Diazepam inhibits cell respiration and induces fragmentation of mitochondrial reticulum

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Diazepam (70–150 μg/ml) significantly inhibits oxygen consumption by pig kidney embryo cells and causes the cellular ATP level to fall. The maximum inhibitory effect develops after 1.5–2.5 h of diazepam treatment. In isolated mitochondria diazepam inhibits respiration in state 2 and 3_u with glutamate and in state 3_u with succinate. Ethylrhodamine staining and electron microscopic study reveal fragmentation of mitochondria in living cells.

Diazepam *Mitochondrial reticulum* *Cell culture* *Respiration* *Membrane potential* *Fluorescent probe*

1. INTRODUCTION

Recently diazepam, a drug commonly used for the treatment of anxiety, was shown to inhibit cell proliferation in tissue culture [1,2]. At the same time because of its chemical structure diazepam should accumulate preferently in lipid-containing parts of the cell. This allows us to speculate that mitochondria are involved in the response to the drug.

We chose three main indexes, allowing us to judge the state of a cellular bioenergetic system. These are: the cellular respiration rate, the intracellular ATP level, and the presence of a proton-motive force on the mitochondrial membrane.

2. MATERIALS AND METHODS

PE cells (pig kidney embryo cells) were grown on coverslips in culture medium 199 supplemented with 10% bovine serum. For measurement of respiration, slips were inserted in a polarographic cell (1.2 ml) [3] with culture medium (3×10^5 cells, pH 7.5). The respiration was measured at 37°C.

For the visualization of mitochondria, slips with cells were incubated with ethylrhodamine (used instead methylrhodamine, rhodamine 123 [4], gift of Dr V. Runov) (10 μg/ml) for 5 min. Coverslips were then rinsed with a rhodamine-free medium and mounted in culture medium in an observation chamber. Stained cells were examined by epifluorescent illumination at 485 nm on an OPTON photomicroscope III equipped with Planapochromate x 63. Photographs were made by using fluorographic film (RF-3) (ASA 1600).

Electron microscopy was done as in [5].

Mitochondria were prepared from rat hind-limb skeletal muscle following closely the routine procedure in [6]. Particles were obtained by sonication of beef heart mitochondria isolated as in [7]. Each sample contained 1 ml medium (120 mM KCl, 5 mM Mops, 5 mM KH_2PO_4 (pH 7.4), $t = 37$°C). Protein was measured as in [8]. Diazepam was used as an alcohol solution of pure diazepam from Gideon Richter, Hungary.

ATP content of PE cells was assayed by the luciferin-luciferase method [9]. ATPase activity was measured by the potentiometric method [10].

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3. RESULTS AND DISCUSSION

Oxygen consumption by PE cells after diazepam addition decreases with time (fig.1a), reaching the minimum rate (close to zero) after 1.5–2.5 h of drug treatment. Longer incubation entails a certain rise in the respiration rate. The addition of an uncoupler (400–600 μM 2,4-dinitrophenol (DNP)) at the point of maximal inhibition greatly activates respiration, for which the same value as observed with DNP alone is registered.

To determine the sites of diazepam action in the respiratory chain, similiar experiments were conducted with isolated mitochondria from skeletal muscle incubated with glutamate as substrate. After diazepam addition (fig.1b) the respiration rate sharply rises and this is gradually followed by respiratory inhibition to a level which is much lower than the initial respiration rate (state 2 [11]). (Much faster and stronger respiratory inhibition by diazepam has been achieved in state 3_u of mitochondria.) At the same time, the subsequent addition of succinate greatly activates the respiration.

In state 2 with succinate as substrate, the respiration of isolated mitochondria is greatly stimulated by diazepam, the respiration rate being close to linear until the oxygen from the incubation media has been exhausted (10–12 min in our experiments). As in the case of glutamate, significant respiratory inhibition takes place in state 3_u and 3. Rather similiar relationships were found in experiments with inside-out sonic submitochondrial particles (fig.1c).

It was also shown that diazepam blocks ATPase activity in isolated muscle mitochondria. 140 μg/ml diazepam proved to inhibit ATPase activity up to 60%.

We cannot give an appropriate explanation of the fact that after diazepam treatment DNP stimulates cellular respiration, taking into account the data obtained with isolated mitochondria. Strong inhibition of NADH oxidation in submitochondrial particles gives reason to suppose that inhibition of mitochondrial respiration cannot be explained in terms of inhibition of substrate transport or particular reactions in the Krebs cycle.

In the PE cell suspension, measurement of the ATP level revealed a decrease from 3.7×10^{-15} to 2.7×10^{-15} mol ATP per cell after diazepam treatment (150 μg/ml, 2 h).

Experiments on the energy-dependent response of the fluorescent probe, ethylrhodamine, show that after 2–20 h incubation of PE cells with 150 μg/ml diazepam cells are able to accumulate ethylrhodamine inside mitochondria. This indicates preservation of the membrane potential on the mitochondrial membrane, but it is impossible to evaluate the magnitude of this potential.

The preservation of mitochondrial staining after diazepam treatment allows us to detect the disrup-

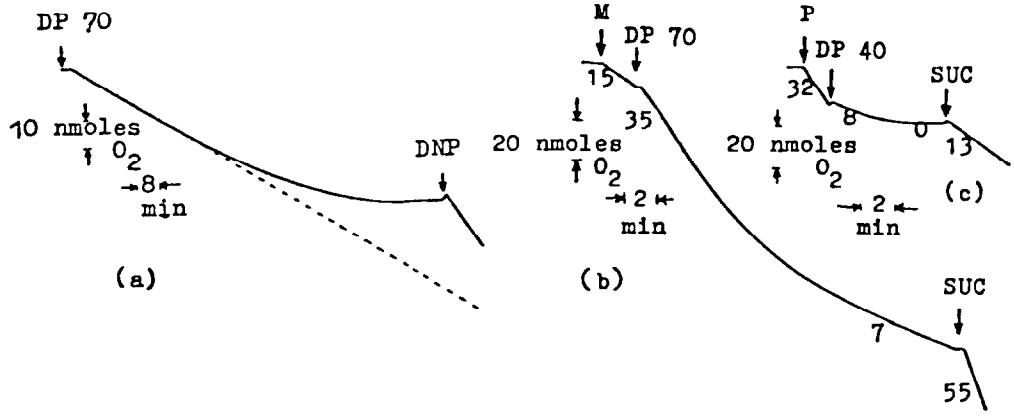

Fig.1. Respiration of pig kidney embryo cells (a), isolated muscle mitochondria (b) and submitochondrial particles (c). DNP (0.3 mM) was added as shown. (Dashed line) Respiration without additions. (DP) Addition of diazepam; figures nearby concentration of diazepam in μg/ml; SUC addition of succinate (2 mM); P addition of submitochondrial particles (0.6 mg protein) from beef heart; (M) addition of mitochondria (0.7 mg protein). The figures below the curves indicate the respiration rates in relative units.

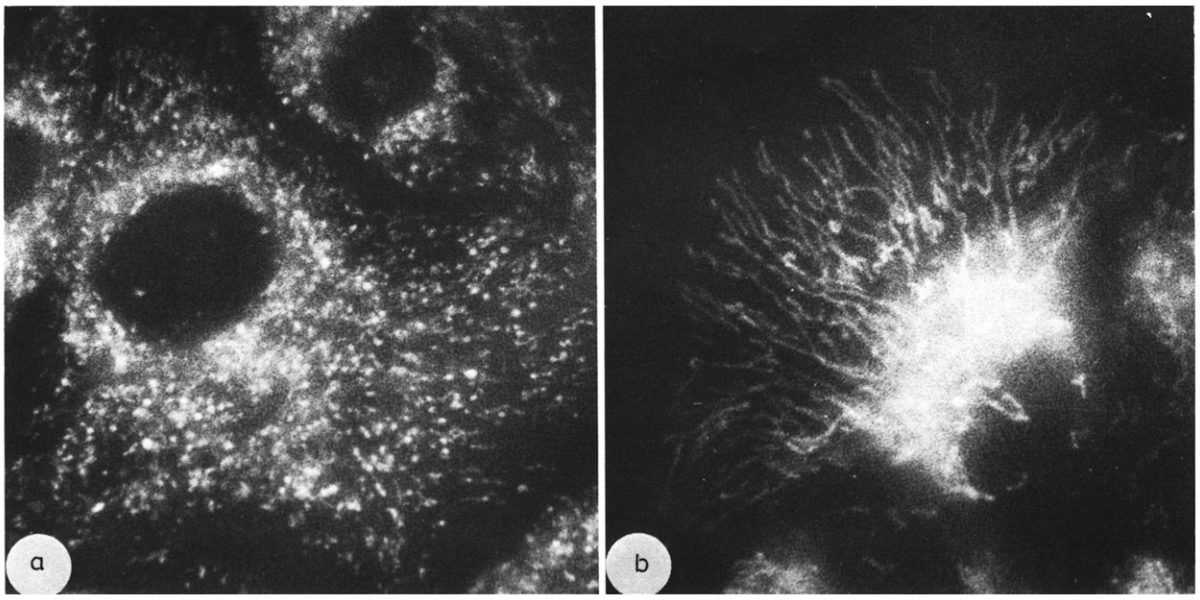

Fig.2. Energy-dependent accumulation of ethylrhodamine in PE cells: (a) cells after diazepam treatment (140 μg/ml, 16 h), (b) control cells ($\times$1530).

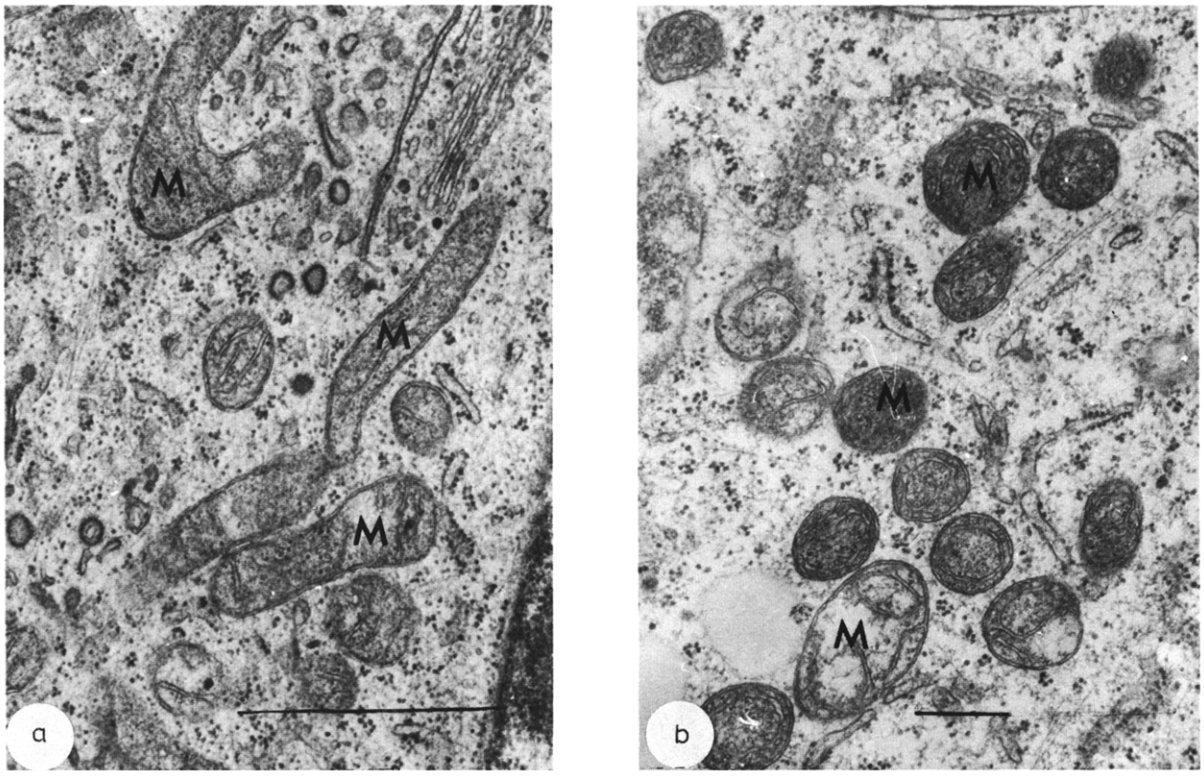

Fig.3. Electron micrographs of ultrathin sections of PE cells: (a) control, (b) after diazepam treatment (150 μg/ml, 20 h). M mitochondria. Bar = 1 μm.

tion of reticulum mitochondriale [12] in the living cells (fig.2a,b). The mitochondria were transformed from elongated, sometimes branched structures into round-shaped vesicles. Although light microscopy does not allow us to evaluate the number of mitochondria in the cell, qualitatively it is obvious that the number of sphere-like mitochondria in the treated cell is much greater than that of normal elongated mitochondria within an intact cell. This result is supported by an electron microscopic study. On ultrathin sections of PE cells the elongated and branched profiles disappear and are replaced by round, slightly oval forms (fig.3a,b).

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