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Electric field-induced fusion of mitochondria

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Fusion of mitochondria in H-medium from rat liver was induced by the application of square-wave voltage with electric field strengths of 1–2.5 kV/cm and duration 100 μs. Electron micrographs showed that the newly fused mitochondria could contain up to five mitoplasts. The fusion yield was close to 12% and respiratory activity was enhanced. The electric field lines did not go through the inner membrane, however, when the electric field strength was greater than 3 kV/cm they did so, resulting in total destruction of the mitochondria.

Electron microscopy; Electrofusion; Respiratory activity; Mitochondria; (Rat liver)

1. INTRODUCTION

Chemical-, virus- or electric field-induced fusion of living cells [1–4] has become a valuable technique in studies of biochemistry, genetic engineering and molecular biology. Until now, except for a paper by Sowers [5] on the fusion of mitochondrial inner membranes and a brief, limited attempt in our laboratory [6], a general study concerning the electric field-induced fusion of mitochondria has been lacking. In particular, attempts to fuse cDNA-loaded vesicles with mitochondria failed to introduce cDNA into the matrix.

Here, we describe an initial investigation undertaken with the goal of devising a method permitting us to introduce, at will, genetic modifications in mitochondria by electrofusion between mitochondria of different origins and then to reintroduce these newly formed mitochondria into rat hepatocytes according to a procedure developed by Mayer et al. [7].

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2. MATERIALS AND METHODS

2.1. *Preparation of rat liver mitochondria*

Liver mitochondria from adult Wistar rats were prepared according to the routine isolation reported in [8] and then resuspended in H-medium [0.07 M sucrose, 0.21 M D-mannitol, 0.002 M Hepes buffer (pH 7.4), and bovine serum albumin (BSA) at 0.5 mg/ml]. All reagents were analytical grade.

Protein concentrations were determined by the Biuret procedure [9] in the presence of sodium cholate and crystalline BSA (from Sigma) was used as a standard.

2.2. *Electrofusion device*

Rectangular pulses of 100 μs duration were fed to two glassy carbon electrodes by means of a Cober 606 P pulse generator. Upon delivery of every pulse both the voltage and current waveforms were recorded on a Nicolet storage oscilloscope.

The electrofusion chamber was designed in such a way that it could be introduced into a centrifuge rotor and thus permit mitochondrial samples to be spun down in situ before experiments and result in the formation of a cylindrical pellet.

The main features of the electrofusion chamber are represented in fig.1. These include the plexiglas core (1) (10 cm length, 1.6 cm external diameter) and the cylindrical electrofusion chamber (3) (0.3 cm diameter) which is surmounted by two glassy carbon electrodes (2) connected to the pulse generator.

Before experiments, an aliquot of the mitochondrial stock suspension in H-medium was pipetted into the fusion chamber and spun down for 5 min at 10000 rpm. The upper electrode, which is free to move vertically inside the electrofusion

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chamber, was then immersed in it in order to make tight contact with the mitochondrial pellet. The distance between the two electrodes was then carefully measured using a cathetometer.

Unless otherwise stated five successive pulses (from 0.5 to 2.8 kV/cm) were fed to the two electrodes with a delay of 5 s to avoid heat accumulation by the Joule effect; in this way the computed rise in temperature within the electrofusion chamber did not exceed 2 degrees.

2.3. *Electron microscopy*

Pulsed mitochondria were incubated at 37°C for at least 1 h to complete membrane resealing and reorganization. This was followed by the procedure in [10]. Thin sections, cut using an LKB ultramicrotome III, were mounted on copper grids and examined with a Siemens Elmiskop 102 electron microscope.

2.4. *Determination of respiratory activity*

Oxidation rates were measured polarographically at 37°C, employing a Clark-type oxygen electrode. Polarographic determination was performed on all mitochondrial samples (in state 4) [11] before and after application of electric field pulses. For each value of external field applied, the percentage of the respiratory recovery of mitochondrial samples was estimated from the ratio of the oxidation rate after application of the external electric field vs that in the absence of an external electric field.

3. RESULTS

When square-wave voltage pulses (<3 kV/cm) were applied to a mitochondrial pellet in H-medium through the electrodes, this resulted in the formation of a certain number of larger mitochondria that have fused with each other as observed by electron microscopy.

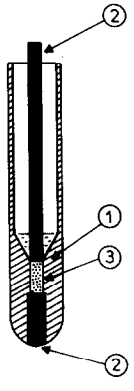


Fig.1. Electrofusion chamber. The core (1) is composed of plexiglas (10 cm long, 1.6 cm external diameter). An aliquot of mitochondrial stock suspension in H-medium was spun down at 10000 rpm in situ for 5 min. The resulting pellet (3) was placed in between two glassy carbon electrodes (2), and different voltage pulses were applied (5 × 100 μs).

In fig.2, different electron micrographs of fused mitochondria are displayed in which one can observe the presence of one to five successive mitoplasts (MT). In fig.2a, a mitochondrion with 2 mitoplasts is shown which is fusing with another mitochondrion. In fig.2a–f, each mitoplast inside a mitochondrion is delimited by a single inner membrane (im) constituting two by two a reticulum (rt) which divides the organelle. At the juncture of mitoplasts with the outer membrane (om) we note the presence of a rather large intermembrane space (ims) with an excess of outer membrane (om) resulting from the fusion of two or several mitochondria.

After fusion has occurred, if fused mitochondria are allowed to stand at 37°C in H-medium for more than 1 h, we can observe the slow intermingling of mitoplast inner membranes (igm) leading to the disappearance of the reticula and formation of a single large mitochondrion (fig.2c,f). This phenomenon is taking place as indicated by the arrow in fig.2c.

The fusion can be followed either by the increase in mean size of the longest mitochondrion diameter after pulsation as shown in fig.3a where the mean size ϕ is plotted vs the number of mitoplasts inside fused mitochondria, or by the index of fusion as given by

$$I = \frac{C(n)}{\sum_{n=1}^{\infty} nC(n)}$$

where $C(n)$ represents the number of fused mitochondria with n mitoplasts (100 mitochondria were counted from electron micrographs). I is a function of applied field strength as shown in fig.3b. It increased for values greater than 1 kV/cm, however when the value of the field strength became too high (>3 kV/cm), mitochondria were completely destroyed. This corresponded to a dramatic increase in current as shown in fig.7. The curves displayed in fig.3b are strongly reminiscent of those obtained for the electrofusion of hepatocytes [12], with two similar characteristics: sigmoidal shape of the threshold for induction of electrofusion and basal level of spontaneous fusion (lack of external electric field) which was close to 3% in our preparation.

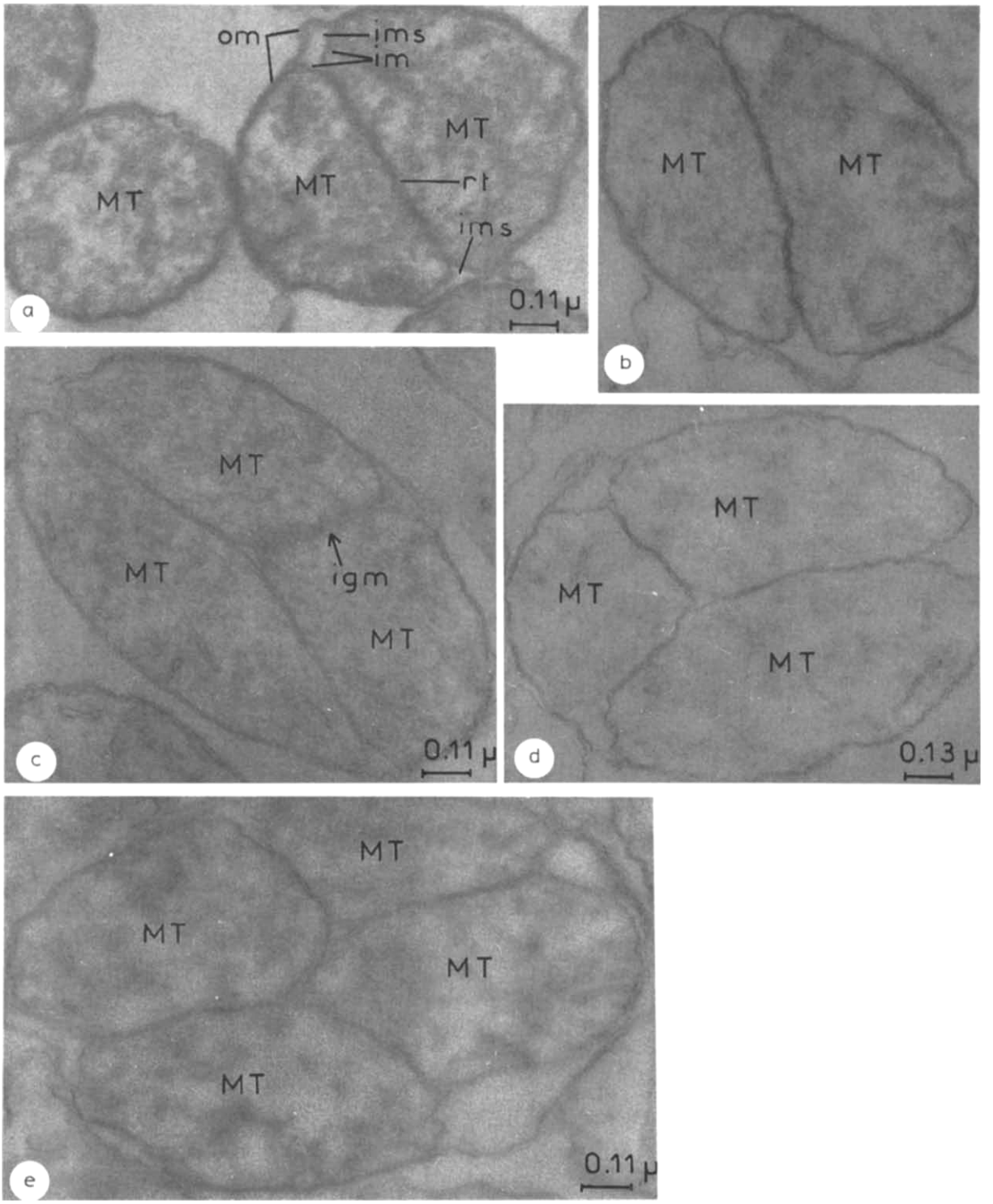
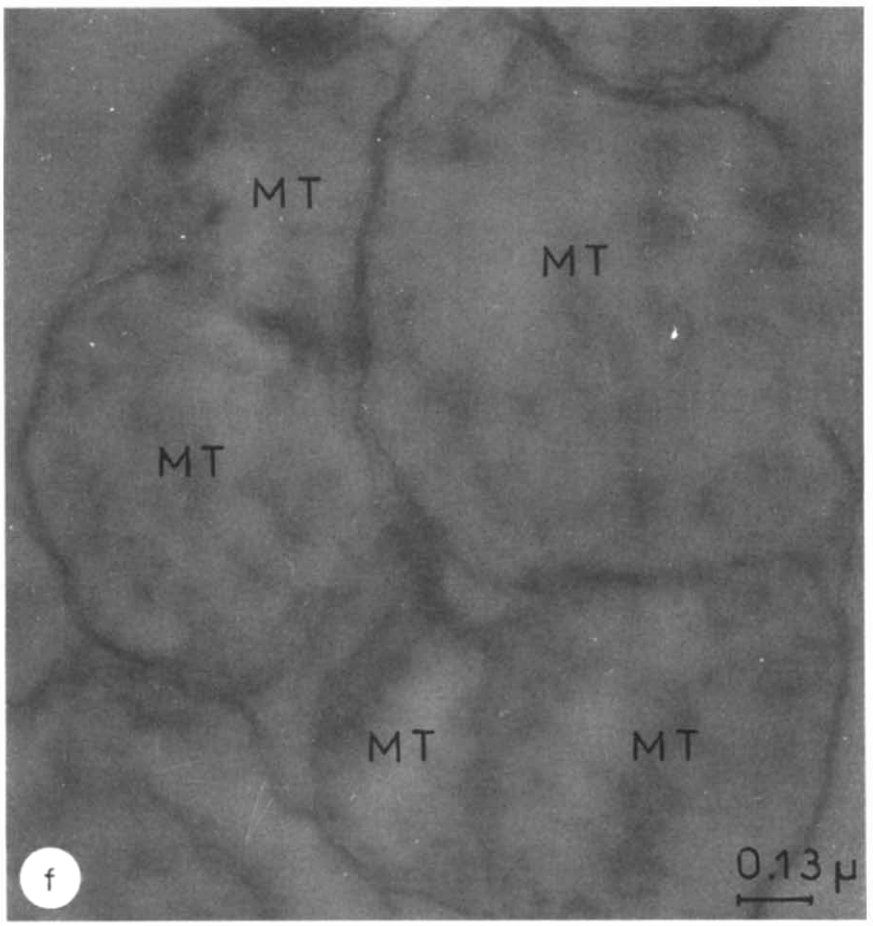
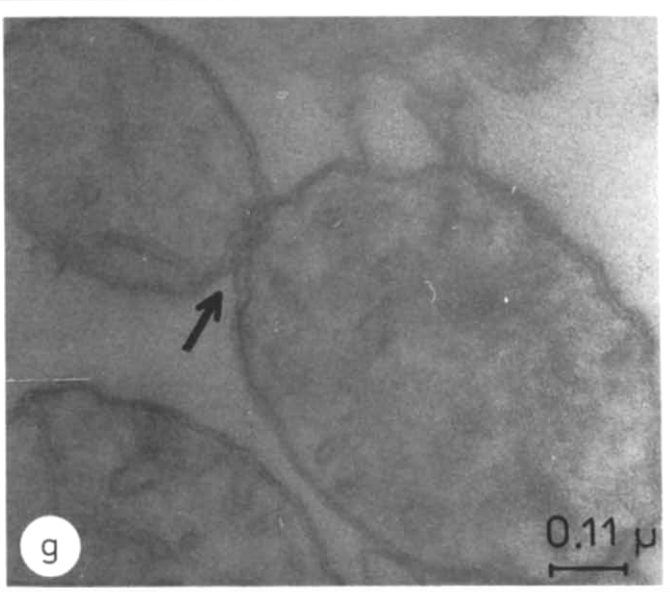


Fig.2. Electron micrographs. Electrofusion of mitochondria in H-medium (pH 5.4); 5 × 100 μs. Before electron microscopy, and after the mitochondrial pellet had been subjected to an external electric field, mitochondria were allowed to stand at 37°C for 1 h for membrane resealing and reorganization. MT, mitoplast; rt, reticulum; om, outer membrane; ims, intermembrane space; im, inner





membrane; igm, intermingling inner membrane. (a) A mitochondrion with 2 mitoplasts undergoing fusion; (b) 2 MT, 1.6 kV/cm; (c,d) 3 MT, 2.5 kV/cm; (e) 4 MT, 2.5 kV/cm; (f) 5 MT, 2.5 kV/cm; (g) electrofusion of two outer membranes indicated by the arrow.

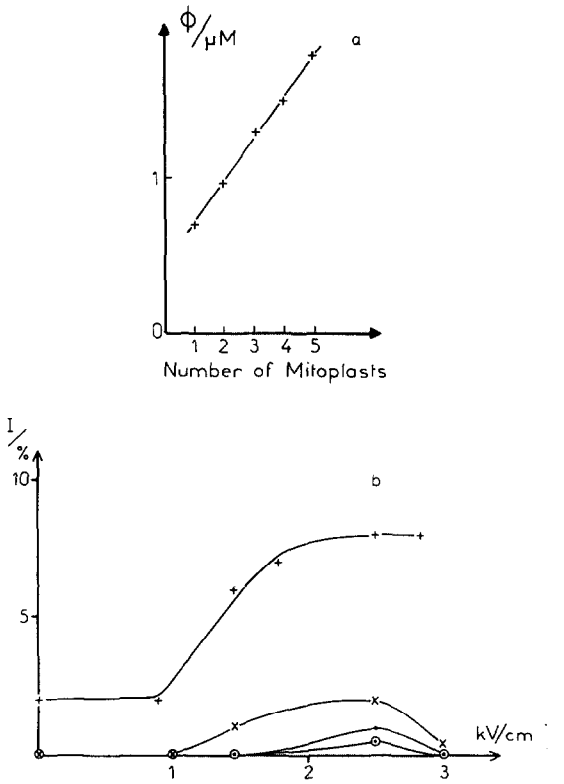


Fig.3. Electrofusion yield. (a) Mean longest diameter of mitochondria plotted vs number of mitoplasts; (b) fusion index (I) plotted vs electric field strength (100 mitochondria were counted):

$$I = \frac{C(n)}{\sum_{n=1}^{\infty} nC(n)}$$

$C(n)$, number of fused mitochondria with n mitoplasts (MT), (+) 2 MT, (×) 3 MT, (•) 4 MT, (⊙) 5 MT.

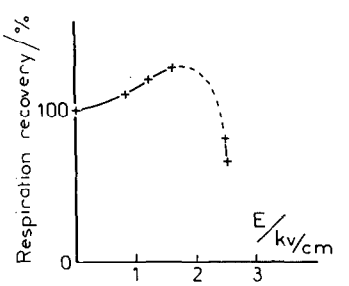


Fig.4. Dependence of respiratory recovery on electric field strength. Mitochondrial suspension in H-medium (pH 7.4) of 20 mg/ml protein sample (50 μl).

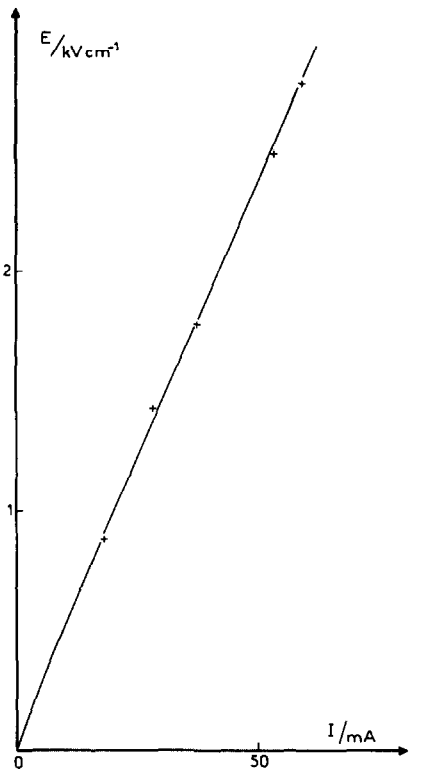


Fig.5. Electric field strength dependence for stationary D.C. current pulse through a mitochondrial pellet (electric field strength < 3 kV/cm).

3.1. *Respiratory activity of pulsed mitochondria*

In fig.4 we have plotted the percentage of respiratory recovery (as defined in section 2) vs electric field strength. The respiratory activity attains a maximum near 2 kV/cm. For electric fields below 2 kV/cm, respiration increases, indicating that not only was the inner membrane undamaged by the electric field but also that the respiratory activity was enhanced. This result is in agreement with those of Hamamoto et al. [13] concerning ATP synthesis driven by an external electric field in rat liver mitochondria and in submitochondrial particles and with the data of Teissié et al. [14]. Above 2 kV/cm, respiratory recovery falls dramatically, this electric field strength corresponding to a maximum induced transmembrane potential close to 100 mV. Such a potential, $\Delta\psi$, is a function of the applied field E, the radius R of the mitochondria, and the position of the point of interest on the membrane surface defined by the

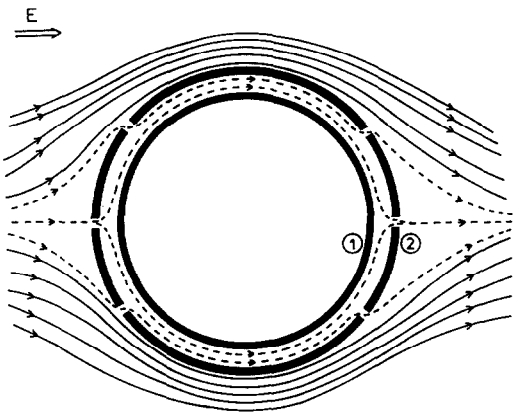


Fig.6. Distribution of electric field lines around and inside a mitochondrion (dielectric double-shell model): (1) inner membrane; (2) outer membrane. (Continuous lines) External electric field lines; (broken lines) electric field lines in intermembrane space. Electric field lines do not go through the mitochondria and current is mainly driven along the external electric field lines for $E < 3$ kV/cm.

angle θ, between the electric field lines and the radius to the point. $\Delta\psi$ is related to these parameters by the well-known relationship [15]:

$$\Delta\psi = 1.5 \times ER\cos\theta$$

When the electric field strength exceeds 3 kV/cm, the structure of the mitochondria is completely destroyed.

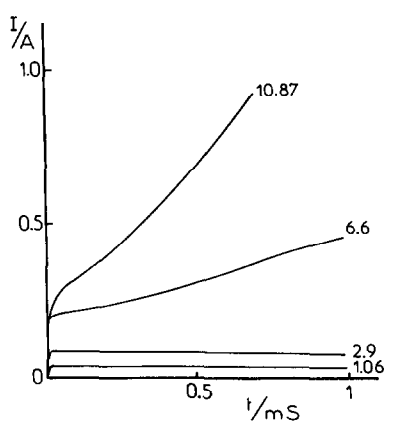


Fig.7. Current dependence on time for different values of electric field (in kV/cm) indicated on each curve. When the electric field strength is greater than 3 kV/cm, the current rises very rapidly with time, corresponding to the total destruction of mitochondria.

4. DISCUSSION

The experiments described above clearly demonstrate the possibility of fusing mitochondria in H-medium by application of an externally transient electric field. This event can occur only if mitochondria are in the form of a pellet so that tight contact can be established between the outer membranes (mitochondria in suspension under similar electric conditions do not fuse). Electron micrographs show clearly that only outer membranes fuse, as indicated by the arrow in fig.2g, this being corroborated by examination of the dependence of the current on electric field strength (fig.5). In fact, the linear dependence of current on the electric field strongly suggests that the electric field lines are very slightly disturbed by voltage pulses of increasing strength (i.e. the conductivity of the pellet remains constant). Current plotted vs electric field strength corresponds to a stationary value of each electric field of less than 3 kV/cm as determined from the time dependence of the current shown in fig.7.

Taking these observations into account, if we assume like most workers that electroporation at sites of membrane contact is responsible for electrofusion [4], only outer membranes are rendered electropermeable, and only a few electric field lines can traverse the intermembrane space as shown in fig.6, in which a mitochondrion is represented by a double-shelled dielectric model [16]. Such behaviour might explain why the conductivity of the mitochondrial pellet remains unchanged irrespective of the electric field strength applied (provided that it remains below 3 kV/cm), since electroporation of the outer membranes gives rise to very few supplementary electric field lines.

When the electric field strength rose above 3 kV/cm, the inner membranes became electropermeable leading to a very rapid rise in current as a function of time (fig.7) and a strong fall in respiratory activity (fig.4). This behaviour probably reflects pore enlargement, resulting in the total destruction of mitochondria.

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