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FUSION OF MITOCHONDRIAL INNER MEMBRANES BY ELECTRIC FIELDS PRODUCES INSIDE-OUT VESICLES

VISUALIZATION BY FREEZE-FRACTURE ELECTRON MICROSCOPY

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The fusion of vesicular-shaped mitochondrial inner membranes was observed by a new approach which combines freeze-fracture electron microscopy and electric field-induced fusion. Results show that membrane events caused by the exposure to the electric field can be time-coordinated with sample freezing for subsequent analysis by freeze-fracture electron microscopy.

Electric field-induced fusion has been reported in many biomembrane systems (for extensive reviews, see Refs. 1–5). The apparatus needed is simple and inexpensive. The procedure yields results which can be immediately monitored by conventional light microscopy. This method of fusion has three additional unique benefits of significant scientific value. First, fusion can be induced without the addition of any exogenous chemicals. Second, fusion can be made to take place simultaneously in membranes in contact with one another. Third, the fact that fusion can be made to take place simultaneously by means of an electrical signal makes possible the coordination of the moment of fusion with the moment of change in another experimental variable.

One procedure by which membranes or cells in suspension can be fused by electric fields is as follows: the suspension is exposed to a relatively weak alternating current field for a period of time sufficient to cause the membranes to become organized into strings or chains of membranes in contact with one another. The number of membrane elements in each one-dimensional array or chain is a function of both the random starting positions and the initial concentration of the membranes. After the membranes become organized into these arrays, a single, relatively high strength direct current pulse is applied which presumably causes membrane pores to develop in the vicinity of membrane-membrane contact [6]. As a result of Brownian motion, the free edges of the pores in different membranes come into contact with one another before the pore closes. Previously separate membrane entities thus become part of a larger membrane continuum.

The significance of the combined use of freeze-fracture electron microscopy and of electronic circuits to coordinate both the application of the pre-freezing electric current waveform and the moment of freezing is that it permits the examination

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Abbreviations: D, diffusion coefficient; d, half distance between electrodes; M, mitochondrial inner membrane spheres; MFP, main fusion product; R.M.S., root-mean-square; S.D., standard deviation; SFP, small vesicular membrane fusion product; t, time.

of rapid changes in membrane geometry with great resolution in time and space.

This preliminary report shows that electric field-induced fusion of spherical-shaped mitochondrial inner membranes in a suspension can be precisely coordinated with quick-freezing of the suspension at a known time after fusion has been initiated.

Isolation and suspension of spherical-shaped mitochondrial inner membranes, and use of the system composed of electrodes, control timers, waveform generation and sample freezing were conducted exactly as previously described [7] with the following changes: a sine wave alternating current (60 Hz) of 100 volts (R.M.S.) was applied to the membrane suspension through the electrodes, and, at exactly 1.0 s after the start of the application of the alternating field, the membrane suspension was frozen. Since the electrode spacing averaged about 0.1 cm (see Ref. 7, Fig. 1), the electric field strength is calculated to be an average of 1000 V/cm. Calculations show that thermal heating (see Ref. 7, Figs. 5 and 6) during the current flow will cause less than a 3–4 deg. C temperature rise in the membrane suspension. From previous data (see Ref. 7, Figs. 1 and 5) the conductivity was estimated by calculation to be $1.8 \cdot 10^{-5} \Omega^{-1} \cdot cm^{-1}$. The diffusion of electrochemical products (at $D = 10^{-5} cm^2 \cdot s^{-1}$) from the electrodes to the center of the membrane suspension ($d = 0.05$ cm) would similarly take place ($d^2 = 4Dt$) only after about 60 s. The frozen samples were processed for freeze-fracture electron microscopy.

Electron micrographs showed a mixture of fracture faces of unfused membrane spheres and cross fractures of very large, roughly elliptical- to cylindrical-shaped membranes (Fig. 1). These cross fractures revealed that the interior compartment was rich in small vesicles. All fused membranes had sizes much greater (e.g. 5–20 μm) than the largest normally-occurring mitochondrial inner membrane sphere (0.5–1.0 μm). It was estimated from electron micrographs that up to 20 individual mitochondrial inner membrane spheres were contributing membrane area to the total area of the main fusion product membrane plus the vesiculation products.

Because the very small vesicles are abundantly

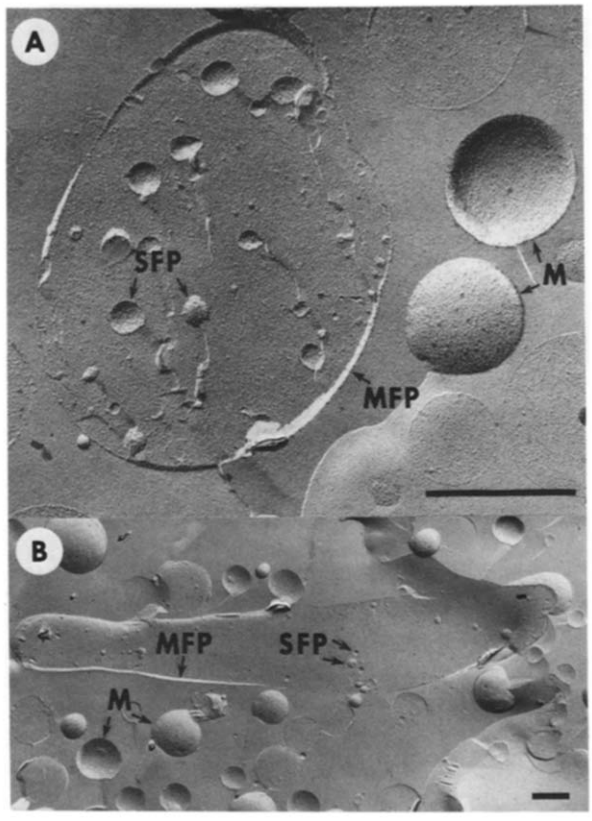

Fig. 1. Electric field-induced fusion of mitochondrial inner membrane spheres (M) into a larger main fusion product (MFP) and many small vesicular membrane fusion products (SFP). (A) Nearly spherical membrane from fusion of small number of mitochondrial inner membranes. (B) Long tubular membrane formed from fusion of a large number of mitochondrial inner membranes. Bars are 1.0 μm.

present inside the interior compartment of the fused membranes and very rarely seen in cross fracture of mitochondrial inner membrane spheres, it must be concluded that they originate by an unknown mechanism from the fusing membranes during fusion. Each of these small vesicles will be referred to as a small fusion product (SFP). Since the fused membrane appears to retain the majority of the membrane surface area contributed by the prefusion components, it will be referred to as the main fusion product (MFP). The small fusion product vesicles had the following three specific characteristics. First, they were much smaller (0.05–0.2 μm) than the smallest naturally-occurring mitochondrial inner membranes (0.5 μm).

Second, more small fusion product vesicles were inside the main fusion product membrane than mitochondrial inner membrane spheres were outside the fused membrane. Lastly, intramembrane particle densities on the convex and concave fracture faces of the spherical-shaped mitochondrial inner membrane were 5270 (±438 S.D.) per μm^2 and 2609 (±750 S.D.) per μm^2, respectively. The intramembrane particle densities on the convex and concave fracture faces of the small fusion products were 2416 (±1288 S.D.) per μm^2 and 5548 (±1306 S.D.) per μm^2, respectively. Thus the ratio of intramembrane particle densities on the two fracture faces of the small fusion products is reversed compared to that found on the spherical-shaped mitochondrial inner membranes. (It has been shown previously [8] that inverted mitochondrial inner membranes have a reversed asymmetry in intramembrane particle density on the two fracture faces as well as a reversal of membrane biochemical properties). This indicates that the membrane fragments from the spherical-shaped mitochondrial inner membranes which gave rise to the small fusion products became inverted during the vesiculation process.

Our observation of inverted vesicles as a byproduct of fusion tends to support a proposed mechanism in which membrane components disorganized at the edges of multiple pores formed by the fusion-inducing electric current pulse reorganize by making contact with edges of corresponding pores on the adjacent membrane (Fig. 17 in Ref. 4 and Fig. 2 in Ref. 9). However, proof of the mechanism will require that freezing of membrane samples be coordinated to take place at a much shorter period of time than the 1.0 s interval used here.

The results of these experiments also indicated that (a) fusion took place in the absence of a relatively high-strength direct current pulse following a prepulse exposure to an alternating current field, and (b) a substantial amount of growth towards the final expected spherical membrane geometry is completed following fusion within 1.0 s since the start of the alternating current exposure. Evidently, the applied alternating current field strength was large enough to not only cause the membranes to rapidly line up, but also to cause perforations to appear within a fraction of the 1.0 s time interval in at least some of the membranes at the points where the membranes are in contact with each other and thereby initiate fusion.

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