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ACTIVATION BY PHOSPHOLIPIDS OF PARTICULATE MITOCHONDRIAL ATPase FROM RAT LIVER

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SUMMARY

1. The activation by phospholipids of particulate mitochondrial ATPase from rat liver was studied on a preparation extracted from submitochondrial particles with cholate.

2. Lysophosphatidylcholine and the highly acidic phospholipids diphosphatidylglycerol, phosphatidylinositol and phosphatidylserine stimulated the ATPase at low concentration (50–60 μg/mg protein); phosphatidylethanolamine was less effective whereas phosphatidylcholine exhibited the lowest activity.

3. The saturation curves of phospholipids acting at low concentration showed in most cases a deviation from normal Michaelis–Menten kinetics. This devation was absent or less manifest with phosphatidylethanolamine and phosphatidylcholine. Competition between phospholipids and oligomycin was seen with acidic phospholipids or mixtures of neutral and acidic phospholipids.

4. Similarly to oligomycin, dicyclohexylcarbodiimide, tributyltin and chlorpromazine competitively inhibited the activation produced by phosphatidylserine, phosphatidylinositol and diphosphatidylglycerol.

5. It is concluded that both the hydrophobic–hydrophilic balance and the negative charge of phospholipids are important in the interaction between phospholipids and protein at the level of particulate mitochondrial ATPase. The competition between phospholipids and inhibitors of mitochondrial ATPase suggests that the activation induced by phospholipids is involved in the inhibitory effect.

INTRODUCTION

The role of phospholipids in the structure and function of cellular membranes raises considerable interest. Acidic phospholipids are especially investigated as potential candidates for several functions, including: (a) activation[1–6], stabilization[7] and structural organization[8] of enzymes associated with membranes; (b) binding of specific activators (epinephrine[9]) and hormone responsiveness[4,5]; (c) discrimination in permeability of univalent cations[10]; (d) interaction with proteins through the negative charge of their head groups[11–13].

Abbreviation: DCCD, *N,N'*-dicyclohexylcarbodiimide.

Recently, evidence was produced[14–17] that phospholipids may be required for the interaction of inhibitors with membrane-associated enzymes.

Phospholipids are known activators of mitochondrial ATPase[18,19]. The reconstitution is profoundly influenced by the method used to reassemble proteins and phospholipids[20].

In this paper the activation of mitochondrial ATPase by phospholipids is further examined using a lipid-depleted preparation of particulate mitochondrial ATPase from rat-liver. Data on the relationship between inhibitors of mitochondrial ATPase and phospholipids are also reported.

MATERIALS AND METHODS

All reagents were commercial grade. After neutralization cholic acid (E. Merck, Darmstadt) gave a colorless solution and was not recrystallized. *N,N'*-Dicyclohexylcarbodiimide (DCCD) (A grade) was from Calbiochem, Tri-*n*-butyltin chloride from Aldrich Chemical Co, and oligomycin from Upjohn. They were used as ethanolic solution. A crude preparation of bovine-brain phospholipids was purified as described[21]. Individual phospholipids were ultrapure phospholipids from General Biochemicals (Chagrin Falls, Ohio). They were all from bovine brain with the exception of lysophosphatidylcholine and one sample of phosphatidylcholine (from eggs). Their purity was checked by thin-layer chromatography using alcaline, neutral and acid solvents as well as the bidimensional system described by Rouser *et al.*[22]. Only phosphatidylethanolamine contained a contamination by an unidentified component, and further purification was achieved using preparative thin-layer chromatography. All phospholipids were dispersed in a 0.25 M sucrose, 10 mM Tris·HCl, 1 mM EDTA (pH 7.5) solution by ultrasonic oscillations. Care was used to keep the pH at a neutral value during and after sonication.

Submitochondrial particles from rat-liver were isolated by the procedure of Kielley and Bronk[23] as described[15]. Extraction of submitochondrial particles with cholate in the presence of a low amount of $(NH_4)_2SO_4$ was performed as described by Kagawa and Racker[18] except that trypsin and urea treatments were omitted. The final sediment was resuspended in 0.25 M sucrose, 10 mM Tris·HCl, 1 mM EDTA (pH 7.5) and stored in small aliquots at —40 °C where it was stable several weeks.

In the reconstitution experiments, about 0.1 mg cholate-extracted fraction was incubated in 0.2 ml of 0.25 M sucrose, 10 mM Tris·HCl, 1 mM EDTA (pH 7.5) containing the desired amount of phospholipids. 0.5 % ethanol with or without the inhibitors was also present. After 5 min at 37 °C, the incubation medium was completed and the ATPase activity followed for 20 min at the same temperature. During this time the reaction was essentially linear. The final composition in a volume of 1.0 ml was: 2.5 mM $MgCl_2$, 3.0 mM ATP·Tris (pH 7.4), 50 mM sucrose, 50 mM Tris·HCl (pH 7.4), 0.2 mM EDTA. The reaction was stopped with 0.25 ml of cold 50 % trichloroacetic acid.

Phosphate was determined according to Fiske and SubbaRow; protein as described by Lowry *et al.*[24] with crystalline serum albumin as standard after dissolving particulate material with sodium deoxycholate. Phospholipid phosphorus was determined after the ashing procedure of Ames and Dubin[25]: 1 μg of phosphorus was

TABLE I

ACTIVITY AND PHOSPHOLIPID COMPOSITION OF DEPLETED PARTICULATE ATPase

Submitochondrial particles from rat-liver were extracted with cholate in presence of 0.1 M $(NH_4)_2SO_4$ (ref. 18). ATPase activity was measured at 37 °C in 50 mM sucrose, 0.2 mM EDTA, 50 mM Tris·HCl, pH 7.4, 2.5 mM $MgCl_2$, 3.0 mM ATP, pH 7.4, 25–30 μg protein submitochondrial particles or 120 μg cholate-extracted fraction. Final volume, 1.0 ml; incubation, 20 min. Reconstitution with phosphatidylserine and other experimental details as outlined in Materials and Methods.

Preparation	*ATPase activity* (*μmoles/mg per h*)	*Phospholipid content* (*mg/mg protein*)	*% Composition of phospholipids*				
			Phosphatidyl-choline	*Phosphatidyl-ethanolamine*	*Phosphatidyl-inositol*	*Diphosphatidyl-glycerol*	*Others*
Original submitochondrial particles	150	0.62	42.9	29.7	6.6	15.6	5.2
Cholate-extracted fraction	3.8	0.054	45.1	30.4	6.2	12.3	6.0
Cholate-extracted fraction + 32 μg phosphatidylserine	28.5	—	—	—	—	—	—

assumed to be contained in 25.3 μg phospholipids[26] except in the case of lysophosphatidylcholine where a molecular weight of 506 was assumed[27].

Phospholipids were extracted overnight at 2 °C with 20 vol. of chloroform–methanol (2:1, v/v), then 30 min at room temperature with the same solution and an additional 30 min at room temperature with chloroform–methanol (2:1, v/v) containing 1 % (v/v) acetic acid. The pooled extracts were washed with 0.2 vol. of 0.040 % $MgCl_2$, taken to dryness and dissolved in chloroform. Qualitative and quantitative estimation of phospholipids were performed as described by Rouser *et al.*[22] using thin-layer chromatography on plates made with Silica Gel HR (Merck) *plus* magnesium acetate as binder.

RESULTS

In agreement with the results of Kagawa and Racker[18] on T-particles from beef heart, in Table I it is shown that the fraction of rat-liver submitochondrial particles precipitated by 33 % $(NH_4)_2SO_4$ after solubilization with cholate, contained less than 10 % of the original content of phospholipids and negligible ATPase activity.

The composition of residual phospholipids was nearly the same as that of the parent submitochondrial particles, which is also shown in Table I. Enrichment of diphosphatidylglycerol was not seen. Measurable amounts of phosphatidylserine were absent in the submitochondrial particles and in the cholate extracted fraction.

TABLE II

ACTIVATING EFFECT OF PHOSPHOLIPIDS ON DEPLETED PARTICULATE ATPase

Reconstitution with phospholipids as outlined in Materials and Methods. Measurement of ATPase as in Table I. A mean of 112 μg protein (range 100–140 μg) cholate-extracted fraction was present in the various experiments. Except where indicated, all phospholipids were from bovine brain. Lineweaver–Burk plots were used to obtain V values. Owing to the non-linearity of Lineweaver–Burk plots in several instances, the amount of phospholipids at 50 % effect was determined in all cases using plots of activation *versus* phospholipid concentration. V is the maximum activity in presence of phospholipids *minus* the activity in their absence. The activity in the absence of phospholipids was on the average of 4.0 μmoles ATP split per mg protein per h in 22 experiments (range 2.0–7.0). The values are means of separate experiments the number of which is given in parenthesis.

Phospholipids	*V* (*$\Delta\mu$moles ATP split/mg per h at 37 °C*)	*Amount of phospholipids at 50 % effect* (*μg/mg protein*)
Total phospholipids from bovine brain	19.3 (2)	347
Phosphatidylserine	23.6 (14)	54
Phosphatidylinositol	21.2 (10)	62
Diphosphatidylglycerol (cardiolipin)	17 (5)	53
Egg lysophosphatidylcholine	16 (4)	
Egg phosphatidylcholine (lecithin)*	12 (2)	490
Phosphatidylethanolamine	11 (2)	265

* In several attempts to obtain titration curves, egg phosphatidylcholine and bovine-brain phosphatidylcholine gave inconsistent and not reproducible figures. Hence these data are to be considered as approximate.

Phosphatidylinositol content in the cholate extracted fraction was variable and difficult to determine owing to the very low amount.

Upon addition of phosphatidylserine a substantial increase in the ATPase activity was elicited, which, however, was not more than 20 % of the original activity. Addition of other phospholipids or removal of cholate by dialysis did not improve the limited reconstitution which is probably to ascribe to loss of solubilized ATPase (F_1). In a similar preparation from bovine-heart T-particles 40 % of the original activity was recovered upon addition of phospholipids[18].

In Table II it is seen that substantial reconstitution of ATPase activity was achieved with all individual phospholipids. However, the highly acidic phospholipids, diphosphatidylglycerol, phosphatidylserine and phosphatidylinositol were more effective than phosphatidylethanolamine which, in turn, was more active than phosphatidylcholine. When highly acidic phospholipids were mixed with phosphatidylcholine in a proportion close to that found in submitochondrial particles (10%) their effectiveness was not reduced. Apparently, the difference among the phospholipids is not only due to the electrostatic charges on the head groups, but also to the hydrophobic-hydrophilic balance. This is indicated by the observation that the neutral phospholipid, lysophosphatidylcholine, displayed high effectiveness. At a limiting amount of phospholipids the stimulation of ATPase remained unchanged on increasing the amount of protein, showing that the activation was not reduced by aspecific binding.

In Fig. 1 kinetic data on the activation by phospholipids in the presence and

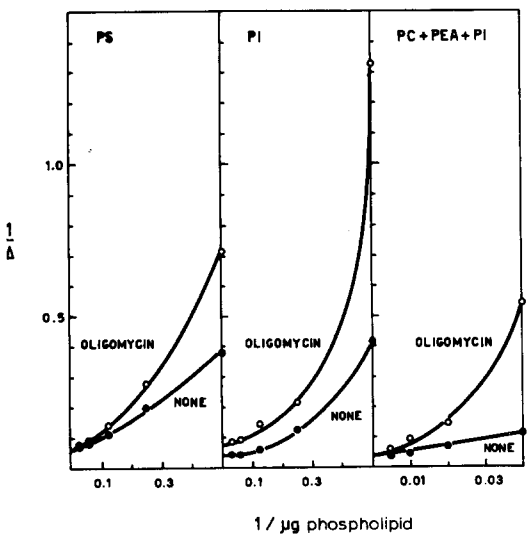

Fig. 1. Reciprocal plots of phospholipid-elicited ATPase activity against phospholipid concentration in presence or absence of oligomycin. PS, bovine-brain phosphatidylserine; PI, bovine-brain phosphatidylinositol; PC + PEA + PI, a mixture of 63 % phosphatidylcholine, 29 % phosphatidylethanolamine and 8 % phosphatidylinositol from bovine brain. Before using the mixture was briefly sonicated. Reconstitution with phospholipids and measurement of ATPase activity as described in Table I and under Materials and Methods. Oligomycin was present at a concentration of 0.2 µg/mg protein (each tube contained 100–130 µg protein). Δ is the ATPase activity in presence of phospholipids *minus* that in their absence.

absence of oligomycin are reported. In a double reciprocal plot of the rate of induced activation as a function of the amount of added phospholipids, phosphatidylserine and phosphatidylinositol yielded curvilinear relationships whereas a mixture of phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol yielded straight lines. Clear divergences from normal kinetics were also exhibited by diphosphatidylglycerol and lysophosphatidylcholine and were seen in the majority of the experiments, though not all. It is possible that this variability was due to the extent of phospholipid depletion, which was different from preparation to preparation. With phosphatidylethanolamine, phosphatidylcholine, the mixture of phospholipids including phosphatidylcholine or pooled bovine-brain phospholipids the deviation from Michaelis–Menten kinetics, if present, was only scarcely manifest.

The stimulation by all phospholipids was sensitive to oligomycin[18] provided the amount of the former was not large enough to produce antagonism[14]. In the presence of the inhibitor the deviation from normal kinetics was more evident. In addition, the linear relationship seen with the mixture of phospholipids was changed by oligomycin, suggesting a reaction between the activator and the inhibitor[28]. Competitive inhibition between oligomycin and phospholipids was manifest but was not present with all phospholipids. Phosphatidylserine and mixtures of phospholipids including acidic members gave more reproducible results.

Appropriate controls showing inhibition after addition of a larger amount of oligomycin, made it unlikely that in the submitochondrial particles from rat liver the removal of oligomycin sensitivity by phospholipids was due to solubilization of the oligomycin-insensitive ATPase.

A number of inhibitors of oxidative phosphorylation (thenoyltrifluoroacetone, antimycin, valinomycin) were without effect on the interaction between mitochondrial particulate ATPase and phospholipids. By contrast, DCCD, tributyltin and chlorpromazine, which are known inhibitors of particulate mitochondrial ATPase, competitively inhibited the activation by phosphatidylserine (Fig. 2). Similar results were obtained with phosphatidylinositol and diphosphatidylglycerol.

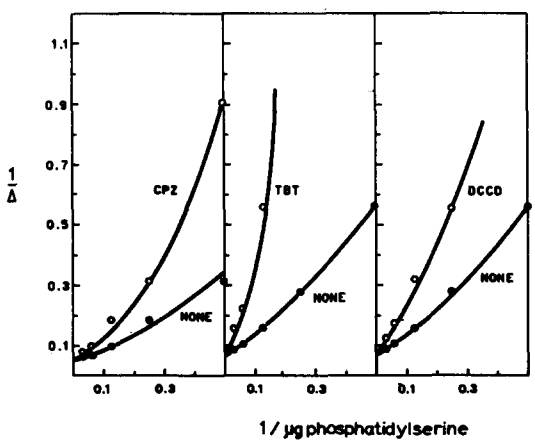

Fig. 2. Competition between phosphatidylserine and inhibitors of mitochondrial particulate ATPase. 20 nmoles per tube chlorpromazine (CPZ); 0.5 nmole per tube tributyltin chloride (TBT); 0.35 nmole per tube DCCD. Experimental condition as in Fig. 1. 114–134 μg protein.

DISCUSSION

The main conclusion of these experiments is that highly acidic phospholipids show a greater effectiveness than phosphatidylethanolamine and phosphatidylcholine in the reactivation of particulate mitochondrial ATPase from rat-liver. The remarkable effectiveness of acidic phospholipids (*cf.* refs 1–7) in maintaining the activity of several enzymes associated with membrane or determining their interaction with lipid-depleted membrane indicates that the properties of these phospholipids are especially suited to meet the need of membrane organization and activity. The high effectiveness of acidic phospholipids is shared by the neutral lysophosphatidylcholine. This indicates the importance of hydrophilic–hydrophobic balance in the activation of mitochondrial ATPase. An effect of lysophospholipids similar to that of acidic phospholipids was found by Sawada *et al.*[6] with protoheme ferro-lyase. Lack of specificity in the activation of particulate mitochondrial ATPase from rat liver by phospholipids was evident. In this respect it behaved like other preparations from bovine heart[18,19].

The phospholipid activation kinetics showed a deviation from normal Michaelis–Menten kinetics which was more evident with phospholipids acting at low concentration (acidic phospholipids and lysophosphatidylcholine). This is reminiscent of the observation of Cunningham and Hager[29] on the activation of crystalline pyruvate oxidase from *Escherichia coli* by phospholipids. Lysophosphatidylcholine, but not phosphatidylcholine, showed a deviation from normal kinetics. Since lysophosphatidylcholine has a finite solubility in water, forming a molecular dispersion at concentration below 450 μM at 25 °C[27], this could be interpreted as an indication that the physical status of phospholipids influences the activation. However, in a preparation of Na^+- and K^+-stimulated ATPase depleted of phospholipids (P. Palatini, F. Dabbeni-Sala and A. Bruni, unpublished) a clear deviation from normal kinetics was seen also with phosphatidylcholine and phosphatidylethanolamine, which form aggregates in water at low concentrations[20]. This suggests that the reason for the unusual kinetics is also in the properties of the insoluble proteic material. Phospholipids added to amorphous aggregates of lipid-free proteins from mitochondrial membrane promote the appearance of membranous structure[31,32]. Cooperativity may become apparent during this transition as new binding sites are exposed when the first micelles of phospholipids are bound.

With membrane-bound enzymes extracted by surface active agents, as in the present case, the situation is further complicated by the presence of residual detergent which could modify the structure of added phospholipids or preclude the binding of the low amounts to the specific site in the protein. Recently Swanljung[33] reported that sigmoidal curves described the activation by diphosphatidylglycerol of particulate mitochondrial ATPase from bovine heart extracted with deoxycholate.

The competition between phospholipids and inhibitors of particulate mitochondrial ATPase indicates that their effects are not independent. The alteration of phospholipid effect produced by oligomycin, DCCD, tributyltin and chlorpromazine could reflect a combination with the activating phospholipid or a modification of their binding sites in the preparation. Both cases can be relevant for understanding the mechanism of inhibition of particulate mitochondrial ATPase.

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