphatidylcholine and phosphatidylethanolamine were the major phospholipids in yolk platelets. Phosphatidylserine and phosphatidylinositol made up about 1% and 9%, respectively, of the total phospholipids. Sphingomyelin represented 4.6% of the total phospholipids, phosphatidic acid only 2%, and diphosphatidylglycerol was undetectable, as shown previously by qualitative chromatography [4]. Phosphatidylcholine plus phosphatidylethanolamine

expressed in μg per subcellular fraction derived from 3000 oocytes or embryos. Values are mean±standard deviation from six independent samples. Protein content at late gastrula stage was similar to that of unfertilized oocyte. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PA, phosphatidic acid; Sph, sphingomyelin; DPG, diphosphatidylglycerol.

Microsomal fraction		Postmicrosomal fraction	
Unfertilized oocyte $N=3$	Late gastrula $N=3$	Unfertilized oocyte $N=9$	Late gastrula $N=6$
43.29±2.71 (55.1)	34.05±2.60 (56.8)	2.95± 0.36 (46.3)	3.33±0.21 (41.4)
8.59±0.43 (22.9)	10.78±1.16 (21.8)	1.92± 0.64 (23.3)	2.35±0.28 (25.4)
2.90±0.43 (7.8)	3.02±0.01 (7.9)	0.53± 0.15 (7.6)	0.54±0.16 (7.6)
2.47±0.80 (4.8)	3.10±0.63 (4.5)	0.57± 0.15 (7.9)	0.70±0.07 (7.9)
0.11±0.04 (1.0)	0.21 (2) (0.6)	0.04± 0.01 (0.3)	0.03±0.01 (0.5)
6.17±1.05 (12.1)	6.22±1.39 (9.4)	1.12± 0.41 (14.4)	1.28±0.11 (16.5)
77.80±6.72	62.6 ±7.74	7.30± 1.58	8.86±1.46
7.1 ±1.1		81.8 ±13.0	
0.7 ±0.2	0.5 ±0.06	8.4 ± 1.7	7.0 ±0.2

represented 78% of the total phospholipids in the mitochondrial fraction, while phosphatidylserine and phosphatidic acid represented approx. 3% and 0.2%, respectively. Diphosphatidylglycerol amounted to about 7% of the total phospholipids and showed a tendency to increase at the gastrula stage. Phosphatidylinositol represented about 7.5% of the fraction. The phospholipid patterns of microsomal and postmicrosomal fractions showed a relatively high sphingomyelin content. The postmicrosomal fraction was found to have: (a) the lowest percent content of phosphatidylcholine; (b) a sphingomyelin content of about 15%; and (c) the highest proportion of phosphatidylserine of all fractions studied. During early development, no striking changes could be detected in the phospholipid patterns of oocytes.

During early embryogenesis, most of the total protein was confined to the yolk platelets (Table I). The postmicrosomal supernatant contained 7% to 10% of the total, whereas only 1.4% to 1.9% was recovered from the mitochondrial fraction. The microsomal fraction yielded 0.5% to 0.7% of the embryonic proteins, which correlates with the small amount of endoplasmic reticulum that has been observed in oocytes and early developing embryos [18].

^{32}P-Labeled phospholipids in oocytes, early embryos, and derived subcellular fractions

This study was designed to follow the uptake of [^{32}P]phosphate into individual phosphoacylglycerols. Table II shows the incorporation of the precursor in total and individual phospholipids during four stages of development: unfertilized and fertilized oocytes, and late blastula and gastrula embryos. Total phospholipids, as well as phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol, showed a steady increase in ^{32}P content during development, while an apparent decrease in phosphatidic acid and minor changes in phosphatidylserine and sphingomyelin were seen.

At the subcellular level, oocyte-labeling was maximal in the mitochondrial fraction, and less in the yolk platelets and postmicrosomal supernatant (Table III). As development continued, radioactive phospholipids increased in all fractions. In the yolk platelet fraction, the increase was about 3.5-fold from oocyte to late gastrula stage, wheres in the mitochondrial fraction, microsomal fraction, and postmicrosomal supernatant, a 2-fold increase was seen in a similar period of time.

The specific activity of each phospholipid during development is given in Fig. 1. A major increase in specific activity was seen in the microsomal fraction. Phosphatidylcholine also displayed high values in the mitochondrial fraction and postmicrosomal supernatant. Phosphatidylcholine, phosphatidylinositol, and phosphatidylethanolamine showed similar increases in the various fractions. The most surprising changes were seen in the mitochondrial and microsomal fractions, where the specific activity of phosphatidic acid decreased. It is also interesting that the ^{32}P-labeling of sphingomyelin in mitochondrial, microsomal,

TABLE II

^{32}P INCORPORATION IN PHOSPHOLIPIDS OF WHOLE OOCYTES AND DEVELOPING EMBRYOS

Values represent nmol of $^{32}P \times 10^{-6}$ per 300 oocytes or embryos.

Phospholipids	Unfertilized oocyte	Fertilized oocyte	Late blastula	Late gastrula
Phosphatidylcholine	103.79±29.4	114.9±33.8	259.6±36.0	237.4±25.0
Phosphatidylethanolamine	28.7 ± 4.0	22.2± 2.3	55.6± 8.6	77.2 (2)
Phosphatidylinositol	15.2 ± 3.9	15.0± 3.7	33.1± 2.1	27.6 (2)
Phosphatidic acid	11.5 ± 3.3	10.0± 2.4	12.4± 5.4	5.1± 3.2
Phosphatidylserine	2.1 ± 0.9	4.1± 1.0	3.3± 1.5	7.0 (2)
Sphingomyelin	3.5 ± 1.6	4.5± 2.4	5.1± 2.1	3.9 (2)
Total	172.6 ±53.8	171.0±37.3	380.4±39.1	353.4±19.0

TABLE III

^{32}P LABELING OF TOTAL PHOSPHOLIPIDS IN SUBCELLULAR FRACTIONS

Subcellular fractions were obtained from about 3000 oocytes or embryos. Data represent mean ± standard deviation. Figures in brackets indicate number of samples. Results are given as nmol ^{32}P.

	Unfertilized oocyte	Fertilized oocyte	Late blastula	Late gastrula
Yolk platelets	227.5 ± 54.0 (7)	252.1 ± 45.1 (4)	868.3 ± 101.1 (4)	794.4 ± 167.3 (3)
Mitochondrial fraction	395.5 ± 83.0 (7)	443.8 ± 48.0 (3)	871.8 ± 106.2 (4)	936.2 ± 91.3 (3)
Microsomal fractions	99.2 ± 46.9 (6)	112.0 ± 35.5 (3)	148.5 (2)	264.5 ± 101.0 (3)
Postmicrosomal supernatant	194.1 ± 55.1 (7)	235.1 ± 54.2 (4)	558.3 ± 161.5 (4)	421.9 ± 76.8 (3)

and postmicrosomal supernatants increased. The specific activity of diphosphatidylglycerol, however, showed only a slight increase during the late gastrula stage. Phosphatidylserine exhibited a marked increase in specific activity in the microsomal fraction.

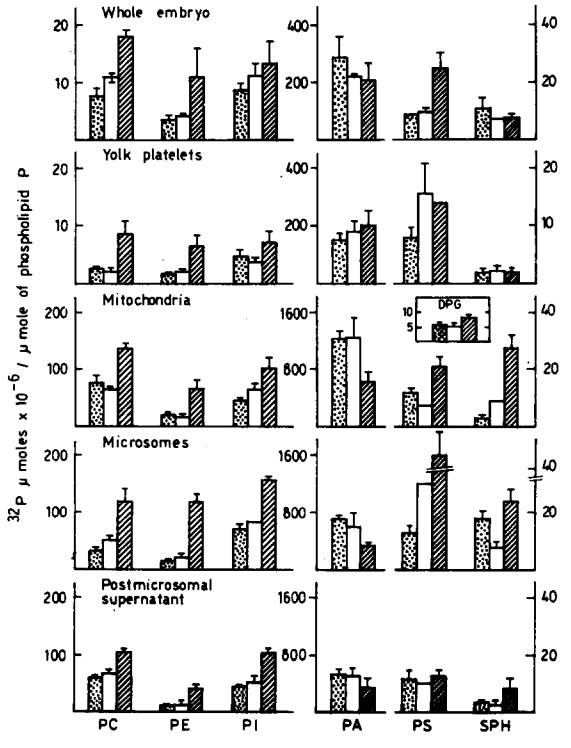

Fig. 1. Specific activities of phospolipids during early amphibian development. Samples were from 300 whole embryos or from subcellular fractions obtained from 3000 embryos. Values are the mean of five different ovulations.

Discussion

The observations reported here concerning the ^{32}P-labeling of individual phospholipids from whole embryos and subcellular fractions during early embryogenesis indicate that active metabolism occurs in spite of the remarkably similar phospholipid profile seen during early stages of development at both the cellular and subcellular levels. This is in agreement with previous observations on the unchanged concentration of total phospholipids [8], phosphatidylcholine, and phosphatidylethanolamine [2] found in eggs during cell cleavage. Subcellular fractions from oocytes showed phospholipid content and distribution similar to fractions from gastrula embryos. However, there were differences in the distribution of individual phospholipids among the analyzed fractions. Phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol were located primarily in yolk platelets. In contrast, 50% of the total phosphatidylserine was distributed between the mitochondrial fraction and postmicrosomal supernatant, with only a very small proportion of this lipid in the microsomal fraction.

The distribution of total phosphatidic acid and sphingomyelin in subcellular fractions was very similar. Mitochondrial and microsomal fractions isolated from oocytes or embryos showed a higher phospholipid content than mitochondrial and microsomal fractions isolated from rabbit liver by Miller and Cornatzer [19] during perinatal development. In agreement with these authors, we found a higher phosphatidylcholine content in the mitochondrial fraction than in the microsomal

fraction, when judged on a protein basis.

The characteristic diphosphatidylglycerol (cardiolipin) content of the mitochondrial fraction seen in mammals [20,21] is also found in amphibian mitochondria. Moreover, this phospholipid must be confined to the mitochondrial fraction, since other fractions are devoid of cardiolipin.

The relatively high amount of enrichment in sphingomyelin found in the postmicrosomal supernatant may be due to the shuttling of phospholipids for use in newly formed membranes and also to the phospholipid exchange that may occur during cell cleavage [4,22]. Hence, the enrichment of this fraction can be related to the transfer of sphingomyelin from the organelle where the phospholipid may be stored (e.g. yolk platelet) or synthesized (microsomal fraction) to the site of membrane formation.

The protein distribution pattern during early embryogenesis, previously reported by our laboratory [4], extends to the microsomal and postmicrosomal supernatant fractions. The reported values are in accordance with those from eggs of the frog, *Xenopus laevis* [23], in which only 12.3% of the total oocyte mass is considered as 'non-yolk' material.

In *Xenopus laevis*, the first new proteins thus far detected evolve during gastrulation,with an increase in the number of new proteins occurring at the neurula stage [24]. Hence, as observed in sea urchin eggs [25], very few proteins are formed during cell cleavage. During early cell cleavage, protein synthesis profiles in these eggs do not change significantly, and new protein synthesis is not detectable until the gastrula stage. In agreement with these studies, we found that the protein content in the whole embryo and in each subcellular fraction was unchanged at least until gastrulation. Since the methods used in this study do not detect minor quantitative changes, no conclusion about protein synthesis can be drawn.

Since yolk platelets contain a higher proportion of proteins and phospholipids than found in oocytes and embryos, nascent membranes may obtain these constituents [26] by a distribution process which may take place at the subcellular location of the phospholipid. Total cell mass [27] and phospholipids have been shown to remain constant during cell cleavage. However, in the yolk platelets [28], membranes do evolve and changes take place. The superficial layers of these inclusions become labile, whereas their central crystalline structural components remain unmodified [29].

Yolk platelets and mitochondrial fractions in early embryos displayed a high level of ^{32}P metabolism, with an enhancement upon gastrulation. Acid-soluble phosphate also becomes labelled rapidly [4] and includes high energy-containing nucleotides [30]. The specific activity of ^{32}P-labeled acid-soluble phosphate diminishes from the period of early development to the gastrula stage [4]. These changes could also be involved in an intracellular distribution of phosphate that may then be used to label lipids.

The increased ^{32}P-labeling of lipid classes at the subcellular level suggests that the existence of an active turnover of the polar moiety of membrane lipids may be related to an endogenous non-lipid pool which is highly labeled with the precursor.

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