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DIFFERENT EFFECTS OF OLIGOMYCIN AND DICYCLOHEXYLCARBODIIMIDE ON ATPases FROM MAMMALIAN CELLS

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SUMMARY

1. The effects of oligomycin and dicyclohexylcarbodiimide on particulate mitochondrial ATPase from rat liver and on several preparations with Na^+,K^+-stimulated ATPase activity were compared.

2. In contrast to oligomycin, dicyclohexylcarbodiimide produced a weak inhibition of Na^+,K^+-stimulated ATPase from rabbit kidney, calf heart and human erythrocyte ghosts. Moreover, it was unable to reproduce the stimulating effect of oligomycin on *p*-nitrophenylphosphatase activity in the presence of limiting amounts of K^+.

3. The Na^+,K^+-stimulated ATPase preparations were found to be more sensitive to oligomycin than generally assumed; the levels for half-maximal inhibition were only 4–40 times higher than those needed for mitochondrial ATPase.

4. Similarly to oligomycin, dicyclohexylcarbodiimide scarcely inhibited particulate mitochondrial ATPase if phospholipids or triolein were included in the incubation medium.

5. It is concluded that oligomycin in a comparable range of concentrations is able to influence several mammalian ATPases associated with membranes, whereas the effect of dicyclohexylcarbodiimide is much more specific for mitochondrial ATPase. Inhibition of other ATPase systems from mammalian cells is produced at concentrations about 1000 times higher. On the basis of the antagonism by phospholipids it is proposed that the effect of both inhibitors is made possible by the presence of phospholipids in these structurally organized ATPase systems.

INTRODUCTION

It is often assumed that oligomycin, introduced by LARDY *et al.*[1], is a specific inhibitor of oxidative phosphorylation. On this ground it is used in experiments with systems containing intact cells in order to study oxidative phosphorylation[2] or to evaluate the contribution of aerobically generated ATP to several energy-requiring processes (spontaneous contraction of cultured heart cells[3], hormone-stimulated lipolysis[4,5], relationship between lipolysis and protein synthesis[6], *etc.*). Although there

Abbreviations: transport ATPase, Na^+, K^+-stimulated ATPase (ATP phosphohydrolase, EC 3.6.1.3); DCCD, *N,N'*-dicyclohexylcarbodiimide.

(1.989 l). On subtracting the volume of the oocyte water (numerically equal to the water content) the volume of 1 kg of dry solids is obtained (0.823 l). Thus the volume percentages of water (compressible volume) and of dry solids (non-compressible volume) are 58.6 and 41.4 %, respectively.

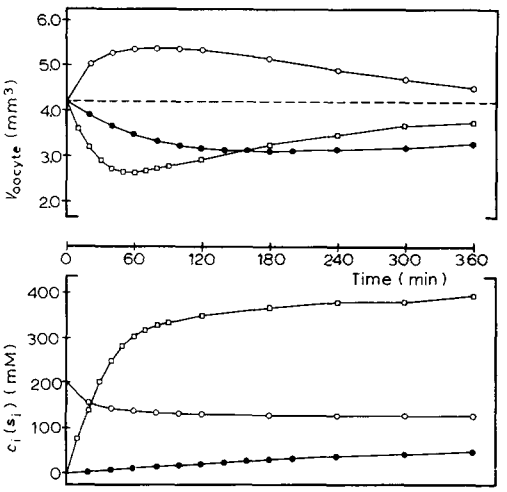

Fig. 4. The time course of the oocyte volume and of the intracellular concentration of the permeating solute calculated on the basis of the mathematical model. ○—○, hypotonic medium, permeating solute KCl (concentration s_i); ●—●, slightly hypertonic medium, permeating solute mannitol (concentration c_i); □—□, strongly hypertonic medium, permeating solute mannitol (concentration c_i).

TABLE III

NUMERICAL PARAMETERS USED IN THE CALCULATION OF THE THEORETICAL CURVES DESCRIBING VOLUME CHANGES

The curves were calculated for a spherical osmometer of the following dimensions: r = 0.1 cm, V_0 (initial) = $4/3 \cdot \pi r^3 = 4.2 \cdot 10^{-3}$ cm^3, $A = 1.3 \cdot 10^{-1}$ cm^2, $A/V = 3/r = 30.0$ cm^{-1}.

Medium	*External concentration of permeating solutes*		*Permeability coefficients*		*Intracellular content of impermeant salts* (*moles* × 10^7)	*Hydrostatic pressure* P_i (*osM* · *cm*$^{-3}$ × 10^4)
	$c_{(mannitol)}$ (*moles* · *cm*$^{-3}$ × 10^4)	$c_{(salts)}$	$k_c(k_s)$ (*sec*$^{-1}$ × 10^4)	k_w (*moles* · *sec* × 10^3)		
Hypertonic (600 mosM)	4.0*, †	—	3.0**	2.5***	8.4	—
Hypertonic (300 mosM)	1.0*, †	—	0.3**	2.5***	8.4	—
Hypotonic (110 mosM)	—	1.06*, †	16.1(i) 0.6(o)	10.0***	8.4	0.4

* $c_{(mannitol)i} = 0$ for $t = 0$; $s_{(salt)o} \approx s_{(K)o} \approx 0$ (Na^+ assumed to be impermeant and $s_{(K)o}$ negligibly low).

** Tentative values.

*** Calculated from initial rates derived from experimental curves.

† Approximated from the experimental figures.

70 % immediately after preparation, and 80–83 % after storage at 4° for a few days.

The Na^+,K^+-stimulated ATPase from calf heart was prepared according to the procedure of MATSUI AND SCHWARTZ[21] with substantial modifications: (a) the enzyme was extracted with sodium deoxycholate for 2 h at 0°. The treatment with deoxycholate was not repeated. (b) The purification with NaI was as follows: 10 min after the addition of the solution containing NaI (which was supplemented with 3 mM dithiothreitol) the suspension was centrifuged for 15 min at 30000 rev./min (Spinco, No. 30 rotor) and the enzyme system, recovered as a floating layer, was immediately resuspended in 0.25 M sucrose, 1 mM dithiothreitol, 1 mM EDTA, 10 mM Tris (pH 7.4), centrifuged (1 h at 30000 rev./min), and washed twice in the same solution. (c) In order to remove residual ouabain-insensitive activity, the final precipitate was resuspended in a solution of 2 mM ATP, 1 mM dithiothreitol, the pH adjusted to 8.0 with few drops of 0.1 M Tris, and subjected to sonic oscillations for 10 min at 0–5°. The suspension was centrifuged for 20 min at 20000 rev./min (Spinco, No. 40 rotor) and the residue discarded. The supernatant was centrifuged for 1 h at 40000 rev./min, yielding a fraction which was resuspended in 0.25 M sucrose, 10 mM Tris, 1 mM EDTA, 1 mM dithiothreitol (pH 7.4), and stored in small aliquots at −35°. The specific activity at 37° in the presence of 100 mM Na^+ and 20 mM K^+ was 15–25 μmoles ATP split per mg protein per h. The inhibition by 0.1 mM ouabain was 95–99 %.

Human erythrocyte ghosts were prepared according to the method of BLOSTEIN[22]. The specific activity at 37° was 0.8–1.4 μmoles ATP split per mg protein per h in the presence of 100 mM Na^+ and 20 mM K^+, and 0.40–0.35 in the absence of these cations.

Analytical procedure

The ATPase activity of submitochondrial particles and transport ATPase preparations was measured at 37° in open tubes with the following incubation medium: 50 mM Tris–HCl (pH 7.4), 2.5 mM $MgCl_2$, 3.0 mM ATP–Tris (pH 7.4), 1 % (v/v) ethanol, 100 mM NaCl, 20 mM KCl. Final vol., 1.0 ml. The incubation time was 20 min for submitochondrial particles and transport ATPase from rabbit kidney and calf heart, 1 h for human erythrocyte ghosts. The amount of enzyme preparation added in the various experiments and minor variations are indicated in the legends to tables and figures.

p-Nitrophenylphosphatase activity of the calf heart transport ATPase preparation was measured at 37° under the conditions described by ROBINSON[23].

The specific activity in the presence of 10 mM KCl was 7–10 μmoles *p*-nitrophenyl phosphate split per mg per h at 37°. The inhibition by 0.1 mM ouabain was 80 %.

Inorganic phosphate was measured according to the method of Fiske and Subbarow, proteins according to the method of LOWRY *et al.*[24], using crystalline bovine serum albumin as standard. Particulate proteins were dissolved with sodium deoxycholate. Phospholipid phosphorus was measured by the method of Fiske and Subbarow after the sample was ashed as described by AMES AND DUBIN[25].

RESULTS

Table I shows the effects of oligomycin and DCCD on mitochondrial ATPase and transport ATPase from various sources using the same experimental conditions.

TABLE I

SENSITIVITY OF ATPases TO OLIGOMYCIN AND DCCD

In all instances 100 mM NaCl, 20 mM KCl, 2.5 mM $MgCl_2$, 3.0 mM ATP–Tris, 50 mM Tris–HCl (pH 7.4), 1 % (v/v) ethanol were present in a final vol. of 1.0 ml. The amount of protein of the various preparations was 25–37 μg for submitochondrial particles, 160–200 μg for calf heart ATPase, 80 μg for rabbit kidney ATPase, 430–1000 μg for human erythrocyte ghosts. In the case of submitochondrial particles and ATPase from calf heart and rabbit kidney the reaction was started by addition of the enzyme preparation; the preparation of erythrocyte ghosts was pre-incubated for 15 min at 37° with the inhibitors, and the reaction was started with ATP. In all cases the reaction was stopped by addition of trichloroacetic acid (final concn., 10 %). Incubation, 20 min at 37°. The values are the means of separate experiments with the range in parentheses. For erythrocyte ghosts the values in the absence of Na^+ and K^+ were subtracted from the total activity.

Preparation	*Amount of inhibitor (nmoles/mg protein) required for 50 % inhibition*			
	Number of experiments	*Oligomycin**	*Number of experiments*	*DCCD***
Rat liver mitochondrial particulate ATPase (submitochondrial particles)	4	0.62 (0.3– 0.9)	3	3.3 (2.7–4.5)
Rabbit kidney transport ATPase	2	23.3 (21.0–25.6)	1	9650
Calf heart transport ATPase	8	11.6 (7.8–13.8)	2	>5800
Human erythrocyte ghosts transport ATPase	4	2.5 (1.2– 3.4)	3	>2000

* Mol. wt. assumed to be 800 (refs. 17, 18).
** Mol. wt. 206.3.

In all instances the preparations were highly sensitive to oligomycin whereas the effect of DCCD was clearly seen on mitochondrial ATPase only. In agreement with previously reported data[11,12], partial inhibition of the transport ATPase was present when DCCD was used at very high concentrations. The effect was slightly but not substantially increased by preincubation for 15 min at 37° in the absence of Na^+.

Transport ATPase preparations were 4–40 times less sensitive to oligomycin than mitochondrial ATPase. This value is lower than others reported previously[26]. On the other hand, the levels of oligomycin for half-maximal inhibition agree with those of JÖBSIS AND VREMAN[27]. In contrast, the sensitivity of mitochondrial ATPase to DCCD was about 1000 times greater than that of transport ATPase preparations.

The amounts of DCCD and oligomycin needed for 50 % inhibition reported here (3.3 and 0.62 nmoles/mg of protein, respectively) are only slightly higher than those reported by BULOS AND RACKER[15] for DCCD and rutamycin (1.6 and 0.29 nmoles/mg of protein, respectively) using 5 min preincubation at 30° of the enzyme preparation (trypsin-treated submitochondrial particles from beef heart mitochondria) in the presence of the inhibitors and a regenerating system during the incubation.

The difference between the effects of oligomycin and DCCD on transport ATPase was confirmed by experiments on *p*-nitrophenylphosphatase activity which is believed to be part of Na^+,K^+-stimulated ATPase[28,29].

It is known[30] that in the presence of limiting amounts of K^+, low concentrations of oligomycin produce a stimulation which is Na^+ dependent and abolished by ouabain.

TABLE II

EFFECT OF OLIGOMYCIN AND DCCD ON *p*-NITROPHENYLPHOSPHATASE ACTIVITY OF TRANSPORT ATPase PREPARATION FROM CALF HEART

Each tube contained in a final vol. of 1.0 ml 50 mM Tris–HCl (pH 7.8), 3 mM $MgCl_2$, 3 mM *p*-nitrophenyl phosphate–Tris, 1 % (v/v) ethanol, 0.1 mM KCl, enzyme preparation, 200 μg of protein. When present, NaCl was added at the concentrations indicated. Incubation, 20 min at 37°.

Additions	*μmoles p-nitrophenyl phosphate split per mg protein per h*	
	Without Na^+	*With 20 mM Na^+*
—	0.77	0.47
100 μM ouabain	0.31	0.30
6 μM oligomycin	0.77	1.15
Oligomycin + ouabain	0.36	0.35
1 μM DCCD	0.77	0.47
10 μM DCCD	0.73	0.43
100 μM DCCD	0.61	0.43
1000 μM DCCD	0.47	0.31

In Table II it is seen that, in contrast to the stimulation produced by oligomycin, DCCD, in a wide range of concentration, was either without effect or produced a slight inhibition at high concentrations.

When tested in the presence of saturating amounts of K^+, oligomycin was essentially devoid of effect, while 30 % inhibition was produced by 1 mM DCCD.

The possibility was considered that the ineffectiveness of DCCD on transport ATPase could be due to a lack of binding to the preparation or to a metabolic transformation. Transport ATPase from calf heart was pretreated with DCCD, centrifuged down and washed. This preparation exhibited normal ATPase activity but, on addition to submitochondrial particles, it inhibited the activity of the latter. Transport ATPase can, therefore, become a carrier of active DCCD which is not irreversibly bound, and can be redistributed in the sensitive preparation.

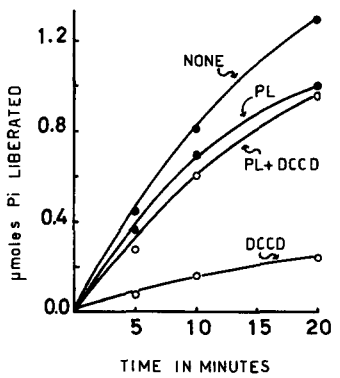

Fig. 1. Antagonism by phospholipids on DCCD-induced inhibition of particulate mitochondrial ATPase. Each tube contained in a final vol. of 1.0 ml: 2.5 mM $MgCl_2$, 3.0 mM ATP–Tris (pH 7.4), 50 mM Tris–HCl (pH 7.4), 50 mM sucrose, 0.2 mM EDTA, 1 % (v/v) ethanol, submitochondrial particles from rat-liver, 37 μg of protein and, where indicated, 1 mg bovine brain phospholipids (PL). DCCD concentration was 0.25 nmole. Temp., 37°. The reaction was started by addition of submitochondrial particles and terminated with trichloroacetic acid (final concn., 10 %).

Considering the similarity between the effect of oligomycin and DCCD it was of interest to investigate whether the antagonism by phospholipids on oligomycin-induced inhibition[31,32] was present also with DCCD. In Fig. 1 it can be seen that at 37° the inhibitory effect of a low concentration of DCCD on mitochondrial ATPase was manifest also when the preincubation procedure usually adopted with this inhibitor was omitted, and that it can be removed by addition of phospholipids. In separate experiments it was observed that the triglyceride triolein can be as effective as phospholipids in the removal of DCCD inhibition.

DISCUSSION

In a comparison between the effects of oligomycin and DCCD on ATPase activities associated with membranes of mammalian cells, a striking difference was found.

An effect of DCCD on mitochondrial ATPase was evident at low concentrations whereas inhibition of other ATPases* occurred only when the inhibitor was used in exceedingly larger amounts (5–10 μmoles/mg protein). Oligomycin, in a comparable range of concentration, was able to influence all the activities under investigation. At present it is not possible to decide whether the oligomycin-induced modifications reflect an identical mechanism of action or are unrelated phenomena. A possibility to be considered is that oligomycin is able to react with and influence some critical points of the arrangement of the membrane (*e.g.* functional protein–phospholipid interaction) which are common to several preparations with ATPase activity and form the basis of the different sequences of reactions.

The antagonism produced on the oligomycin-induced inhibition by phospholipids is consistent with this hypothesis. As suggested previously[31,32], this would indicate the possibility that oligomycin reaches the functional proteins of the membrane through the phospholipid channel, where it can be bound and dissolved.

The fact that also DCCD can be antagonized by phospholipids suggests a more general role of these compounds in the interaction of lipophilic inhibitors with organized biological systems.

In this context, the finding[33] is relevant that oligomycin can inhibit the reconstituted D(−)-β-hydroxybutyrate dehydrogenase, an enzyme which is critically dependent on the interaction between the functional protein and lecithin-containing phospholipids.

Finally, it is clear that the effect of oligomycin in intact cells is not selective and has to be used with caution when testing the role of oxidative phosphorylation in systems containing intact cells. Inhibition of the active extrusion of Na^+ accompanied by a regain of extracellular K^+ is apt to occur since this process is linked to the transport ATPase which is very sensitive to oligomycin and is located in the plasma membrane, i.e. the first barrier oligomycin must come across. The use of DCCD which is devoid of effect on transport ATPase is possibly advantageous to this purpose, provided low concentrations and an appropriate temperature of incubation are used in order to avoid aspecific effects and delay in the onset of inhibition.

* Unpublished experiments by Dr. F. Carpenedo in this laboratory showed that a high concentration of DCCD (5 μmoles/mg protein) produced a partial inhibition also on Ca^{2+}-stimulated ATPase in isolated vesicles from rabbit skeletal muscle sarcoplasmic reticulum.

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