REVERSAL BY PHOSPHOLIPIDS OF THE OLIGOMYCIN INDUCED INHIBITION OF MEMBRANE ASSOCIATED ADENOSINTRIPHOSPHATASES

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Summary. The inhibitory effect of oligomycin on sodium, potassium-stimulated ATPase and on mitochondrial ATPase was removed by the addition of purified phospholipids to the incubation medium. Ouabain effect was not modified. Phospholipids with isoelectric ionic portion (particularly lecithins) were found more active than phosphatidylethanolamines and bovine brain phosphatidylserine. Differences in the activity of lecithins from various sources were also seen. This indicates that the composition of hydrophobic fatty acid chains influences the reversal of oligomycin effect. The antagonism by the external added phospholipids may reflect the affinity of oligomycin with the phospholipids of the enzyme preparation.

Oligomycin has been shown (1,2,3) to inhibit both particulate mitochondrial ATPase and the sodium, potassium-stimulated ATPase (transport ATPase). Studies on the mechanism of action of this antibiotic are of considerable interest in order to explore possible similarities in the operations of the two systems located in different cellular membranes.

In this report it is shown that some of the phospholipids present in high amount in the cellular membranes can reverse the inhibition by oligomycin of both mitochondrial and transport ATPases. This points to the crucial role of phospholipids in the effect of oligomycin, an indication in line with recent observations (4) showing marked differences among individual phospholipids in the activation and restoration of rutamycin sensitivity to mitochondrial ATPase.

Materials and methods. The transport ATPase was prepared from beef heart by a modification (5) of Matsui and Schwartz (6) procedure. Submitochondrial particles were prepared, as described by Kielley and Bronk (7), from rat liver mitochondria isolated in 0.25 M sucrose, 2 mM Tris-HCl pH 7.4 according to Hogeboom (8). Soya

bean and rat liver mitochondrial phospholipids were prepared and purified as described previously (9). Bovine brain lecithin and phosphatidylethanolamine were a kind gift by Fidia Farmaceutici, Abano (Italy). Egg lecithin was purchased from Sigma, phosphatidylserine and sphingomyelin from General Biochemicals. The purified phospholipids were suspended, homogenized in 0.25 M sucrose, 1 mM EDTA, 10 mM Tris.HCl pH 7.4 and "solubilized" by sonication using a Biosonik III Sonifier. The concentration of phospholipids was calculated after determination of total phosphorus according to the method indicated elsewhere (9). The conditions of measure of the ATPase activities are given in the legends to Tables and Figures. Proteins were estimated by the Lowry et al. (10) procedure.

Results and Discussion. In Table I it is seen that the oligomycin effect on

TABLE I

Antagonism between oligomycin and phospholipids on transport ATPase

Each tube contained in a final volume of 1.0 ml, 100 mM NaCl, 20 mM KCl, 2.5mM $MgCl_2$, 2.5 mM ATP.Tris pH 7.4, 50 mM Tris.HCl pH 7.4, 25 mM sucrose, 0.1 mM EDTA, 130-250 µg of enzyme proteins. 20 min incubation at 37°C. The reaction was terminated by the addition of trichloroacetic acid. Oligomycin concentration was 6 µg/ml (24-46 µg/mg proteins). The order of addition was: phospholipids or detergent in 0.1 ml of 0.25 M sucrose, 10 mM Tris.HCl pH 7.4, 1 mM EDTA, the enzyme and then oligomycin. After 5 min at room temperature the incubation medium was completed.

Additions	µmoles ATP split/mg protein/h		
	without oligomycin	with oligomycin	%inhib.
none	19.6	5.5	71.94
2.5 mg soya bean phospholipids	17.8	12.8	28.09
2.4 mg bovine brain lecithin	18.6	17.8	4.31
2.5 mg bovine brain phosph.ethanolamine	16.9	9.2	45.57
none	25.0	9.1	63.60
0.6 mg bovine brain lecithin	25.0	20.0	20.00
0.6 mg Tween 80	28.0	17.2	38.58
none	16.8	6.4	61.91
0.5 mg soya bean lecithin	13.6	10.7	21.33
0.5 mg egg lecithin	16.5	11.2	32.13
0.5 mg soya bean phosph.ethanolamine	16.0	7.7	51.88
0.5 mg bovine brain phosph. serine	12.4	7.7	37.91

transport ATPase can be partially removed by the addition of pooled soya bean phospholipids "solubilized" by sonic oscillation. Differences were seen when individual phospholipids from various sources were tested. The lecithins were more active than phosphatidylethanolamines and bovine brain phosphatidylserine.

Among phospholipids with isoelectric polar group, bovine brain sphingomyelin could not be tested because it produced strong inhibitory effect on this ATPase activity. The antagonism by lecithins is especially significant considering the high amount of this phospholipids in the cellular membranes (11,12,13).

Similarly to other situations (12,14,15) in which some of the effects of phospholipids on membrane-associated ATPases was duplicated by detergents, non-ionic detergents such as Tween 80, Lubrol WC and Emasol were found effective antagonists of oligomycin. In agreement with previous results of Tanaka and Strickland (16) the inhibition by ouabain was not changed by the addition of phospholipids.

In Table II it is seen that the addition of purified phospholipids can remove the effect of oligomycin also on submitochondrial particles. Previous findings of Kagawa and Racker (14) have shown that washing a rutamycin inhibited preparation of submitochondrial particles with pooled soya bean phospholipids, the activity was partially restored and the bound rutamycin removed.

Also on submitochondrial particles phospholipids with isoelectric ionic portion (particularly mitochondrial and bovine brain lecithins) were highly active; phosphatidylethanolamines and non-ionic detergents were much less effective, phosphatidylserine inhibited the activity of mitochondrial ATPase.

Moreover, detergents and bovine brain lecithin showed a completely different effect when 2,4-dinitrophenol-stimulated ATPase in intact mitochondria was tested. The former inactivated the ATPase, the latter produced a significant reversal of the oligomycin induced inhibition. The difference between lecithin and detergents on intact mitochondria and submitochondrial particles clearly shows that the phospholipid effect is not necessarily due to detergent activity.

A titration curve for lecithin is given in Fig. 1. On transport ATPase the antagonism is already manifest at low amount (0.4 mg/mg proteins) but maximal activity is reached at 9-10 mg/mg proteins. The possibility is to be considered that not all the added lecithin is partecipating in the antagonism with oligomycin but only the amount which, after sonication, is dispersed in aggregates of

TABLE II

Antagonism between phospholipids and oligomycin on particulate mitochondrial ATPase

Each tube contained in a final volume of 1.0 ml (a)submitochondrial particles from rat liver mitochondria 31 µg proteins, 100 mM NaCl, 20 mM KCl, 2.5 mM $MgCl_2$, 3.2 mM ATP.Tris pH 7.4, 50 mM Tris.HCl pH 7.4, 25 mM sucrose, 0.1 mM EDTA; (b) intact rat liver mitochondria 122 µg proteins, 100 mM NaCl, 20 mM KCl, 0.1 mM 2,4-dinitrophenol, 3.2 mM ATP.Tris pH 7.4, 50 mM Tris.HCl pH 7.4, 50 mM sucrose, 0.2 mM EDTA.The oligomycin concentration was 0.05 µg/ml (1.6 µg/mg protein of submitochondrial particles) and 0.2 µg/ml (1.6 µg/mg protein of rat liver mitochondria). Incubation, 20 min at 37°C. The reaction was started by the addition of the enzyme preparations and terminated by the addition of trichloroacetic acid.

Additions	µmoles ATP split/mg protein/h		
	without oligomycin	with oligomycin	%inhib.
(a) submitochondrial particles			
none	92	12.4	86.53
0.4 mg bovine brain lecithin	84	75	10.72
0.4 mg bovine brain phosph.ethanolamine	80	25	68.75
0.4 mg Tween 80	84	33	60.72
none	87.5	7.8	91.09
0.5 mg mitochondrial lecithin	83	79	4.82
0.5 mg egg lecithin	80	48	40.00
0.3 mg soya bean lecithin	88.5	31.4	64.52
0.3 mg soya bean phosph.ethanolamine	83	17.4	79.04
0.3 mg bovine brain phosph.serine	31.4	17.4	-
0.2 mg bovine brain sphingomyelin	76	39.4	48.16
(b) rat liver mitochondria			
none	25.8	2.0	92.25
0.5 mg bovine brain lecithin	25.8	12.9	50.00
0.5 mg Tween 80	9.8	4.0	-

appropriate size. Comparable results were obtained with bovine brain lecithin using submitochondrial particles.

In Table III it is seen that preincubation of transport ATPase with oligomycin followed by centrifugation and washing, resulted in a strongly inhibited preparation. The activity was restored by the addition of lecithin. When preincubation was made in the presence of oligomycin plus lecithin, the resulting preparation was fully active in the absence of subsequent addition of the phospholipid.

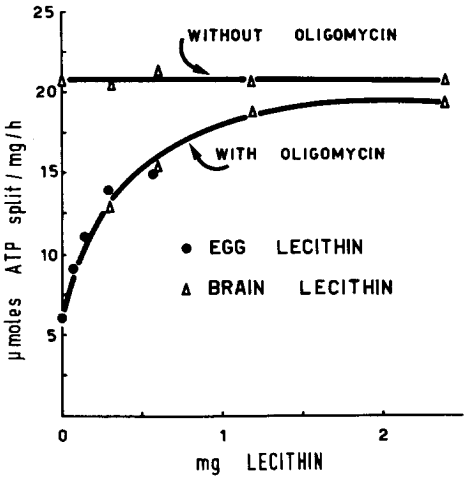

Fig. 1 Effect of increasing amounts of lecithin on the inhibition of transport ATPase by oligomycin. Experimental conditions as in Table I. Oligomycin was 6 µg/ml (46 µg/mg proteins).

TABLE III

Pretreatment of transport ATPase with oligomycin

An aliquot of the enzyme preparation (650 µg proteins) was incubated at room temperature for 5 min in 0.6 ml of 0.21 M sucrose, 8 mM Tris.HCl pH 7.4, 0.8 mM EDTA, 1.7 mM ATP.Tris pH 7.4, 0.2 mM dithiothreitol with the addition indicated below (60 µg oligomycin, 11 mg bovine brain lecithin or both). After centrifugation 10 min at 20.000 RPM (Spinco N°40 rotor) and washing, the sediments were resuspended in 0.2 ml of the sucrose, Tris, EDTA solution and the activity tested as described in Table I in the presence or absence of 2.3 mg bovine brain lecithin.

Addition during the pretreatment	µmoles ATP split/mg protein/h	
	without lecithin	with lecithin
none	23.5	20.8
oligomycin	6.2	19.0
lecithin	22.4	19.6
oligomycin + lecithin	18.8	18.8

This was taken as indication that lecithin inhibited the binding of oligomycin to the enzyme preparation.

Previous observations (14) have shown that oligomycin needs some structural

organization, in which phospholipids are required, in order to produce inhibitory effect on mitochondrial ATPase. From the results reported in this paper it can be thought that an essential step in the effect of oligomycin is the interaction with the phospholipids of the enzyme preparation. The aggregates formed by the added phospholipids could constitute a system which effectively competes for the inhibitor also when it is already bound to the enzyme complex.

The polar head of phospholipids has a clear influence on the interaction between oligomycin and external phospholipids. Phospholipids forming in water aggregates without a net charge at physiological pH (lecithin and sphingomyelin) can interact much better. On the other hand the differences in the activity among the various lecithins suggest that the hydrophobic fatty acid chains are also important. It is possible that the degree of saturation of the fatty acid constituents, which directly affects the temperature transition and permeability of the lecithin aggregates in water (17) becomes a determining factor in the interaction with oligomycin. On this ground, it is conceivable that the quantitative and qualitative composition of the phospholipids present in the membrane-associated ATPases may regulate the extent of the inhibition produced by the antibiotic.

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