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REGULATION OF PHOSPHOLIPID-ATPase COMPLEX INTERACTION BY THE ADENINE NUCLEOTIDE CARRIER

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(1) The effect of phospholipids on a preparation containing the ATPase complex and the adenine nucleotide carrier is studied in the presence of ligands known to affect the conformation of these components of the mitochondrial inner membrane. (2) When ATPase activity is abolished by phospholipid depletion, the reactivation induced by phosphatidylcholine is prevented by the simultaneous addition of ATP. ADP partially reproduces the ATP effect. AMP, GTP, UTP and P_i are ineffective. (3) The influence of ATP is associated with reduced phospholipid binding to the membrane fragments and is reversible. The ATP effect on reconstitution is not manifest when phosphatidylcholine is added together with negatively charged phospholipids. (4) Carboxyatractyloside does not modify the phospholipid-ATPase complex interaction but bongkrekic acid is as effective as ATP. In the presence of ADP, the influence of bongkrekic acid is considerably increased. (5) It is concluded that the binding of ATP to the adenine nucleotide carrier enables the complex to select between the charged and uncharged phospholipids. As a result of the carrier conformational change, the ATPase complex is induced to prefer a negatively charged phospholipid environment.

Introduction

Phospholipids of the mitochondrial inner membrane show different effectiveness in stimulation of the ATPase complex [1–5]. Thus, diphosphatidylglycerol and other negatively charged phospholipids increase ATPase activity when added to the isoelectric phosphatidylcholine. This differential activation suggests the existence of mechanisms promoting the association of negatively charged phospholipids with the ATPase complex when required by the functional activity. A mechanism based on the specific location of phospholipids and the ATPase complex subunits may be indicated by the asymmetric distribution of these components in the mitochondrial inner membrane. According to the pattern of phospholipid distribution [6,7], diphosphatidylglycerol is predominant in the inner half of the bilayer, a location allowing close contact with F_1-ATPase, the catalytic segment of the complex. Due to extensive F_1-ATPase conformational changes, variable connections may be established between the enzyme and the activating phospholipid.

Recently [8], it has been found that the interaction of phosphatidylcholine with the ATPase complex is prevented when the preparation contains the adenine nucleotide carrier. This finding indicates the proximity between these two components of the mitochondrial inner membrane and suggests that the adenine nucleotide carrier may influence the phos-

Abbreviations: Mes, 4-morpholineethanesulfonic acid; Ado-*PP*[NH]*P*, adenosine 5′-[β,γ-imido]triphosphate.

pholipid-induced activation of the ATPase complex. In accord, when these two components are separated by Triton X-100 [9] or a short-chain phosphatidylcholine [10], the ATPase complex becomes less stable and the oligomycin sensitivity may be lost. Although the adenine nucleotide carrier and ATPase complex are obtained as separate entities and are shown to perform independently their funcitons in reconstituted systems [11,12], their functional association in situ is likely [13–15].

In the present paper, the phospholipid-ATPase complex interaction has been examined under the influence of ligands known to induce conformational changes in F_1-ATPase and in the adenine nucleotide carrier.

Materials and Methods

Egg phosphatidylcholine and bovine brain phosphatidylserine were prepared according to conventional procedure. Egg lysophosphatidylcholine was purchased from Sigma. Dielaidoylphosphatidylcholine was generously donated by Professor De Gier, University of Utrecht, and [^{3}H]dipalmitoylphosphatidylcholine by Dr. C. Montecucco, University of Padua. The purity of phospholipids was tested by thin-layer chromatography. Dispersions of individual phospholipids or their mixtures were prepared (1–2 mg/ml) by sonication in 25 mM Tris-Mes (pH 7.0).

Bonkrekic acid was kindly provided by Professor Berends, University of Delft. Ammonia-Sephadex particles [16] were obtained from bovine heart mitochondria. From these particles the phospholipid-deficient ATPase complex was prepared by extraction with cholate [17]. The residual content of phospholipids was 0.1 μmol/mg protein, approx. 10% of the initial amount. Experiments were done also with the ATPase complex prepared according to the method of Serrano et al. [18], omitting the lysophosphatidylcholine-deoxycholate treatment.

Standard reconstitution procedure. 1 mg of phospholipid-deficient ATPase complex from ammonia-Sephadex particles was incubated for 40 min at 0°C in 1.0 ml of 25 mM Tris-Mes (pH 7.0) containing the phospholipids and appropriate additions. The sample was layered on top of 3.5 ml of 0.25 M sucrose, 25 mM Tris-Mes (pH 7.0) and centrifuged for 20 min at 0°C at 30 000 rev./min in a Beckman SW 50 swinging-bucket rotor. The sediment was collected, washed once and tested for ATPase activity and phospholipid content. When more accurate estimation of phospholipid binding was desired the phospholipids were supplemented with 1% [^{3}H]dipalmitoylphosphatidylcholine (final radioactivity, 5.10^5 dpm/μmol). After sonication, the mixture was used in the reconstitution procedure. The radioactivity was measured in the sediment and in the supernatant using a liquid scintillation counter.

Analytical procedure. To detect the ATP degradation when this compound was added during the reconstitution of the ATPase complex with phospholipids, the sample was centrifuged for 20 min at 0°C at 40 000 rev./min. The supernatant was collected, supplemented with 0.6 M perchloric acid and centrifuged. After neutralization with 10% KOH in 1 M Tris, the ADP content was determined following the oxidation of NADH in the presence of phospho*enol*pyruvate, pyruvate kinase and lactate dehydrogenase. The ATPase activity was tested at 37°C in 0.6 ml of 167 mM sucrose, 7 mM Tris-Mes, 0.6 mg bovine serum albumin, 2.7 mM phospho*enol*pyruvate (monopotassium salt), 20 μg pyruvate kinase, 4 mM ATP, 4 mM $MgCl_2$, 20 μg reconstituted ATPase complex (final pH 7.2 at 37°C). Protein, P_i and phospholipid phosphorus were determined according to conventional procedures (see Ref. 3).

Results

In Table I it is shown that the addition of ATP during incubation of the ATPase complex with phosphatidylcholine prevented the reactivation by 60%. The effect of ATP was observed when the reconstitution procedure was performed at 37, 20 and 0°C but at the lowest temperature it was more manifest and reproducible. Since egg phosphatidylcholine has a gel-to-fluid state transition below 0°C, 40 min incubation at 0°C were routinely employed.

Among the nucleotide triphosphates, ATP was the most effective. ADP induced some inhibition whereas AMP, GTP, UTP and P_i were ineffective. Appropriate controls in the presence of glucose and hexokinase excluded the possibility that the limited ADP effect originated from some ATP formed in situ. Conversely, the ATP effect was not modified by the addition of phosho*enol*pyruvate and pyruvate kinase. The ATP-

TABLE I

INHIBITION BY ATP OF PHOSPHATIDYLCHOLINE-INDUCED REACTIVATION

The phospholipid-deficient ATPase complex was reconstituted (Materials and Methods) with phospholipids in the presence and absence of 3 mM of the indicated adenine nucleotide. The amounts of phospholipids were 2 μmol/mg phosphatidylcholine, 0.5 μmol/mg phosphatidylcholine/phosphatidylserine (1 : 1, mol/mol), 2 μmol/mg phosphatidylcholine/lysophosphatidylcholine (2 : 1, mol/mol). The conditions for the ATPase activity assay were 167 mM sucrose, 7 mM Tris-Mes, 0.6 mg bovine serum albumin, 2.7 mM phospho*enol*pyruvate (monopotassium salt), 20 μg pyruvate kinase, 4 mM ATP, 4 mM $MgCl_2$, 20 μg ATPase complex. Final pH 7.2 at 37°C. Final volume 0.6 ml. Incubation for 10 min at 37°C.

Phospholipid	Nucleotide added during reconstitution	ATPase activity ($\mu mol \cdot mg^{-1} \cdot min^{-1}$)
None	–	0.10
None	ATP	0.10
Phosphatidylcholine	–	2.21
Phosphatidylcholine	ATP	0.85
Phosphatidylcholine	ADP	1.70
Phosphatidylcholine	AMP	2.00
Phosphatidylcholine, phosphatidylserine	–	3.41
Phosphatidylcholine, phosphatidylserine	ATP	3.28
Phosphatidylcholine, lysophosphatidylcholine	–	1.27
Phosphatidylcholine, lysophosphatidylcholine	ATP	1.33

induced inhibition was usually tested on the ATPase complex obtained from ammonia-Sephadex particles in order to exclude the involvement of the ATPase inhibitor protein [19]. However, equal effectiveness of ATP was observed in preparations from EDTA and pyrophosphate submitochondrial particles. Similar results were obtained using the ATPase complex prepared according to the procedure of Serrano et al. [18], omitting the final lysophosphatidylcholine-deoxycholate extraction. In all these preparations the values of ATP-induced inhibition were directly related to the extent of phospholipid depletion. In this respect, the procedure of Kagawa and Racker [17] was found preferable.

The effect of ATP was not influenced by EDTA and did not require magnesium. This was at variance with the MgATP-induced inactivation of membrane-bound F_1-ATPase described earlier [20,21]. Since magnesium was not required, a further distinctive feature was the negligible ATP hydrolysis. Under the standard conditions of reconstitution employed here (1 mg ATPase complex, 3 μmol ATP, 2 μmol egg phosphatidylcholine, incubated for 40 min at 0°C in 1 ml Tris buffer), only 0.4% of the added ATP could be found as ADP in enzymatic spectrophotometric determinations (see Materials and Methods). The mean value was 12 nmol (range 0–30 nmol) in five experiments.

When the structure of the phophatidylcholine bilayer was modified by addition of the acidic phosphatidylserine or the hydrophilic lysophosphatidylcholine, the influence of ATP was no longer detected. In these experiments phosphatidylserine was preferred to diphosphatidylglycerol in order to minimize the solubilization of F_1-ATPase [4]. Furthermore, phosphatidylserine yields 1 charge/molecule at neutral pH and forms stable bilayer structures. With phosphatidylcholine/phosphatidylserine mixtures, substantial removal of the ATP effect was already achieved after the addition of 30 mol% phosphatidylserine.

The role of phosphatidylcholine was further defined by the following observations. (a) The ATP effect was maximally manifest at low phosphatidylcholine-to-protein ratios (up to 2 μmol/mg protein). This amount of phosphatidylcholine produced only suboptimal reactivation of the ATPase complex. (b) A fluid state of the phosphatidylcholine bilayer was required. This was established with dielaidoylphosphatidylcholine that has a transition temperature at 13°C. When 2 μmol of this phospholipid were added to 1 mg lipid-depleted ATPase complex and the

TABLE II

INHIBITION OF PHOSPHATIDYLCHOLINE BINDING

The phospholipid-deficient ATPase complex was reconstituted (Materials and Methods) with phospholipids in the absence and presence of 3 mM of the indicated nucleotide. The phospholipids in organic solvent were supplemented with 1% [^{3}H]dipalmitoylphosphatidylcholine, taken to dryness and sonicated in 25 mM Tris-Mes (pH 7.0). Other conditions as in Table I.

Phospholipid added	Nucleotide	ATPase activity (μmol · mg^{-1} · min^{-1})	Phospholipid binding (μmol/mg protein)
Phosphatidylcholine	–	1.41	0.32
Phosphatidylcholine	ATP	0.76	0.17
Phosphatidylcholine	Ado *PP*[NH]*P*	0.34	0.20
Phosphatidylcholine	AMP	1.41	0.29
Phosphatidylcholine, phosphatidylserine	–	3.22	0.58
Phosphatidylcholine, phosphatidylserine	ATP	3.80	0.58
Phosphatidylcholine, lysophosphatidylcholine	–	1.07	1.04
Phosphatidylcholine, lysophosphatidylcholine	ATP	1.23	1.14

reconstitution step performed at 0°C, a large amount of the phospholipid aggregated with the ATPase complex (1.5 μmol/mg protein). No effect of ATP was recorded. When the reconstitution was performed at 20°C and therefore above the transition temperature, a lower amount of the phospholipid was bound (0.5 μmol/mg protein) and the ATP-induced inhibition was manifest. The greater binding of the phospholipid in the gel state was in agreement with previous data on dipalmitoylphosphatidylcholine [22].

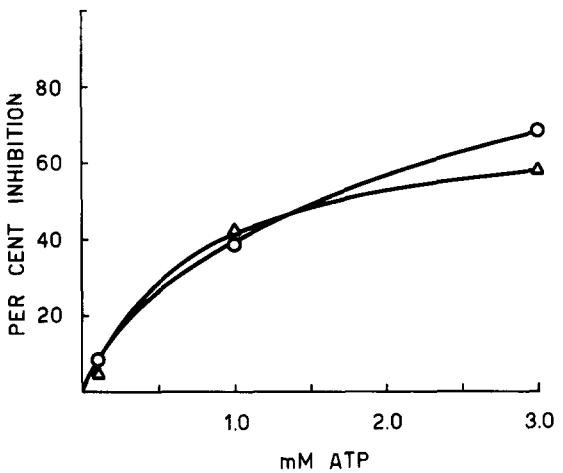

Fig. 1. Inhibition of ATPase activity and phospholipid binding. The reconstitution was performed as described in Materials and Methods using 2 μmol/mg protein egg phosphatidylcholine and the indicated amount of ATP. The variations in ionic strength due to the different ATP concentrations were balanced with NaCl. The pospholipid binding was assessed by chemical determination of total phosphorus. (o———o) ATPase activity, (△———△) phospholipid binding.

Since these results indicated a relationship between the effect of ATP and the extent of phosphatidylcholine binding, the influence of ATP on this process was examined using labelled liposomes. In Table II it is seen that the presence of ATP inhibited the association of phosphatidylcholine with the preparation. The effect disappeared upon the addition of phosphatidylserine or lysophosphatidylcholine. When Ado*PP*[NH]*P* substituted for ATP, together with the expected inhibition of ATPase activity, significant inhibition of phospholipid binding was similarly produced. The experiment of Fig. 1 showed that the ATP effects on the ATPase activity and on the phosphatidylcholine binding were directly related although the quantitative values of inhibition did not correspond in all the experiments. Half-maximal effect on the two processes was produced at approx. 0.8 mM ATP. Similar ATP concentrations were required to detect ATP-induced conformational changes in submitochondrial particles [23] and to obtain optimal reconstitution of urea particles from rat liver mitochondria [24]. In agreement with the conclusion of a primary ATP effect on phosphatidylcholine binding, in Table III it is seen that a second incubation of the ATPase complex with phospholipids after the removal of ATP restored the ATPase activ-

TABLE III

REVERSAL OF ATP EFFECT

The phospholipid-deficient ATPase complex was reconstituted (Materials and Methods) with 2 μmol/mg phosphatidylcholine in the absence and presence of 3 mM ATP. After washing, the reconstituted complexes were incubated a second time with the indicated phospholipid in the absence of ATP. The conditions for the second incubation were 10 min at 37°C with 3.0 μmol/mg phosphatidylcholine or 0.5 μmol/mg phosphatidylserine in 0.2 ml of 0.25 M sucrose, 25 mM Tris-Mes (pH 7.2). Other conditions as in Table I.

First incubation with phospholipids	Second incubation with phospholipids	ATPase activity ($\mu mol \cdot mg^{-1} \cdot min^{-1}$)
Phosphatidylcholine	–	1.32
Phosphatidylcholine, ATP	–	0.36
Phosphatidylcholine	phosphatidylcholine	1.85
Phosphatidylcholine, ATP	phosphatidylcholine	1.78
Phosphatidylcholine	phosphatidylserine	3.32
Phosphatidylcholine, ATP	phosphatidylserine	3.00

ity. These results clearly showed that inactivation of F_1-ATPase was not involved in this ATP effect. Since ATP is a ligand of F_1-ATPase and of the adenine nucleotide carrier, these experiments did not allow us to distinguish between the role of two components. The effect of Ado*PP*[NH]*P* was also inconclusive. In accord with the structural requirements for adenine nucleotide binding and transport [25,26], this compound, although transported at low efficiency, could still associate with the carrier and trigger the conformation unfavorable for ATPase complex reactivation. More information was obtained by studying the influence of inhibitory ligands and the effect of ATP on F_1-ATPase-depleted membranes.

In contrast to carboxyatractyloside, bongkrekic acid effectively reproduced the influence of ATP (Table IV). Full effectiveness of bonkrekic acid was at 50 nmol/mg protein, an amount slightly higher than that usually employed. However, a large fraction of this lipophilic inhibitor is expected to be incorporated in the excess of phospholipids present during the incubation. At equal inhibition of phospholipid binding, the effect on the ATPase reactivation was more manifest with bongkrekic acid than with ATP. Furthermore, only partial restoration of ATPase activity followed a second phosphatidylcholine addi-

TABLE IV

EFFECT OF BONGKREKIC ACID

0.5 mg phospholipid-deficient ATPase complex was reconstituted (Materials and Methods) with 2 μmol/mg phosphatidylcholine or 0.5 μmol/mg phosphatidylcholine/phosphatidylserine in the absence or presence of the indicated additions. Final volume 0.5 ml. Where indicated, a second incubation with phosphatidylcholine was performed as described in Table II. The binding of phospholipids was assessed by chemical determination of total phosphorus after the final washing. The values were corrected for the initial phospholipid content (0.1 μmol/mg protein).

Addition during reactivation	ATPase activity ($\mu mol \cdot mg^{-1} \cdot min^{-1}$)	Phospholipid binding (μmol/mg)
Phosphatidylcholine	1.54	0.32
plus 3 mM ATP	0.78	0.14
plus 50 μM bongkrekic acid	0.54	0.16
plus 50 μM bongkrekic acid (second phosphatidylcholine addition)	1.08	–
plus 10 μM bongkrekic acid	1.28	0.26
plus 10 μm bongkrekic acid, 3 mM ADP	0.76	0.15
Phosphatidylcholine/phosphatidylserine	3.42	0.46
plus 50 μM bongkrekic acid	3.88	0.47

tion. These findings most likely reflected the tighter binding of the inhibitor ligand to the carrier. In agreement with an increased of affinity of ADP for the carrier in the presence of bongkrekic acid [27], ADP and a small amount of bongkrekic acid together prevented the reconstitution of the ATPase complex. Separate controls confirmed that bongkrekic acid did not affect the ATPase activity of undepleted submitochondrial particles.

After treatment of the preparation with 3.5 mM NaBr and washing [28], sodium dodecyl sulfate polyacrylamide gel electrophoresis and ATPase activity served as controls for F_1-ATPase depletion. The resulting membrane fragments were able to bind purified F_1-ATPase and to reconstitute a phospholipid-dependent ATPase complex sensitive to oligomycin. When the depleted membrane fragments were incubated for 40 min at 0°C with 2 μmol/mg phosphatidylcholine, the phospholipid binding was 0.6 μmol/mg and 3 mM ATP yielded 50% inhibition. AMP was ineffective.

Discussion

Structural and functional relationships are established between membrane proteins and the phospholipid bilayer. Extrinsic [29] or intrinsic [30,31] proteins induce phospholipid phase separation. Conversely, the lipid phase influences the protein conformation [32]. In agreement with this mutual influence, this paper shows that a protein conformational change induced by ATP decreases the interaction of the ATPase complex with isoelectric phospholipids and induces a preference for negatively charged phospholipid structures. At the same time negative phospholipids elicit optimal ATPase complex activity. Viewed in a more general context, these findings show that the binding of hydrophilic ligands to a membrane receptor initiates a conformational change leading to the alteration of phospholipid-protein interactions. Considerable membrane reorganization may follow through phospholipid phase separation or asymmetric distribution. Indeed, extensive ultrastructural changes are observed in the mitochondrial membrane under the influence of ATP and ADP [33–35]. The functional consequences of ATP-induced membrane reorganization may include new relationship between the ATPase complex and other intrinsic proteins showing the same phospholipid preference. In the light of recent views on the role of short-range interactions in oxidative phosphorylation [36], it is relevant that the ATPase complex and cytochrome oxidase [37] may associate together in clusters containing anionic phospholipids. The specificity of ATP, lack of magnesium requirement and effectiveness of bongkrekic acid show that the regulation of phospholipid-protein interactions is not a function of the ATPase complex subunits. Rather, they show that the adenine nucleotide carrier is associated with the preparation and is responsible for the discrimination between isoelectric and negatively charged phospholipids. The effect of bongkrekic acid and the greater activity of ATP in comparison to ADP show that the carrier conformation suitable for selecting between phospholipids can be triggered from the matrix side. In this connection it is significant that acidic phospholipids activate the translocase activity [38,39] and that the accumulation of negative charges in the matrix side of the carrier orients toward ATP extrusion [11,35]. The role of the adenine nucleotide carrier in the ATP- and ADP-induced ultrastructural transition of mitochondrial membranes is documented by the inhibitory effect of atractyloside [33–35]. In agreement with the involvement of the adenine nucleotide carrier in the selection of phospholipids, control experiments on the reactivation of complex V failed to show any influence of ATP and bongkrekic acid. This purified preparation of the ATPase complex does not contain the adenine nucleotide carrier [12].

Two properties of negatively charged phospholipids underlie the carrier-dependent phospholipid selection. The first is the possibility of increasing the lipid-protein association through electrostatic bonds. Significantly, the carrier is a cationic protein [40] and increased phospholipid binding is observed upon the addition of phosphatidylserine to phosphatidylcholine. The second is the possibility of assembling expanded bilayer structures. This can be inferred from the effect of lysophosphatidylcholine that reverses the ATP effect in spite of the isoelectric polar head. Lysophosphatidylcholine incorporation is known to produce bilayer expansion, permeability increase and elevation of free monomer concentration [41]. These modifications are suitable for promoting the association between proteins and phospholipids.

It can be concluded that the ATP-induced conformational change prevents protein penetration into the isoelectric bilayer because of tight phospholipid organization. The same limitation is not found in negatively charged phospholipid structures due to the expanded bilayer and electrostatic interactions. The observed inactivity of phosphatidylcholine liposomes in reconstitution experiments with the purified adenine nucleotide carrier [38] or partially purified ATPase complex [42] may find an explanation on this basis. Since the proportion of diphosphatidylglycerol increases in the residual phospholipids of the ATPase complex after extraction with cholate [17], the amount of this acidic phospholipid may become sufficient to reduce the influence of ATP in preparations partially depleted of phospholipids. In accord, the ATP-induced effect is dependent upon the extent of phospholipid depletion. The conclusion of an ATP-induced inhibition of protein penetration into the phosphatidylcholine bilayer is consistent with decreased binding of this phospholipid under the influence of ATP, with the increase in nucleotide influence at low temperature and in fluid phosphatidylcholine bilayers. At low temperature the increase of lipid-lipid interactions and packing density enhances the efficiency of the lipid mechanical barrier to protein penetration. On the other hand, when the isoelectric bilayer is in the fluid state the possibility of interaction with protein maximally relies on penetration. In the gel state the interaction with protein can be promoted by the structural defects inherent in this lipid organization [43]. In our experiments with dielaidoylphosphatidylcholine this has been manifest with the high phospholipid content of complexes reconstituted below the transition temperature.

In summary, the results of this paper confirm the functional and structural association between the ATPase complex and adenine nucleotide carrier [8, 15]. Moreover, they show that the reactivation of the ATPase complex by phosphatidylcholine not only depends upon the length and fluidity of acyl chains [3,22], but also on the conformation of the protein present in the preparation.

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