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Mitochondrial lipids in *Bufo arenarum* full-grown oocytes

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Summary

Both the content and composition of polar and neutral lipids from the mitochondrial fraction of ovarian full-grown *Bufo arenarum* oocytes were analysed in the present study. Triacylglycerols (TAG) represent 33% of the total lipids, followed by phosphatidylcholine (PC), free fatty acids (FFA) and phosphatidylethanolamine (PE). Diphosphatidylglycerol (DPG) or cardiolipin, a specific component of the inner mitochondrial membrane, represents about 4% of the total lipid content. Palmitic (16:0) and arachidonic (20:4n6) acids are the most abundant fatty acids in PC and PE, respectively. DPG is enriched in fatty acids with carbon chain lengths of 18, the principal component being linoleic acid. In phosphatidylinositol, 20:4n6 and stearic acid (18:0) represent about 72 mol% of the total acyl group level. The main fatty acids in TAG are linoleic (18:2), oleic (18:1), and palmitic acids. The fatty acid composition of FFA and diacylglycerols is similar, 16:0 being the most abundant acyl group. PE is the most unsaturated lipid and sphingomyelin has the lowest unsaturation index.

Keywords: Amphibian oocytes, Fatty acid composition, Mitochondria, Neutral lipids, Phospholipids

Introduction

During the development of amphibian oocytes many thousands of different organelles and macromolecular species are formed and positioned in the cell (Davidson, 1976). The full-grown oocyte of the toad *Bufo arenarum*, stage V according to Valdéz Toledo & Pisanó (1980), represents female germinal material that has reached the last growth stage in the ovarian follicle. The oocyte accumulates, through different synthetic processes, the substances that it will use during early development. In addition, the oocyte contains an abundant amount of vitello organized as yolk platelets that are asymmetrically distributed through the cytoplasm. These organelles have been generally considered as mere reservoirs of raw materials (Karasaki, 1963) but their vesicular structure may be indicative of an active participation either in lipogenesis or in membrane biogenesis (Alonso, 1989).

The occurrence of discontinuous mitochondrial synthesis during *Xenopus* oogenesis has been suggested. Mitochondria are accumulated and aggregated during the previtellogenic period as a mitochondrial cloud and are dispersed throughout the cytoplasm during vitellogenesis (Balinsky & Devis, 1963; Van Blerkom & Runner, 1984). Similarly, a recent study in *Bufo arenarum* oogenesis has demonstrated that the relative mitochondrial population of stage III oocytes (late vitellogenesis) is larger than that of stage V (Bruzzone *et al*., 2003). In *Xenopus*, their level remains constant through maturation, fertilization and early development (Webb & Smith, 1977; Callen *et al*., 1980). The maternal contribution is enough to maintain oxidative phosphorylation in early embryogenesis (Larsson *et al*., 1998). Mitochondria located within the mitochondrial cloud are highly active compared with mitochondria dispersed within the cytoplasm, thus suggesting that the mitochondrial cloud is a region of high activity in frog oocytes (Wilding *et al*., 2001).

A structural relationship between the mitochondria and the yolk platelets in amphibian and crustacean oocytes has been proposed (Ward, 1962; Massover, 1971; Vallejo *et al*., 1979; Warner *et al*., 2002). Also, a functional relationship between these organelles has been suggested in *Bufo arenarum* oocytes. At fertilization time, platelet triacylglycerols undergo a massive breakdown and efficient metabolization through β-oxidation, a mechanism that takes place in mitochondria (Alonso *et al*., 1986).

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Recently, a detailed analysis of lipids has been carried out in yolk platelets from *Bufo arenarum* oocytes (Buschiazzo *et al*., 2003). The aim of the present study was to analyse the content and composition of neutral and polar lipids in mitochondrial fractions.

Materials and methods

Experimental system

Mature females of the toad *Bufo arenarum* Hensel, 1867 were used. They were captured from the surroundings of Bahía Blanca city and maintained under laboratory conditions. The ovarian tissue was surgically removed and transferred to ND 96 solution (96 mM NaCl, 1 mM $MgCl_2$, 2 mM KCl, 5 mM HEPES; pH 7.4; Sigma, St Louis, MO). Full-grown oocytes (1.7–1.8 mm in diameter), stage V according to Valdéz Toledo & Pisanó (1980), were isolated with watchmaker's forceps under a stereotaxic microscope just before use.

Subcellular fractionation

Oocyte homogenates were prepared in 50 mM Tris-HCl buffer, pH 7.4, containing 10^{-3} M EDTA and 0.3 M sucrose according to the procedure described by Bonini de Romanelli *et al*. (1981). All the experimental steps were carried out at 0–4 °C. The yolk platelet fraction was sedimented at 1500 *g* for 20 min. Two washings were done at the same velocity during 10 min. The supernatants were centrifuged at 20 000 *g* for 20 min. The pellet was rehomogenized in the same medium and centrifuged at 20 000 *g* for 15 min. The washing was repeated, after which the mitochondrial fraction was sedimented.

Lipid analysis

Lipid extraction was performed following Folch *et al*. (1957). Phospholipids were separated by two-dimensional thin-layer chromatography (TLC) on silica gel H according to Rouser *et al*. (1970). Neutral lipids were separated by monodimensional TLC on silica gel G using hexane/diethylether/acetic acid (80:20:1, by volume). Phospholipids were identified after exposure of the plates to iodine vapours and quantified by phosphorus analysis after digestion with perchloric acid (Rouser *et al*., 1970).

For the determination of fatty acid composition, the lipids were separated by TLC as described above, and spots were visualized under ultraviolet light after spraying with 2′-7′-dichlorofluorescein 0.2% in methanol. Fatty acid methyl esters were prepared according to the method of Morrison & Smith (1964) using boron trifluoride (14% w/v in methanol; Sigma, St Louis, MO). Methyl esters were purified by TLC using hexane/diethylether (95:5, by volume) on silica gel G prewashed with methanol/diethylether (75:25, by volume). Fatty acid analysis was carried out by temperature-programmed gas-liquid chromatography (GLC) using 21:0 methyl ester as internal standard. Two glass columns (2 m × 0.2 cm i.d.) packed with 10% SP2330 on 80–120 Chromosorb WAW (Supelco, Bellefonte, PA) were connected to two flame ionization detectors operated in the dual-differential mode. The initial and final oven temperatures were 160 and 220 °C, respectively, and the rate of increase was 5 °C/min. Injector and detector temperatures were 220 and 230 °C, respectively. The carrier gas was N_2 (30 ml/min). Chromatograms were quantified with a CDS-111 Varian integrator (Palo Alto, CA). Peaks were identified by comparison of retention times with those of standards. This procedure led to a tentative identification of polyunsaturated fatty acids. The unsaturation index (UI) was determined as the sum of moles% of individual unsaturated fatty acids times the number of double bonds.

During all the procedures (lipid extraction, solvent evaporation, TLC spotting, drying and spraying of the TLC plates, and derivatization), the lipids were kept in an N_2 atmosphere and at low temperature. All organic solvents were of analytical grade.

Other analytical methods

Proteins were determined according to Lowry *et al*. (1951) after extraction with 1 N NaOH using crystalline bovine serum albumin as standard.

Results and discussion

Biochemical studies have been carried out in mitochondrial fractions from oocytes in different species (Petrucci & Cesare, 2000; Stojkovic *et al*., 2001), yet little is known about their lipid composition. The present study provides the first detailed lipid analysis of mitochondria of *B. arenarum* full-grown oocytes. The content and composition of the mitochondrial neutral and polar lipids were analysed.

Phospholipid content of the mitochondrial fraction is shown in Fig. 1. The total phospholipid amount in mitochondria is 208.87 ± 9.68 nmol/mg protein. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the main phospholipids followed by diphosphatidylglycerol (DPG) or cardiolipin, phosphatidylinositol (PI) and sphingomyelin (SM). Phosphatidylserine (PS) and phosphatidic acid (PA) are minor components. When the results from the present study were compared with those related to mitochondrial phospholipids of ovulated oocytes of *Bufo arenarum* (Alonso *et al*., 1982), a similar percentage

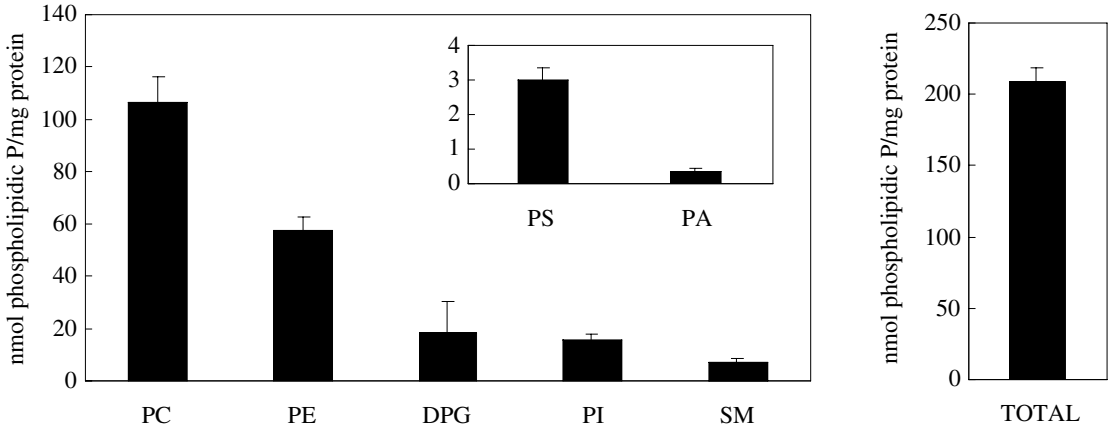

Figure 1 Content of phospholipids in the mitochondrial fraction from *Bufo arenarum* full-grown oocytes. Phospholipidic phosphorus was measured according to Rouser *et al*. (1970). Results are presented as nanomoles of phosphorus per milligram protein and are mean values ± SD from four independent samples. PC, phosphatidylcholine; PE, phosphatidylethanolamine; DPG, diphosphatidylglycerol; PI, phosphatidylinositol; SM, sphingomyelin; PS, phosphatidylserine; PA, phosphatidic acid.

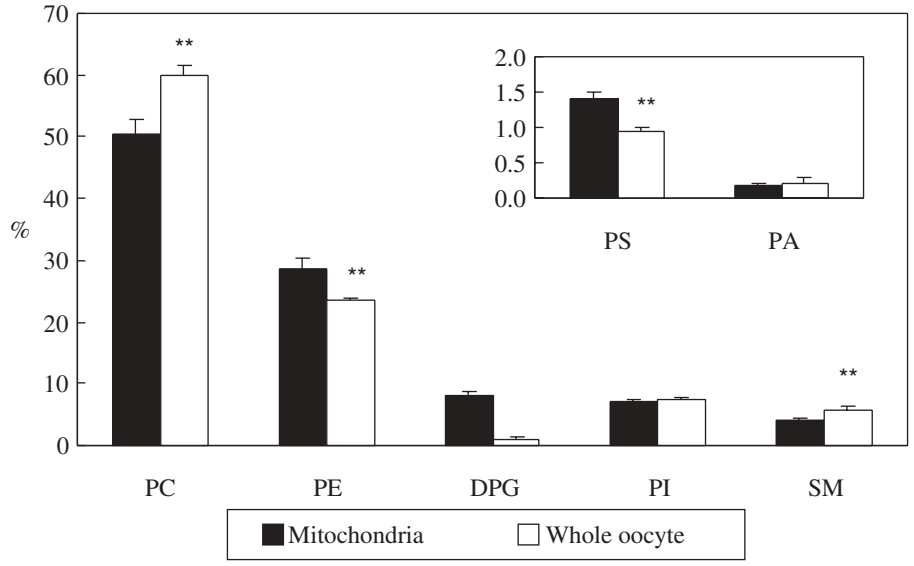

Figure 2 Phospholipid percentage distribution in *Bufo arenarum* mitochondrial fraction and full-grown oocytes. Data correspond to mean values ± SD of four independent samples. Asterisks indicate significant differences between these two groups ($p < 0.01$). PC, phosphatidylcholine; PE, phosphatidylethanolamine; DPG, diphosphatidylglycerol; PI, phosphatidylinositol; SM, sphingomyelin; PS, phosphatidylserine; PA, phosphatidic acid.

distribution was observed. These results may indicate that the meiosis reinitiating process and oviductal secretions do not overall alter the mitochondrial phospholipid profile.

Phospholipid distribution is similar in whole full-grown oocytes and in their mitochondrial fractions (Fig. 2). In both cases, PC and PE are about 80% of the total content as in full-grown oocytes of *Xenopus laevis* (Alonso *et al*., 1987). It is evident that the mitochondrial fraction is enriched in DPG, a specific component of the inner mitochondrial membrane that represents about 8% of the total phospholipid content. The sum of choline lipids, PC and SM represents about 60% of the total level as in other biological systems (White, 1973).

The fatty acid composition of PC, PE, DPG and PI in the mitochondrial fraction is shown in Table 1. In PC, palmitic (16:0), linoleic (18:2), arachidonic (20:4n6) and oleic (18:1) acids are the most abundant fatty acids, representing about 71 mol% of the total amount. In PE, the main acyl group is 20:4n6, which represents 24 mol% of the total fatty acids. In addition, important levels of stearic acid (18:0), 18:1 and 18:2 were observed.

DPG is enriched in fatty acids with carbon chain lengths of 18, linoleic acid being the principal component, as shown in mitochondrial fractions of

Table 1 Fatty acid composition of phosphatidylcholine, phosphatidylethanolamine, diphosphatidylglycerol and phosphatidylinositol in mitochondria from full-grown *Bufo arenarum* oocytes

	PC	PE	DPG	PI
Fatty acids		(mol %)		
16:0	26.15 ± 1.33	7.71 ± 1.06	4.55 ± 0.36	8.86 ± 0.98
16:1	8.68 ± 1.85	1.83 ± 0.23	2.03 ± 0.22	1.18 ± 0.21
17:0	0.95 ± 0.11	1.33 ± 0.18	0.26 ± 0.06	1.42 ± 0.03
18:0	6.29 ± 0.46	18.90 ± 1.34	1.43 ± 0.18	36.63 ± 3.73
18:1	13.72 ± 0.45	14.69 ± 1.33	14.90 ± 0.58	6.72 ± 0.86
18:2	16.27 ± 0.70	15.42 ± 0.41	65.15 ± 1.25	3.79 ± 0.39
18:3n3	2.07 ± 0.12	2.48 ± 0.39	7.96 ± 0.48	0.30 ± 0.01
20:3n6	0.93 ± 0.06	0.45 ± 0.22	0.59 ± 0.05	–
20:4n6	14.97 ± 1.71	23.95 ± 1.79	2.11 ± 0.42	35.46 ± 3.75
20:5n3	3.19 ± 0.44	5.08 ± 0.92	–	1.52 ± 0.10
22:5n6	0.16 ± 0.02	0.16 ± 0.06	–	0.21 ± 0.01
22:5n3	3.20 ± 0.48	4.18 ± 0.83	1.02 ± 0.10	2.69 ± 0.13
22:6n3	3.42 ± 0.20	3.83 ± 0.82	–	1.22 ± 0.08
SFA	33.39 ± 1.83	27.94 ± 2.33	6.24 ± 0.43	46.91 ± 3.96
MUFA	22.40 ± 1.63	16.52 ± 1.53	16.93 ± 0.79	7.90 ± 1.06
PUFA	44.21 ± 2.74	55.55 ± 3.79	76.83 ± 0.65	45.19 ± 4.29
UI	189.07 ± 10.19	231.60 ± 17.83	188.06 ± 1.12	196.71 ± 16.26

Data are mean values ± SD of four independent samples.
PC, phosphatidylcholine; PE, phosphatidylethanolamine; DPG, diphosphatidylglycerol; PI, phosphatidylinositol; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; UI, unsaturation index obtained as described in Materials and Methods.

ovulated oocytes and early embryos of *Bufo arenarum* (Bonini de Romanelli *et al*., 1981). A similar composition has been registered in the majority of animal tissues and higher plants (Comte *et al*., 1976; Landriscina *et al*., 1976; Ledwoch *et al*., 1979; Schlame *et al*., 2000). In contrast, in yeast palmitoleic acid (16:1) and 18:1 seem to represent the main quantities, whereas in bacteria DPG possesses saturated and unsaturated fatty acids of 14 and 18 carbons (Jacovcic *et al*., 1971; Hoch, 1992).

As for PI, the compositional analysis (Table 1) demonstrates that 20:4n6 and 18:0 are the main acyl groups, which represent approximately 72 mol% of the total level. Significant amounts of 16:0 and 18:1 were also found. This fatty acid profile is similar to that described for this phospholipid in mitochondria from *Bufo arenarum* ovulated oocytes (Bonini de Romanelli *et al*., 1981). Arachidonic and stearic acids are the main acyl components of PI in other tissues of mammals and amphibians (Baker & Thompson, 1972).

The UI of PE is higher than that of PC, DPG and PI. There is an important contribution of polyunsaturated fatty acids (PUFA) in DPG, while in PI similar proportions of saturated fatty acids (SFA) and PUFA are observed.

Table 2 shows the compositional analysis of SM, PS and PA. SM has a characteristic fatty acid profile. It mainly contains saturated fatty acids (about 77 mol% of the total acyl groups), the most abundant ones being 18:0 and 16:0. Nervonic acid (24:1) is a typical fatty acid that represents about 7 mol%. SM has a high contribution of SFA. In PS, 18:0 is the predominant acyl group followed by 16:0 and 20:4n6. As regards PA, 16:0 and 18:0 represent approximately 46 mol% of the total content. Similar levels of 20:4n6 and 18:1 were

Table 2 Fatty acid composition of sphingomyelin, phosphatidylserine and phosphatidic acid in mitochondria from full-grown *Bufo arenarum* oocytes

Fatty acids	SM (mol %)	Fatty acids	PS (mol %)	PA (mol %)
15:1	1.63 ± 0.25	16:0	23.13 ± 1.91	25.08 ± 6.48
16:0	24.79 ± 4.42	16:1	5.59 ± 1.69	6.63 ± 0.72
18:0	35.63 ± 2.85	17:0	–	1.64 ± 0.52
18:1	6.38 ± 1.23	18:0	33.72 ± 4.39	21.53 ± 5.73
18:2	7.31 ± 0.29	18:1	8.98 ± 1.26	14.73 ± 1.75
20:0	1.69 ± 0.07	18:2	10.66 ± 2.86	9.66 ± 0.49
24:0	5.24 ± 0.55	18:3n6	–	5.07 ± 1.54
24:1	7.56 ± 0.51	20:4n6	13.27 ± 3.21	15.66 ± 2.36
26:0	9.77 ± 1.22	20:5n3	4.64 ± 1.21	–
SFA	77.12 ± 0.19	SFA	56.85 ± 2.96	48.26 ± 5.26
MUFA	15.57 ± 0.47	MUFA	14.57 ± 1.14	21.36 ± 2.37
PUFA	7.31 ± 0.29	PUFA	28.57 ± 1.94	30.39 ± 3.51
UI	30.38 ± 0.18	UI	118.36 ± 8.21	124.30 ± 13.43

Data are mean values ± SD of four independent samples.
SM, sphingomyelin; PS, phosphatidylserine; PA, phosphatidic acid. Other details as in Table 1.

Table 3 Fatty acid composition of neutral lipids in mitochondria from full-grown *Bufo arenarum* oocytes

Fatty acids	TAG	DAG	FFA
	(nmol/mg protein)		
14:0	7.13 ± 1.01	1.07 ± 0.18	2.44 ± 0.74
15:0	3.64 ± 0.20	0.83 ± 0.04	1.85 ± 0.57
15:1	2.16 ± 0.59	0.22 ± 0.09	–
16:0	82.45 ± 6.33	7.33 ± 0.12	18.71 ± 5.55
16:1	44.25 ± 5.56	3.05 ± 0.09	8.21 ± 0.80
17:0	4.83 ± 0.48	0.52 ± 0.15	1.30 ± 0.24
17:1	6.98 ± 0.57	–	1.33 ± 0.10
18:0	13.50 ± 1.05	3.44 ± 0.42	9.19 ± 2.55
18:1	82.79 ± 6.93	5.54 ± 0.48	12.18 ± 2.38
18:2	113.90 ± 9.56	4.12 ± 0.11	12.22 ± 5.36
18:3n6	4.87 ± 0.60	0.37 ± 0.04	0.54 ± 0.21
18:3n3	38.80 ± 2.16	1.38 ± 0.23	6.83 ± 1.94
20:3n6	3.98 ± 0.04	–	0.23 ± 0.04
20:4n6	17.79 ± 1.09	1.25 ± 0.65	3.35 ± 0.62
20:4n3	2.60 ± 0.98	–	0.41 ± 0.17
20:5n3	6.92 ± 1.81	–	1.01 ± 0.59
22:5n6	0.82 ± 0.13	–	–
22:5n3	4.72 ± 0.80	0.30 ± 0.05	0.52 ± 0.20
22:6n3	3.65 ± 0.60	0.42 ± 0.12	0.48 ± 0.09
Total	148.59 ± 8.85	14.92 ± 0.57	80.80 ± 8.48
SFA	111.55 ± 8.61	13.19 ± 0.21	33.48 ± 9.31
MUFA	136.17 ± 10.59	8.81 ± 0.48	21.72 ± 2.37
PUFA	198.05 ± 8.21	7.83 ± 0.66	25.59 ± 7.21
UI	157.11 ± 3.33	110.70 ± 5.86	123.00 ± 24.12

Data are mean values ± SD of four independent samples. Total amount of triacylglycerol fatty acids is divided by 3 and of diacylglycerol fatty acids is divided by 2.
TAG, triacylglycerols; DAG, diacylglycerols; FFA, free fatty acids. Other details as in Table 1.

determined. There were no differences in UI when PS and PA were compared.

The content and composition of neutral lipids is shown in Table 3. Triacylglycerols (TAG) represent about 61% of the total content of neutral lipids while diacylglycerols (DAG) and free fatty acids (FFA) represent 6% and 33%, respectively.

The main fatty acids in TAG are 18:2, 18:1 and 16:0. Similar levels of 16:1 and linolenic acid (18:3) were found. The fatty acid profile is similar to that observed in TAG from stage III oocytes (Valdéz Toledo & Pisanó, 1980), in which a relatively larger mitochondrial population with respect to other oogenetic stages has been reported (Bruzzone *et al.*, 2003). In DAG, 16:0 is the main fatty acid followed by 18:1, 18:2, 18:0 and 16:1. In both lipids, significant levels of 20:4n6 were determined. The acyl distribution of FFA is similar to that described for DAG, 16:0 also being the main fatty acid. The UI of TAG is higher than that of FFA and DAG, mainly because of the contribution of PUFA. The high values found in neutral lipids of 16:0, 18:1 and 18:2, could serve as a reservoir pool of metabolic precursors because these fatty acids are required as substrates for elongation and desaturation. They may also function as combustible lipids via β-oxidation.

In general, little is known about unusual fatty acids such as 14:0, 15:0, 15:1, 17:0 and 17:1 in the different biological systems. Their presence in mitochondrial lipids could be indicative of an oxidative mechanism different from that of β-oxidation. It may then also be possible that some of these unusual fatty acids are synthesized through propyonyl-CoA.

Altogether, the results of the present study show that TAG are the most abundant lipids in mitochondria followed by PC. PE and FFA are found in similar quantities. DPG represent a significant 4% of the total mitochondrial lipids. PE is the most unsaturated lipid and SM shows the lowest UI.

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