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THE ACTIVATION OF MITOCHONDRIAL PARTICULATE ATPase BY LIPOSOMES OF DIACYLPHOSPHOLIPIDS

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SUMMARY

1. The stimulation of mitochondrial particulate ATPase by natural phosphatidylcholines was studied in a phospholipid-free preparation from bovine heart obtained by extraction with cholate. The low stimulatory activity of these phospholipids was increased by: (a) introduction of negatively charged amphiphatic substances into the zwitterionic liposomes; (b) addition of Cl^- to the incubation medium.

2. Liposomes of acidic phospholipids or phosphatidylcholine containing anionic amphipaths prevented the ATP plus Mg^{2+}-induced decrease of ATPase activity in Mg–ATP particles which have a high content of ATPase inhibitor.

3. Acidic phospholipids, in contrast to phosphatidylcholine, prevented the inhibition of soluble and particulate ATPase by purified ATPase inhibitor.

4. It is concluded that the structure of the lipid–water interface is of primary importance in the stimulation produced by liposomes of diacylphospholipids on particulate mitochondrial ATPase. This effect is associated with the removal of inhibition induced by endogenous ATPase inhibitor on phospholipid-depleted preparations.

INTRODUCTION

Differences have been found in the activation of mitochondrial particulate ATPase by various phospholipids [1, 3]. Strongly acidic phospholipids, as well as lysophosphatidylcholine, were more effective than phosphatidylethanolamine and phosphatidylcholine [2]. The different physicochemical properties of these phospholipids are well known [4–7]. Dawson [8], and Bangham and Dawson [9] showed that the ineffectiveness of phosphatidylcholine to serve as a substrate for phospholipase B from *Penicillium notatum* could be overcome by changing the properties of the lipid–water interface (i.e. by introducing amphipathic substances which imparted negative charges to the isoelectric liposomes). This procedure allowed further investigations on the interaction between phospholipids and mitochondrial particulate ATPase. It will be shown that the introduction of a negative charge greatly improves the low stimulation induced by isoelectric liposomes of phosphatidylcholine. Data suggesting antagonism between negatively charged liposomes of diacylphospholipids and ATPase inhibitor will also be presented.

MATERIALS AND METHODS

Sodium dodecylsulfate (Sigma) was recrystallized from 95% ethanol, sodium dicetylphosphate (Sigma) from ethoxy-ethanol and ethylhexadecyldimethylammonium bromide (Matheson, Coleman, Bell) from benzene. Bovine heart diphosphatidylglycerol was obtained from Sigma, a crude preparation of egg phosphatidylcholine was kindly donated by Libam (Montegrotto Terme, Italy) and purified further in a column of silicic acid (Bio Rad). Bovine brain phosphatidylcholine and other purified phospholipids were from General Biochemicals. In all instances, only one spot was detected on thin-layer chromatography [10].

Liposomes were prepared in 0.25 M sucrose, 10 mM Tris–HCl, 1 mM EDTA (pH 7.4) by sonication at 0 °C in a probe-tip apparatus (Biosonik III) under a stream of pure N_2. Phosphatidylcholine and sphingomyelin were sonicated for 1 h, acidic phospholipids for 5–10 min and this was followed by 30 min centrifugation at 40 000 rev./min (Spinco, No. 40 rotor). Homogeneous preparations of liposomes of phosphatidylcholine using Sepharose 4B filtration were prepared according to Huang [11].

Sodium dodecylsulfate or ethylhexadecyldimethylammonium bromide were added as aqueous solutions to the dipersion of phosphatidylcholine to give 0.1 mg per mg of phospholipid. The mixture was sonicated, centrifuged and dialyzed for 2 h at room temperature followed by 6 h at 0 °C against two changes of 50 vol. of 0.25 M sucrose, 10 mM Tris–HCl, 1 mM EDTA (pH 7.4) with mixing maintained by a stream of N_2. Mixtures of oleic or dicetylphosphoric acid with phosphatidylcholine were obtained from the compounds dissolved in chloroform. After removal of the solvent, the mixtures were sonicated as described above.

Bovine heart mitochondria [12], submitochondrial particles prepared in the presence of pyrophosphate [13], Mg^{2+} and ATP [14], NH_3 followed by Sephadex filtration (AS-particles) [15], ATPase inhibitor [16] and soluble ATPase (F_1) [16] were isolated according to the published procedures. Phospholipid-depleted, mitochondrial particulate ATPase was obtained from the particles prepared in the presence of pyrophosphate by a modification of the procedure devised by Kagawa and Racker [17]. 5 mg/ml submitochondrial particles were extracted for 1 h at 0 °C with 1 mg cholate per mg protein in a medium which was 0.22 M in sucrose, 1 M KCl and 1.5 mM in dithiothreitol, 9 mM Tris–HCl and 0.9 mM EDTA (pH 8.0). After 20 min centrifugation at 0 °C (40 000 rev./min, Spinco No. 40 rotor), the supernatant was fractionated at 0 °C with a saturated solution of $(NH_4)_2SO_4$ to take the fraction precipitated between 30 and 50% saturation. This was dissolved in 0.25 M sucrose, 1 mM EDTA, 10 mM Tris–HCl (pH 7.4) at a concentration of about 7.0 mg/ml and centrifuged for 20 min at 40 000 rev./min to discard any sedimentable material. The particulate ATPase was precipitated twice with 50% saturated $(NH_4)_2SO_4$ and resuspended in the sucrose–EDTA–Tris solution in which yielded a barely turbid suspension that was stored at −40 °C. The yield was around 20% of the starting materials in terms of protein content. The amount of phospholipids varied in seven preparations between 0.09 and 0.04 mg/mg protein (7–14% of the phospholipid content of the original submitochondrial particles). The activity was 0.0–0.1 μmoles ATP split/mg protein per min at 37 °C, but rose to 2.8–4.0 in the presence of 1 mg/mg protein total bovine brain phospholipids and was about 95% inhibited by oligomycin.

The dry-ashing procedure [18] was used to determine the phospholipid phosphorus which was multiplied by 25.3 to obtain the concentration of diacylphospholipids [19]. The molecular weight of egg lysophosphatidylcholine was assumed to be 506 [20]. P_i [21] and protein [22] were determined according to standard procedures.

Reactivation of ATPase activity by phospholipids was determined by adding 20–25 μg of the depleted enzyme preparation to 0.3 ml of 0.17 M sucrose, 0.7 mM EDTA and 7 mM Tris–HCl (pH 7.4) containing the phospholipids. After 5 min at 37 °C, the medium was supplemented with the other components to contain in a final volume of 1.0 ml, 50 mM sucrose, 0.2 mM EDTA, 50 mM Tris–HCl (pH 7.4), 2.5 mM $MgCl_2$, 3 mM ATP, 2 mM phosphoenolpyruvate and 15 μg pyruvate kinase (320 units/mg). The reaction was followed for 20 min and stopped by the addition of 0.25 ml cold 50% trichloroacetic acid. Variations are indicated in the Tables and Figures.

RESULTS

As with rat liver particulate ATPase [2], the preparation from bovine heart was stimulated by low amounts of strongly acidic phospholipids and lysophosphatidylcholine (50% effect at 0.1–0.15 mg/ml protein). The stimulation was inhibited by oligomycin, but the effect of the inhibitor disappeared when the amount of acidic phospholipid was increased, as opposed to its effect on rat liver preparation. This was correlated with the phospholipid-induced solubilization of oligomycin-insensitive ATPase [23].

As shown in Fig. 1, phosphatidylcholine produced only a low stimulation. In spite of further purification of the phospholipid by thin-layer chromatography and preparation of homogeneous liposomes by Sepharose filtration [11], titration curves with this phospholipid were frequently irregular with alternate figures of lower and higher activations. Addition of a small amount of bovine serum albumin eliminated the irregular pattern and occasionally produced a further reduction of stimulation. Since acidic proteins like bovine serum albumin and ovalbumin do not bind isoelectric liposomes at neutral pH [24–26] and natural phosphatidylcholines do not form molecular solutions [4, 5, 7], this observation illustrated the difficulty of eliminating residual traces of lysophosphatidylcholine or fatty acids, which are known activators of mitochondrial particulate ATPase [17] and readily bound [27, 28] by these proteins. In order to evaluate the interference by these compounds, controls with a low amount (0.3 mg) of albumin were routinely included in the experiments with phosphatidylcholine. This amount of albumin did not inhibit the activation induced by mixed liposomes of bovine brain phospholipids, whereas it strongly retarded the activation by lysophosphatidylcholine, which has a finite solubility in water and is able to stimulate the ATPase at concentrations below the critical micellar concentration.

To explore whether isoelectric liposomes of phosphatidylcholine acquire stimulating activity on ATPase after introduction of negative charges, the anionic amphipathic dicetylphosphate was used. This is a water-insoluble compound which imparts a negative charge to the liposomes of phosphatidylcholine when mixed in suitable proportions [9]. By itself, it was found to be devoid of any stimulating effect

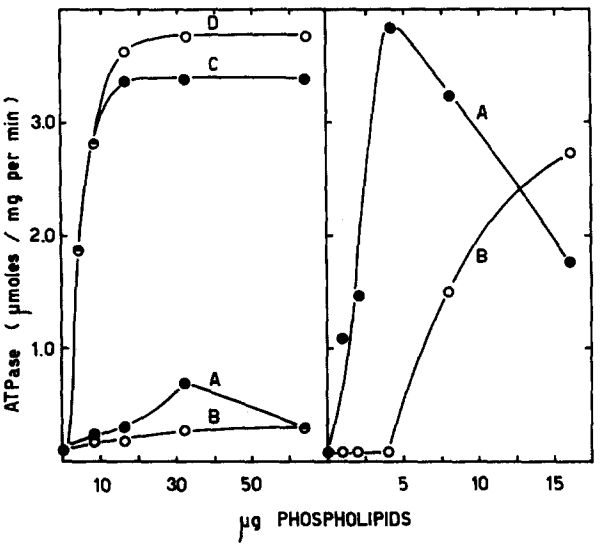


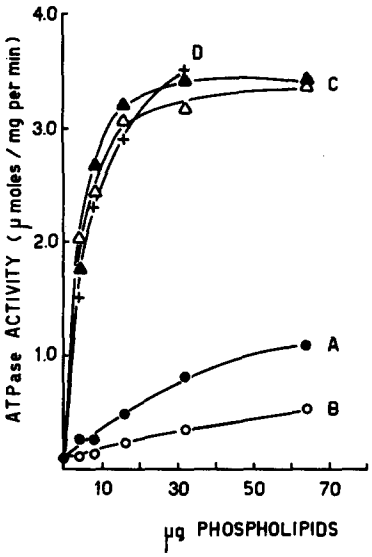
Fig. 1. Activation of ATPase by phospholipids. (Left): A, egg phosphatidylcholine; B, the same plus 0.3 mg bovine serum albumin; C, mixed phospholipids from bovine brain; D, the same plus albumin. (Right): A, egg lysophosphatidylcholine; B, the same plus albumin. 25 μg enzyme were incubated for 5 min at 37 °C with the phospholipids in 0.3 ml of 0.17 M sucrose, 7 mM Tris–HCl and 0.7 mM EDTA (pH 7.3) with or without the albumin. After this preincubation, the ATPase activity was measured as described under Materials and Methods.

on particulate ATPase.

In Fig. 2, it is seen that mixed liposomes of phosphatidylcholine and dicetylphosphate produced a clear stimulation of ATPase activity which was not reduced, by even large amounts of albumin. The induced ATPase activity was 90% inhibited by oligomycin. Similar observations were made with other anionic amphipathic compounds (dodecylsulfate and oleate). In these cases, however, the results were less convincing because the activity of mixed liposomes was progressively reduced by increasing amounts of albumin. This suggested that free molecules of these soluble anionic amphipaths were in equilibrium with mixed liposomes. Furthermore oleate had a strong stimulating activity when used alone. The role of negative charges was confirmed by the observation that on the introduction of positive charges in the liposomes of phosphatidylcholine by the use of the cationic–amphipathic ethylhexadecyldimethylammonium, no activation of ATPase followed.

In agreement with these results, it was found that bovine brain sphingomyelin, which forms isoelectric or positively charged liposomes [29], had no stimulating effect.

In Fig. 3, it is seen that the activity of phosphatidylcholine was greater in a medium containing increasing amounts of NaCl. The NaCl-induced activity was oligomycin sensitive. Bovine serum albumin did not modify this activation, nor did this concentration of salt inhibit the binding of lysophosphatidylcholine, or oleate to albumin. Separate experiments showed that the effect of NaCl was neither reproduced by a corresponding concentration of glucose nor eliminated by preparing liposomes in a medium containing the same concentration of NaCl. Osmotic modi-



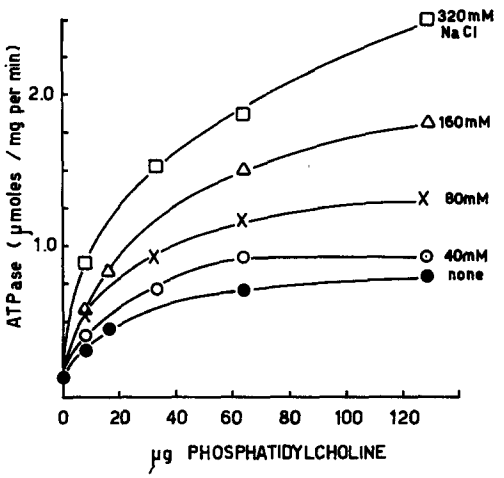


Fig. 2. Stimulation by negatively charged liposomes of phosphatidylcholine. A, egg-phosphatidylcholine; B, the same plus 0.3 or 4.0 mg bovine serum albumin; C, phosphatidylcholine containing 20 % (w/w) dicetylphosphate without or with 0.3 mg albumin; D, the same plus 4.0 mg albumin. The preparation of mixed liposomes of egg phosphatidylcholine and dicetylphosphate and the measurement of ATPase activity are described under Materials and Methods. 25 μg particulate ATPase were incubated for 5 min at 37 °C with the liposomes in 0.3 ml of 0.17 M sucrose, 7 mM Tris–HCl and 0.7 mM EDTA (pH 7.3) with or without bovine serum albumin.

Fig. 3. Effect of phosphatidylcholine in the presence of NaCl. 25 μg particulate ATPase were incubated for 5 min at 37 °C in 0.3 ml of 0.17 M sucrose, 7 mM Tris–HCl, 0.7 mM EDTA and 0.3 mg bovine serum albumin (pH 7.3), in the presence of the indicated amount of NaCl and bovine brain phosphatidylcholine. Measurement of ATPase activity as described under Materials and Methods.

fication of liposomes, therefore, did not seem to be involved. NaCl used without phospholipids did not increase the activity of phospholipid-depleted particulate ATPase. Of the other salts tested (Table I), NaCl, KCl and choline chloride were all effective, suggesting that the cation component of salts was not involved. Among the sodium salts of halides, the following order of effectiveness was found: $Cl^- > Br^- > F^- > I^-$. Ac^- and also SO_4^{2-} were active, but were not so effective as Cl^-. Anions are known to accelerate cold inactivation of soluble ATPase [30]. Furthermore, several organic and inorganic anions are capable of preventing the interaction of ATPase inhibitor with soluble and particulate ATPase [31, 16]. As a result, activation of soluble ATPase by anions has been demonstrated [32]. On the basis of these findings, it was thought that Cl^- increased the effectiveness of phosphatidylcholine, thereby altering the association between the ATPase inhibitor and the ATPase. The negative charge would enable anionic liposomes to achieve the same effect without the assistance of added anions. This possibility was tested employing Mg–ATP particles [14], which have a high content of ATPase inhibitor and AS particles [15], whose content of ATPase inhibitor is low.

In Table II, it is seen that preincubation of Mg–ATP particles in a medium with a low concentration of Cl^- and appropriate amounts of ATP and Mg^{2+}, resulted

TABLE I

EFFECT OF SALTS ON THE STIMULATION BY PHOSPHATIDYLCHOLINE

In the first incubation, 25 μg particulate ATPase were incubated for 5 min at 37 °C with salts and 120 μg bovine brain phosphatidylcholine in the conditions described in Fig. 3. In the second incubation, the ATPase was determined as described under Materials and Methods.

Additions during first incubation	ATPase activity (μmoles/mg per min)
–	0.09
Phosphatidylcholine	0.70
plus 320 mM NaCl	2.42
plus 320 mM KCl	2.32
plus 320 mM choline chloride	2.42
plus 320 mM sodium acetate	1.36
–	0.13
Phosphatidylcholine	0.48
plus 160 mM NaCl	1.32
plus 160 mM NaBr	1.14
plus 160 mM NaF	0.83
plus 160 mM NaI	0.35

TABLE II

REVERSAL BY ANIONIC LIPOSOMES OF ATP, Mg^{2+}-INDUCED INHIBITION OF ATPase ACTIVITY

0.3 ml of 0.17 M sucrose, 7 mM Tris–HCl, 0.7 mM EDTA and 0.3 mg bovine serum albumin (pH 7.3) containing 50 μg ATP–Mg particles were incubated for 10 min at 37 °C with the additions indicated (first incubation). The medium was then brought to a final volume of 1.0 ml to give 50 mM sucrose, 50 mM Tris–HCl, 0.2 mM EDTA, 3.0 mM ATP, 2.5 mM $MgCl_2$, 0.3 mg albumin, 2 mM phosphoenolpyruvate, 15 μg pyruvate kinase (pH 7.3) and the incubation followed for an additional 10 min at 37 °C. The reaction was stopped with 0.25 ml 50 % trichloroacetic acid. Appropriate controls were allowed to correct for the small amount of phosphate liberated in the first 10 min when ATP and Mg^{2+} were present. Oligomycin, 1 μg/ml. Egg phosphatidylcholine.

Additions during first incubation	ATPase activity (μmoles/mg per min)	
	Without oligomycin	With oligomycin
–	2.0	0.2
1 mM ATP, Mg^{2+}	1.1	0.2
plus 50 μg phosphatidylcholine	1.2	0.2
plus 50 μg phosphatidylcholine containing 20 % dicetylphosphate	2.0	0.2
1 mM ATP, Mg^{2+}	0.8	0.2
plus 50 μg phosphatidylcholine	0.9	0.2
plus 25 μg phosphatidylserine	1.8	0.2
plus 150 mM NaCl	1.5	0.2
plus NaCl and phosphatidylserine	1.6	0.2

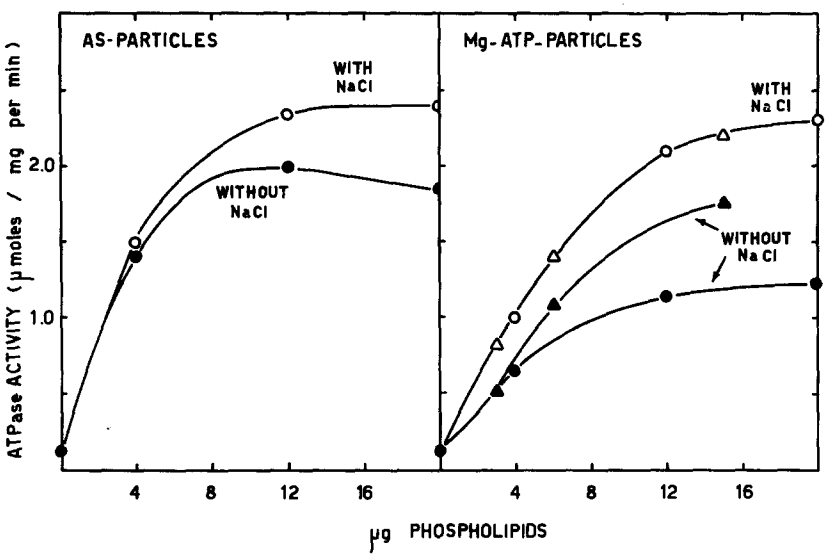


Fig. 4. Activation of ATPase in phospholipid-depleted AS particles and Mg–ATP particles. 10 mg particles were extracted with cholate as described under Materials and Methods. The fraction precipitated at 50 % saturated solution of $(NH_4)_2SO_4$ was washed once with 0.25 M sucrose, 10 mM Tris–HCl and 1 mM EDTA (pH 7.4). Reactivation by phospholipids was determined as described under Materials and Methods with 20 μg AS particles or 48 μg Mg–ATP particles in the presence or absence of 300 mM NaCl during the 5 min preincubation. ●—●, bovine brain phosphatidylserine without NaCl; ○—○, phosphatidylserine plus NaCl; ▲—▲, bovine heart diphosphatidylglycerol without NaCl; △—△, diphosphatidylglycerol plus NaCl.

in a decrease of the ATPase activity. This was in agreement with the observation [16] that ATP and Mg^{2+} were required for the association of ATPase with the ATPase inhibitor and indicated that a similar effect was induced under these conditions. The addition of a regenerating system (phosphoenolpyruvate and pyruvate kinase) did not change the effect of ATP and Mg^{2+}. The addition of phosphatidylcholine during the preincubation did not modify the effect of ATP and Mg^{2+}, but significant stimulation of ATPase activity was seen with liposomes of phosphatidylcholine containing dicetylphosphate. Accordingly, the acidic phosphatidylserine was effective in producing an oligomycin-sensitive stimulation of ATPase activity. As expected, NaCl used alone reproduced the effect of anionic liposomes, but the stimulation was not additive to that of phosphatidylserine. By using AS particles, preincubation with ATP and Mg^{2+} failed to reduce the ATPase activity and, therefore, stimulation by anionic phospholipids was not detected. The experiment of Fig. 4 provides further indications that in order to fully activate mitochondrial-particulate ATPase, liposomes of diacylphospholipids need to remove the inhibition produced by the endogenous ATPase inhibitor. Phospholipid-depleted AS particles were stimulated by phosphatidylserine without the assistance of Cl^-, but the same preparation derived from Mg–ATP particles required Cl^- to be stimulated to a maximum extent. With diphosphatidylglycerol, which is more acidic than phosphatidylserine, the requirement was less striking. Direct evidence of antagonism between ATPase inhibitor and anionic phospholipids was obtained in experiments with purified inhibitor. As is shown

TABLE III

ANTAGONISM BETWEEN PHOSPHOLIPIDS AND ATPase INHIBITOR

16 μg AS particles were added to 0.3 ml of a medium containing ATPase inhibitor (0.1 μg Expts 1 and 2, 0.05 μg Expt 3), 0.17 M sucrose, 7 mM Tris–2-(*N*-morpholino)ethanesulfonic acid), 0.3 mg bovine serum albumin, 0.7 mM EDTA, 2 mM phosphoenolpyruvate, 10 μg pyruvate kinase, 4 mM ATP, 4 mM $MgCl_2$ and the indicated amount of phospholipids (final pH, 7.2). After 10 min at 37 °C, the reaction was stopped with 0.1 ml 50 % trichloroacetic acid.

Expt No.	Phospholipid	ATPase (μmoles/mg per min)	
		Without inhibitor	With inhibitor
1	—	5.9	2.0
	50 μg phosphatidylcholine	6.0	1.6
	100 μg phosphatidylcholine	5.2	1.6
	25 μg phosphatidylserine	6.0	4.0
	50 μg phosphatidylserine	4.7	4.6
2	—	5.1	2.3
	50 μg phosphatidylcholine	4.7	2.1
	10 μg phosphatidylserine	4.5	3.0
	10 μg diphosphatidylglycerol	4.4	4.5
3	—	4.5	2.7
	5 μg phosphatidylcholine	4.6	2.7
	5 μg phosphatidylethanolamine	4.7	3.2
	5 μg phosphatidylserine	4.5	3.2
	5 μg phosphatidylinositol	4.4	3.2
	5 μg diphosphatidylglycerol	4.8	4.4

in Table III, acidic phospholipids, in contrast to phosphatidylcholine, prevented the inhibition induced by the ATPase inhibitor on AS particles. It was of interest to note that diphosphatidylglycerol, which was the most effective in the stimulation of phospholipid-depleted Mg–ATP particles and in producing solubilization of mitochondrial ATPase [23], was also the most effective in preventing the effect of ATPase inhibitor. The phospholipid-induced ATPase in the presence of the inhibitor was oligomycin sensitive. The antagonism by anionic phospholipids was also seen when soluble ATPase was substituted for AS particles. In spite of its potent stimulating effect on particulate ATPase, lysophosphatidylcholine was devoid of effect when tested on Mg–ATP particles and ATPase inhibitor. This established a clear difference between it and the acidic phospholipids.

DISCUSSION

A phospholipid-depleted preparation of mitochondrial particulate ATPase was stimulated to a significant extent by phosphatidylcholine only when anionic amphipathic substances gave a negative charge to the liposomes. This explains the great effectiveness shown by anionic phospholipids [2] and constitutes evidence that a negative charge is a prerequisite for the effect of insoluble diacylphospholipids. By contrast, soluble lysophospholipids were activated also with an isoelectric ionic group. Although the stimulation of oligomycin-sensitive ATPase in a phospholipid-

depleted preparation is apparently the same with diacylphospholipids and lysophosphatidylcholine, significant differences can be put in evidence in other systems. Only diacylphospholipids reversed the ATP plus Mg^{2+}-induced inhibition of ATPase in Mg–ATP submitochondrial particles and prevented the effect of ATPase inhibitor. This suggests a different mechanism of action between the two classes of compounds which would be consistent with their different physicochemical properties [7] and with the observation that lysophospholipids are not detected [33] (or detected only in trace amounts [34]) in the mitochondrial inner membrane. The stimulation of ATPase by both anionic liposomes of diacylphospholipids and isoelectric lysophosphatidylcholine can be compared with the activity of phospholipase B from *P. notatum* [9]: lysophospholipids are easily hydrolyzed whereas diacylphospholipid liposomes need a negative charge. The analogy suggests similar requirements for the formation of a lipoproteic complex in the two cases. As discussed by Bangham and Dawson [9] and Dawson [6], the possibility has to be considered that the activation of phosphatidylcholine by anionic amphipathic substances is due to the capability of negative charges to produce dispersion of phospholipid particles. Formation of smaller liposomes would be the consequence of electrostatic repulsion. The ineffectiveness of cationic amphipaths makes this possibility unlikely. Moreover, the activation by phosphatidylcholine was not improved by preparation of small and homogeneous liposomes by prolonged sonication and Sepharose filtration [11].

The observation that the effectiveness of diacylphospholipids is lower when the content of ATPase inhibitor in the preparation is high, together with the capability to prevent the effect of ATPase inhibitor, suggest that the activation of particulate ATPase is associated with removal of inhibition produced by this endogenous component. This implies that the masking of ATPase activity, which follows the removal of phospholipids [17], is accompanied by reinforcement of association between F_1 and inhibitor. In recent research [35], the existence of reversible states in which F_1 and the inhibitor are more or less associated have been postulated. It was shown that ADP and coupled electron transport favor the dissociation of ATPase from the inhibitor, whereas the reverse effect is induced by ATP and Mg^{2+} (see also refs 31, 36 and 16).

Influences on the association of ATPase with the inhibitor can be expected also by the structural components of particulate ATPase. This would contribute to explaining the change in the properties of F_1 when it is included in the membrane [37]. Pullman and Monroy [31] have shown that inhibitor protected the soluble ATPase against cold inactivation. The antagonism between phospholipids and inhibitor is in line with this possibility and suggests that these components help to maintain a high turnover of F_1 through an effect on the association with the ATPase inhibitor. Although further investigations are required to clarify this point, it is possible that activation and inhibition of ATPase activity can be produced through an influence on this intrinsic mechanism of regulation. Antagonism between phospholipids and specific inhibitors of particulate ATPase (oligomycin, dicyclohexylcarbodiimide) has been demonstrated [2, 38].

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