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Effect of phospholipase-D on rat kidney mitochondria*

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Abstract. Incubation of purified rat kidney mitochondrial fraction with phospholipase-D resulted in the accumulation of phosphatidic acid in the membrane due to the degradation of membrane-bound phosphatidylcholine, -serine and -ethanolamine Simultaneously with the hydrolysis of the phospholipids, cholesterol and protein were released from the mitochondrial membrane into the medium, and binding of Ca^{2+} by mitochondrial membranes increased. Phospholipase D-treated mitochondrial fraction exhibited increased swelling *in vitro* in the early stages of incubation (15 min) after which the mitochondria were ruptured. Membrane-bound adenosine triphosphatase was partially inactivated and the enzyme activity was not significantly restored by incubation with sonicated dispersions of phosphatidylcholine, -serine and cholesterol. These results indicate that removal of choline, serine and ethanolamine from membrane-bound phospholipids disrupt phospholipid-cholesterol and phospholipid-protein association and affect functions of the membrane.

Keywords. Mitochondria; phospholipase-D; phosphatidylcholine; phosphatidylethanolamine; phosphatidylserine; swelling; adenosine triphosphatase; Ca^{2+} binding.

Introduction

It is universally recognised that ionic as well as apolar interactions are involved in lipid protein association in biological membranes. Phospholipases have been employed as extrinsic aids to elucidate the role of polar and apolar interactions in the organisation of structure and function of membranes. Considerable work has been carried out on the action of phospholipases A and C on red blood cells, microsomal and mitochondrial fractions of mammalian tissues and other organelles. This has helped in formulation of membrane models (Lenard and Singer, 1966; Singer and Nicholson, 1972). However, there is paucity of information on the action of phospholipase-D on biological membranes. Fragmentory information on the effect of phospholipase-D (EC 3.1.4.4) on the activity of membrane-bound enzymes of microsomal fraction and sarcoplasmic reticulum is available (Martonossi *et al*., 1969; Elling and Di Augustine, 1971; Kapoor *et al*.,

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1972; 1974). We have studied the effect of phospholipase-D on the membrane-bound lipids, membrane morphology, Ca^{2+} binding, swelling *in vitro*, and adenosine triphosphatase activity of the purified mitochondrial fraction of rat kidney.

Methods

Preparation of mitochondrial fraction and phospholipase-D

Adult male albino rats, Charles Foster Strain (100–150 g body wt) maintained on stock diet of the animal house of the Institute, were used in this study. The animals were fasted for 24 h before sacrificing by decapitation. The kidneys were excised and mitochondrial fraction, whose purity was established by electron microscopy and assay of marker enzymes, was prepared according to Zaidi *et al.* (1976). Phospholipase-D was prepared from cabbage according to the method of Yang *et al.* (1967) and phospholipase-D activity was assayed according to Davidson and Long (1958). The enzyme preparation used in the present study was free from proteolytic or phospholipase A or C activity.

Swelling, ATPase activity and Ca^{2+} binding

In vitro swelling of the mitochondrial fraction was studied according to Lehninger (1959) and total ATPase activity was assayed according to Post and Sen (1967) For studying Ca^{2+} binding, 5 mg mitochondrial fraction was incubated with phospholipase-D in the presence of $^{45}Ca^{2+}$ (as a solution of calcium chloride in dilute HCl, 30mCi/g Ca, Bhabha Atomic Research. Centre, Bombay). The reaction was stopped by centrifugation at 20,000 g for 10 min at 0° C and the radioactivity in the mitochondrial pellets in protein precipitated at 22·6% trichloroacetic acid, in extracted lipids and various phospholipids (resolved by thin layer chromatography) was assayed in a Packard Tricarb liquid scintillation counter. Suspensions of washed mitochondrial pellets and trichloroacetic acid precipitable protein were applied on Whatman No. 1 paper strips which were dipped in vials containing scintillation fluid (PPO 0·4%, POPOP 0·01% in freshly distilled toluene) while lipid samples and scrapings of silica gel from thin layer plates were directly added to the vials.

Electron microscopy

Samples of mitochondrial pellets, untreated or treated with phospholipase-D, were fixed in Caulfield's fixative and embedded in a mixture of araldite and epon and their ultra-thin sections were examined in a Hitachi electron microscope (HUIIE) after staining withuranyl acet ate and lead acetate at an accelerating voltage of 75 kV.

Extraction and analysis of mitochondrial lipids

The lipids of membrane pellets and the lipids and bases of membrane-bound phospholipids released into the incubation medium were prepared and analysed as reported by Kapoor *et al.* (1974) and Zaidi *et al.* (1976).

Results and discussion

Degradation of membrane lipids

Table 1 shows that incubation of the mitochondrial fraction with phospholipase-D leads to degradation of membrane-bound phosphatidylcholine, -ethanolamme, -serine and to some extent of cardiolipin also. Nearly 90% of membrane-bound phosphatidylcholine and 39% and 33% of phosphatidyl-ethanolamine and -seline were hydrolysed and phosphatidic acid accumulated. Water-soluble bases choline, ethanolamine and serine were released into the medium but no lipid P was detected in the medium. Although the degradation of membrane-bound phospholipids was complete in 15 min release of cholesterol from membrane was observed at 15 and 60 min of incubation. This pattern is in agreement with the mode of action of this enzyme on isolated phospholipids (Davidson and Long, 1958) and membrane-bound phospholipids of microsomal fraction (Elling and Di Augustine, 1971; Kapoor *et al.*, 1972, 1974). Finean (cited by Kavanau, 1965) has postulated hydrogen bond association between the hydroxyl of cholesterol and nitrogen of phospholipid molecules in biological membranes, and it seems that removal of nitrogenous bases from the mitochondrial membranes during degradation with phospholipase-D loosens the packing of membrane cholesterol and facilitates release from the membranes.

Table 1. Effect of phospholipase-D on lipid composition of rat kidney mitochondria

Lipid constituent (μmol/mg mitochondrial protein)	Treatment with phospholipase-D (min)		
	0	15	60
Phosphatidylcholine (PC)	0·30±0·007	0·03±0·001	0·03±0·001
Phosphatidylethanolamine (PE)	0·23±0·003	0·14±0·004	0·14±0·002
Phosphatidylserine (PS)	0·06±0·003	0·04±0·002	0·04±0·001
Cardiolipin	0·12±0·009	0·10±0·003	0·09±0·001
Phosphatidic acid (PA)	0·07±0·004	0·40±0·012	0·41±0·002
Cholesterol	0·04±0·002	0·03±0·002	0·02±0·003

Phospholipase D 5 units; 0·2 M acetate buffer (pH 5·6); 100 μM $CaCl_2$ and mitochondrial protein (6mg), in a final volume 10 ml (0 25 M sucrose) was incubated at 25° C for 15 min Reaction was stopped by centriguation at 20,000 g for 10 min at 0° C. All values are mean ± SE of five experiments.

Mitochondrial swellingand membrane morphology

During 15 min of incubation with phospholipase-D, there was considerable increase in mitochondrial swelling *in vitro* (untreated 7% and phospholipase D-treated 14%) as observed by reduction in absorbance of the mitochondrial suspension (figure 1) and as seen in the electron micrographs (figure 2a). After 15 min of treatment with phospholipase-D the absorbance of the mitochondrial suspension

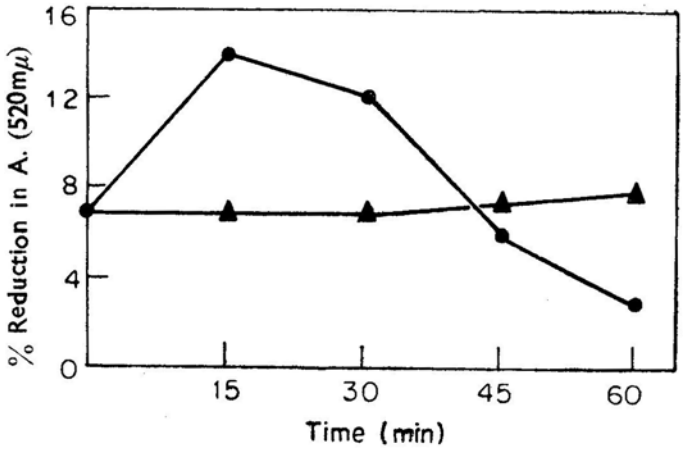

Figure 1. Effect of phospholipase D treatment on mitochondrial swelling *in vitro.* Incubations as in table 1.

Mitochondrial pellets (2 mg protein) obtained after incubation at specific time suspended in 3·0ml of 0·15 M KCl + 20mM Tris-HCl PH 7·4, and absorbancy at 520 nm recorded at 0 min and after incubation for 30 min at 30°.

started increasing, this indicated the rupture of mitochondria which was confirmed by electron micrographs (figure 2b) Maximal swelling of the mitochondria and hydrolysis of membrane phospholipids coincided at 15 min of incubation.

Mitochondrial ATPase

Total ATPase of the mitochondrial fraction was inactivated by 26% after treatment with phospholipase-D; reconstitution with phosphatidylcholine or phosphatidylserine (10,umol per 5 mg mitochondrial protein) restored the ATPase activity by 6 and 9% respectively, while reconstitution with cholesterol had no effect (table 2).

Calcium uptake

Incubation of the mitochondria with phospholipase-D resulted in continuous increase in the uptake of $^{45}Ca^{2+}$ by digested membranes all through the 60 min incubation (table 3). In the mitochondrial pellets obtained after 60 min digestion with phospholipase-D, 72·3% of the uptake was in the lipids, and 22·6°/in trichloroacetic acid precipitable proteins. Distribution of the radioactivity in various phospholipids resolved on thin layer chromatography showed that maximum binding was in the phosphatidic acid (see footnote to table 3).

From the above results it is clear that the action of phospholipase-D on mitochondrial fraction of rat kidney is quite different that of phospholipase C although both the enzymes remove polar head groups of membrane-bound phospholipids Removal of nitrogenous bases from the membrane-bound phospholipids leads to release of membrane cholesterol and protein but phosphatidc acid, the main product of degradation, remains bound to the membranes This causes increased swelling of mitochondria and their ultimate rupture and increased calcium-binding as well as partial inactivation of ATPase.

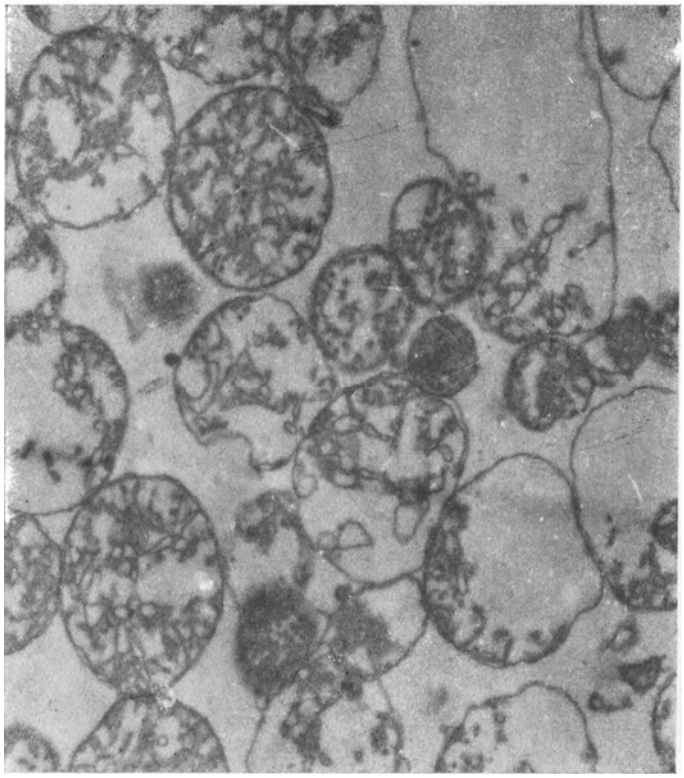

Figure 2a. Electron micrograph of normal mitochondria (× 14,800),

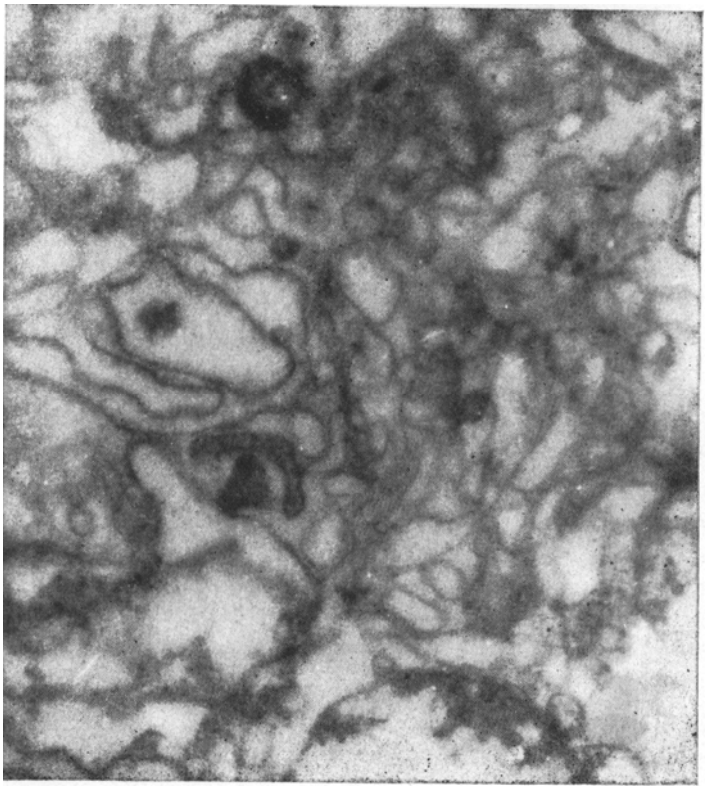

Figure 2b. Electron micrograph of ruptured mitochondria after treatment with phospholipase-D for 60 min (× 36,000).

Table 2. Effect of phospholipase-D on ATPase activity.

Additions	ATPase activity
None	3·4
Phospholipase-D	2·5
Phospholipase-D treated, reconstituted with :	
PC	2·6
PS	2·7
Cholesterol	2·5

Mitochondrial pellets (5mg protein) obtained after treatment with phospholipase-D as in table 1, resuspended in sucrose, 10 μM PC or PS or cholesterol added as sonicated dispersions in Tris-HCl pH 7·4, final volume 10 ml (0·25 M sucrose) and incubated for 90 min at 30° C, and centrifuged again. ATPase was assayed in the pellets. Activity was expressed μmol P_i: released/mg protein/15 min at 37° C. The data represent average of duplicate assays.

Table 3. $^{45}Ca^{2+}$ binding to mitochondrial fraction treated with phospholipase-D

Phospholipase-D treatment (min)	$^{45}Ca^{2+}$ binding (cpm/mg mitochondrial protein)
0	780
10	5770
20	9980
40	10900
*60	11238

Incubation as in table 1, 0·4 μCi $^{45}Ca^{2+}$ added.

* Incorporation in the lipids was 72·3% of the total and 22·6% trichloroacetic acid precipitable protein. % Distribution of incorporation in lipids was : PC 2·5, PE + PS 11·2 phosphatidic acid 78.0 and cardiolipin 8·8,

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