Journal of Bioenergetics and Biomembranes, Vol. 22, No. 1, 1990

The Abnormal-Shaped Mitochondria in Thymus Lymphocytes Treated with Inhibitors of Mitochondrial Energetics

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Received September 15, 1989; accepted September 26, 1989

Abstract

The effects of uncouplers (DNP, FCCP), oligomycin, and rotenone on the energetics and mitochondrial ultrastructure in lymphocytes have been studied. We confirmed the previous observations done on Ehrlich ascites and cardiomyocyte culture cells that uncouplers and respiratory inhibitors cause the appearance of ringlike and dumbbell-like mitochondria. It is shown that this effect does not correlate with decrease in ATP concentration, changes in oxygen consumption, or condensation of the mitochondrial matrix. FCCP (2 μM) is more effective in the induction of abnormal-form mitochondria than 240 μM DNP, oligomycin, or rotenone. Combined treatment with DNP, oligomycin, and rotenone or with DNP and rotenone produces an effect as strong as 2 μm FCCP. DNP (240 μM) and FCCP (2 μM) have a similar effect on respiration and intracellular ATP, but only the latter induces condensation of the mitochondrial matrix.

Key Words: Mitochondrial ultrastructure; ATP; uncouplers; abnormal-form mitochondria; lymphocytes.

Introduction

Changes in the form and size of mitochondria are one of the initial responses of cells to many different effectors (Smith and Ord, 1983). To clarify what features of cell metabolism are reflected in such structural modification, it is necessary to investigate the interrelationships between changes in mitochondrial morphology and metabolism.

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0145-479X/90/0200-0051$06.00/0

Abnormal-form mitochondria resembling rings and dumbbells on ultrasections have been observed in some physiological states, the dumbbell form being associated with dividing mitochondria (Bell and Muhlethaler, 1964; Olson, 1973; Endress and Sjolund, 1976; Osafune *et al.*, 1975. On the other hand, mitochondria in the form of rings and dumbbells have been detected also in cells incubated in the presence of uncouplers and the respiratory inhibitors (Buffa *et al.*, 1967; Laiho and Trump, 1975).

These mitochondria were observed in heart culture myoblasts after 1 h incubation with different uncouplers that exerted 1.5–2 times stimulation of oxygen consumption (Buffa *et al.*, 1967); however, such a long incubation with uncouplers may induce nonspecific changes in cellular metabolism. "Ring-form condensation" of mitochondria was described for Ehrlich ascites tumor cells after incubation with high concentrations of DNP,[4] antimycin A, and monoiodoacetate; under these conditions a significant decrease in ATP concentration was shown (Laiho and Trump, 1975). Thus, it is not yet clear why these particular forms appear—whether they are due to the condensation of matrix to the fall in ATP concentration, etc.

Thymus lymphocytes represent a convenient test object for a parallel study of mitochondrial ultrastructure and energetics in intact cells. The respiration of nonactivated lymphocytes is entirely due to mitochondria. They are well permeable to hydrophobic substances. Mitochondrial energetics inhibitors supress the respiration of lymphocytes and isolated mitochondria at similar concentrations (if one relates the concentration of hydrophobic inhibitors to that of cytochromes) (Cittadini *et al.*, 1975; Gulyaeva *et al.*, 1985). Thymus lymphocytes can be easily obtained without damaging the integrity of cytoplasmic membranes. All this allows one to study the ultrastructure and activity of mitochondria shortly after the addition of inhibitors.

In this paper we compared the effects of uncouplers (DNP and FCCP), rotenone, and oligomycin on the mitochondrial ultrastructure and energetics in the intact lymphocytes.

Methods

Lymphocytes were isolated as described earlier (Gulyaeva *et al.*, 1985). The isolation medium contained 145 mM NaCl, 5.6 mM KCl, 8 mM MOPS and 10 mM pyruvate, or 10 mM glucose, pH 7.4. Respiration of lymphocytes (10^8 cells/ml) was measured at 37°C by a Clark-type oxygen electrode. Corresponding inhibitors were added after 2 min of such incubation.

[4] Abbreviations: DNP, 2,4-dinitrophenol; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; MOPS, morpholinopropane sulfonate.

After another 2 min aliquots were collected for ATP assay and electron microscopy. ATP assay was performed as described by Brand and Felber (1984). For electron microscopy, the cells were fixed with 2.5% glutaraldehyde in incubation medium (pH 7.4) for 1 h. These preparations were postfixed with 1% OsO_4, dehydrated gradually with ethanol, and embedded in Epon-812. Ultrathin sections were cut on an LKB-111 instrument (Sweden) and stained with lead according to Reynolds (Reynolds, 1963). Photographs were obtained on a Hitachi-12 electron microscope.

DNP, oligomycin, MOPS (Serva), FCCP, rotenone (Sigma), and glutaraldehyde (Merk) were used in the experiments. Rotenone, oligomycin, and FCCP were dissolved in twice-distilled ethanol.

Results

Morphologically, the mitochondria in thymus lymphocyte suspension (Fig. 1A) differ from mitochondria of thymus tissue only in that they have a denser matrix. Other characteristics of their ultrastructure are similar: cristae are mutually parallel, and mitocondria on ultrathin sections have an elongated or round form.

DNP (40 μM) or FCCP (0.4 μM), which exerted a maximal (and equal) stimulation on the respiration rate, caused but a weak decrease in the matrix density or none at all (not shown).

At higher concentrations, the uncouplers inhibited oxygen consumption. In the presence of 240 μM DNP or 2 μM FCCP, the respiration rate was approximately a third of the maximal one. Yet the uncouplers at these concentrations affected differently the morphology of mitochondria (Fig. 1B, C).

In the presence of 240 μM DNP, in most of the experiments the matrix became more electron-translucent and, in some cases, some swelling of mitochondria was observed. These results were reproduced when the incubation medium was supplemented with 2 mM NaH_2PO_4, 0.4 mM $CaCl_2$, and 0.4 mM $MgCl_2$. FCCP (2 μM) produced little increase in the density of the matrix. Both uncouplers caused local expansions of the intermembrane space.

We conducted a series of experiments to determine whether the DNP-induced mitochondrial swelling would change with a further lowering of $\Delta\bar{\mu}H$. To suppress $\Delta\bar{\mu}H$ generation by the respiratory chain, rotenone was used; and to prevent $\Delta\bar{\mu}H$ generation at the expense of hydrolysis of the glycolytic ATP, oligomycin was added. Both inhibitors were taken at concentrations exerting a maximum inhibitory effect on lymphocyte respiration.

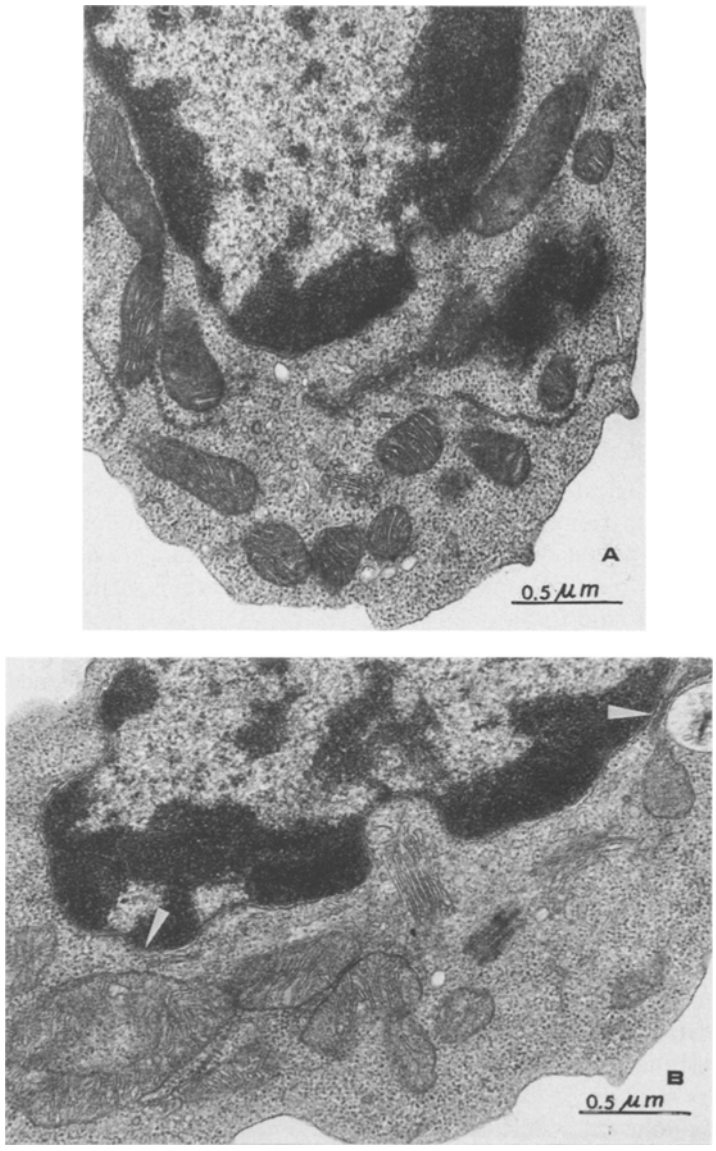

Fig. 1. Effect of mitochondrial energetics inhibitors on mitochondrial ultrastructure in lymphocytes. Incubation medium contained 145 mM NaCl, 5.5 mM KCl, 8 mM MOPS, 10 mM pyruvate, pH 7.4, 37°C. Cells were incubated for 2 min in a polarographic cell; then 240 μM DNP (B), 2 μM FCCP (C), oligomycin (0.2 μg/ml), 1 μM rotenone, and 240 μM DNP (D), and 10 μM FCCP (E) were added. After 2 min incubation with these inhibitors, cells were fixed with glutaraldehyde. Arrows indicate ultrasections of ring-shaped and dumbbell-shaped mitochondria. (A) Without addition of inhibitors. For details, see Methods.

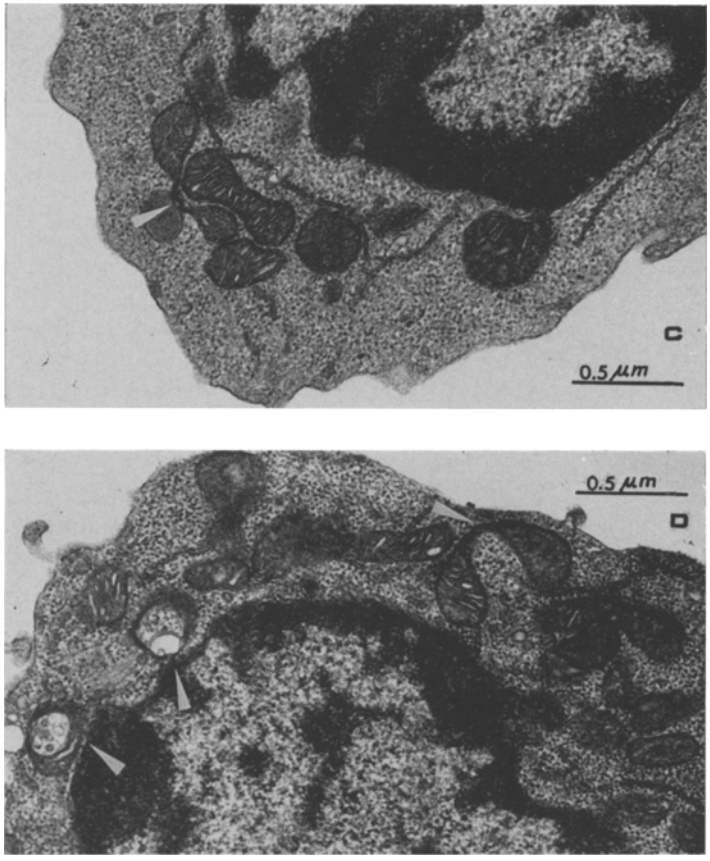

Fig. 1. Continued.

Rotenone (1 μM) has little or no effect on the mitochondrial ultrastructure. Oligomycin (0.2 μg/ml) caused mitochondria to swell; the density of the matrix was lower than that in control cells. Mitochondria remained swollen when acted upon both by oligomycin and by rotenone. The 240 μM DNP-induced decrease in mitochondrial matrix density persisted in the presence of oligomycin (Table I). The addition of oligomycin, rotenone, and 240 μM DNP abolished the mitochondrial swelling and even induced a condensation of the matrix (Fig. 1D). The effect of these inhibitors was similar to that of 2 μM FCCP.

Earlier we showed (Markova *et al.*, 1989) that in isolated rat liver mitochondria, incubated in a medium containing 120 mM KCl and 10 mM phosphate in the absence of Ca^{2+} binding agents, 240 μM DNP induced a

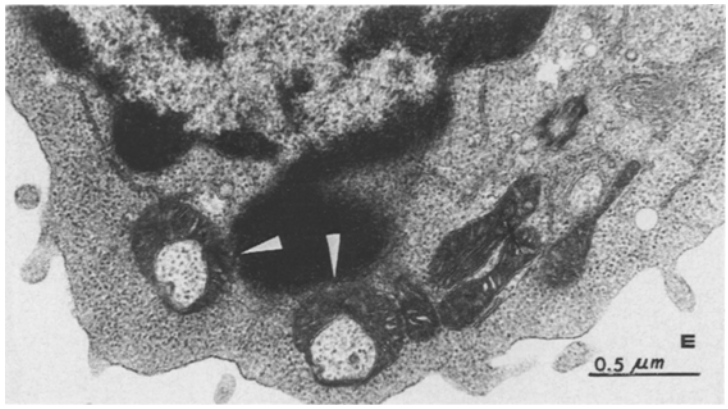

Fig. 1. Continued.

stronger swelling than 2 μM FCCP, even though both uncouplers had the same effect on the rate of mitochondrial respiration. These data show that the different effects of DNP and FCCP are due not to peculiarities of their interaction with cells but to the difference in their action on the morphology of mitochondria.

A 2-min incubation of lymphocytes in the presence of 240 μM DNP and 2 μM FCCP resulted not only in a change in density of the mitochondrial matrix but also in abnormal mitochondrial forms which, on ultrathin sections, resembled rings and dumbbells in shape (Fig. 1). To assay this

Table I. Effect of Mitochondrial Energetics Inhibitors on Ultrastructure of Mitochondria in Lymphocytes[a]

Inhibitors	Changes in matrix density	*N*
Control	—	0
Oligomycin	Decrease	0
Rotenone	No change	1–2
DNP	Small decrease	
Oligomycin and DNP	Small decrease	5–12
Oligomycin and rotenone	Small decrease	
Rotenone and DNP	No change	
Oligomycin, rotenone, and DNP	Small increase	17–25
FCCP, 2 μM	Small increase	
FCCP, 10 μM	Strong increase	40–50

[a]The number of abnormal-form mitochondria per 200 cell ultrasections (*N*) was counted. The same count was taken three times, and similar results were obtained. Additions: 1 μM rotenone, 0.2 μg/ml oligomycin, and 240 μM DNP. Incubation medium was as in Fig. 1. For other conditions see Methods.

effect, we counted the number of abnormal mitochondrial profiles on 200 cell ultrasections (Table I).

Incubation of lymphocytes without mitochondrial energetics inhibitors or in the presence of oligomycin alone did not produce abnormal-form mitochondria in any of the preparations examined. Most frequently, such mitochondria occurred when lymphocytes were incubated in the presence of FCCP or when they were treated by oligomycin (0.2 μg/ml), 1 μM rotenone and 240 μM DNP, or by 240 μM DNP in the presence of 1 μM rotenone. The intermediate effect was observed in samples with 240 μM DNP, with oligomycin and rotenone, or with 240 μM DNP and oligomycin. The effect of 10 μM FCCP proved to be stronger than that of 2 μM FCCP (Table I, Fig. 1E).

All these inhibitors accounted for a significant drop in the intracellular concentration of ATP. However, frequency of occurrence of abnormal-form mitochondria as hardly a result of the fall in concentration of intracellular ATP. Indeed, the ATP concentration falls in about the same measure under the action of 240 μM DNP or 2.4 μM FCCP (from 403 + 12 in the control to 129 + 5 and 125 + 5 pmol ATP/10^6 cells, respectively), and under the action of oligomycin or oligomycin with 240 μM DNP (to 187 + 8 and 193 + 12 pmol ATP/10^6 cells, respectively). On the other hand, ultrathin sections of lymphocytes incubated in the presence of oligomycin did not show ring-shaped and dumbbell-shaped mitochondria, which occurred however, in the presence of oligomycin with DNP; the effect of 2 μM FCCP was stronger than that of 240 μM DNP (Table I).

As one can see in Table I, the occurrence of abnormal-form mitochondria is sometimes accompanied by condensation of the mitochondrial matrix. In other cases such mitochondria appear when there is no condensation of the matrix or even when the matrix becomes more electron-translucent.

Discussion

Our data on the condensation of the mitochondrial matrix in lymphocytes incubated with 2–10 μM FCCP and the swelling of mitochondria under the action of oligomycin concurs with the results described for other cells (Smith and Ord, 1983; Laiho and Trump, 1975; Leikina *et al.*, 1982; Rydzynsky and Cieciura, 1980). These effects are explained by $\Delta\psi_m$-dependent ion fluxes.

However, in the experiments with 240 μM DNP we observed a decrease of the matrix density (Fig. 1) while the $\Delta\psi_m$ decreased (Gulyaeva *et al.*, 1985; Mokhova and Rozovskaya, 1986. In other cells uncouplers could induce a swelling of mitochondria as well if much longer incubation times were used (Smith and Ord, 1983).

Uncoupler-induced mitochondrial swelling (Lehninger, 1962; Hackenbrock, 1968) is a very complicated cascade of events. Such swelling may be connected with a decrease in the rigidity of mitochondrial membranes (Skulachev, 1972) and/or with an increase in the concentration of osmotic-active substances with high molecular mass. The swelling is Ca^{2+}-dependent and there is evidence that lysophospholipids and mitochondrial deenergization potentiate this process (Igbavboa and Pfeiffer, 1988). In this context it is important that lymphocytes from rat thymus have especially high activity of phospholipase A_2 (Jacqueline and Jacques, 1984; Goppelt-Struebe *et al.*, 1986).

Three-dimensional reconstruction of mitochondria from serial ultrasections showed that ring-shaped and dumbbell-shaped mitochondrial profiles result from the local convergence of the membrane, owing to the cuplike invagination of its surface (Konoshenko *et al.*, 1986). However, the appearance of abnormal-shaped mitochondria is hardly a result of matrix condensation: DNP or oligomycin in the presence of rotenone caused no condensation of the matrix, yet they induced the abnormal-shaped mitochondria (Table I).

It is noteworthy that the occurrence of the abnormal-shaped mitochondria is increased when 240 μM DNP-containing medium is supplemented with rotenone or with rotenone and oligomycin (Table I). This is in agreement with the assumption that a decrease in $\Delta\mu H$ is essential.

Thus we have confirmed the observations of Buffa *et al.* (1967) and Laiho and Trump (1975) that uncouplers and respiratory inhibitors may induce ringlike and dumbbell-like mitochondria (in ultrasections). Our data suggest that the occurrence of the abnormal-shaped mitochondria in cells cannot be explained by a decrease in intracellular ATP concentration or by condensation of the mitochondrial matrix and that a lowering of $\Delta\tilde{\mu}H$ might be involved in this process.

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