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Cytoskeletal reorganization during electric-field-induced fusion of Chinese hamster ovary cells grown in monolayers

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Mammalian cells were shown to fuse after direct electric pulsation of the plated cells in culture. The extent of fusion was controlled by the duration of the post-pulse incubation. Formation of polynucleated cells was slow, even at 37°C. Pre-pulse incubation with colchicine increased the fusion yield slightly. Cytoskeletal organization during the post-pulse incubation was observed using immunofluorescence techniques. Microfilaments were unaffected, but microtubules disappeared during the first minutes following the pulse, and then reformed on subsequent incubation.

Introduction

External electric field-induced cell permeabilization and associated fusion are powerful tools that are gaining widespread application in cell biology and biotechnology [1–3]. This technique presents a lot of advantages when compared to the well known poly(ethylene glycol) treatment approach: synchronicity, no lethality, no contamination by chemical additives, higher yield. However, the molecular mechanisms underlying the electrically induced processes are still very poorly understood. Most of the published investigations have analyzed processes taking place in the plasma membrane. Numerous aqueous pores have been thought to be induced in the membranes [4,5]. Pore formation has been explained on thermodynamic considerations [6,7], but very few experimental results have been reported [8]. A recent electron microscopic study showed some transient alterations of the plasma membrane of pronase-treated erythrocytes [9]. The exact localization of these putative pores was not specified. They were observed in the lipid matrix [10], but may be associated with alterations in a transmembraneous protein [11]. Direct involvement of the field in cell fusion, as proposed by Zimmermann [3] is, however, less likely. The field appears to mediate a structural change in the membrane which confers fusogenic properties. Fusion is obtained if the separated cells are permeabilized by the field, and then brought into contact [12,13].

Many other features of electric field-induced cell permeabilization are poorly understood. The lifetime of permeabilization is short (less than 15 s) in large unilamellar pure lipid vesicles [10], in agreement with the results on lipid bilayers [14], but is quite long in intact cells [15]. A dramatic geometrical change of the cells in suspension occurs during electrofusion [16,17], and in some cases intracellular contents may be lost [3]. In plated cells, the nuclei are found clustered in the middle of the polykaryon.

The aim of the present study was to investigate experimentally the processes affecting Chinese hamster ovary cells over a longer time span (minutes) than the duration of the pulse (μs).

Materials and Methods

Cell culture

Chinese hamster ovary cells (CHO) have been adopted for use in a large number of somatic cell genetics laboratories (see Ref. 18 for a review). We selected the WTT clone which was kindly given to us by Prof. Zalta (this institute). This strain differs from the parent CHO-K1 in being not strictly anchorage-dependent [18]. It grows plated in monolayer (generation time 16–18 h) but has been adapted for suspension culture (generation time 22–24 h). Cells grown in suspension can be replated very easily.

In the present experiments, cells were grown plated on culture dishes (Nunc, Denmark) in Eagle's minimum medium (MEM 0111, Eurobio, France) supplemented with 6% new born calf serum (Boehringer, F.R.G.), antibiotics and glutamine, at 37°C in an air/CO_2 (95 : 5) atmosphere.

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Electrofusion

The electrofusion technique using the system developed in this laboratory or using a CNRS electropulsator (marketed by Jouan) has been described in a previous publication [19]. The electric field pulses were applied directly to the cells growing on the culture dishes (Nunc, Denmark), at a confluent density (800 per mm^2). For the immunofluorescence experiments, the cells were plated on sterile glass coverslips (18 × 18 mm) (Polylabo, France) which were dipped into the bottom of the culture dish. Before the electric pulse treatment, the culture medium was replaced by a pulsing medium (10 mM phosphate buffer (pH 7.4), 1 mM $MgCl_2$, 250 mM sucrose) which has been shown to be well suited for electrofusion [20]. The homogeneous field was applied via two parallel stainless-steel electrodes, 5 mm apart, except for the immunostaining experiments where it was increased to 18 mm in order to treat all the cells on the coverslip. Five repetitive square wave pulses (1.2 kV/cm, 100 μS) were applied; these electrical conditions were shown to be optimal to trigger the fusion with this cell strain [19]. The pulse (intensity, duration) was checked on line with an oscilloscope (Enertec, France). After pulsing, the cells were re-incubated in the culture medium at 37°C unless otherwise specified. Sterility was ensured by operation under a laminar flow hood (ESI, France).

The extent of fusion was evaluated by the polynucleation index which is the percentage of the number of nuclei in polynucleated cells to the total number of nuclei:

$$I(\text{in } \%) = 100 \sum_{n=2}^{\infty} nC_n / \sum_{n=1}^{\infty} nC_n$$

where C_n is the number of cells where n nuclei are present [19]. Some polynucleated cells were always present in the strain we used [19].

On-line video-monitoring of the fusion process

Cells were pulsed as described previously. The culture dish where the cells were grown and pulsed was brought on the heated stage of an inverted microscope (Diavert, Phaco 32× objective, Leitz, F.R.G.). The temperature was kept at 37°C. The cells were observed by means of a video network (JVC S100 SECAM color camera, JVC TM 90 PSN monitor, Philips VR 2334 V2000 magnetoscope). The process was video-taped during 60 min. Due to the technology of the magnetoscope, it was very easy to follow the events affecting the culture during the post-pulse incubation by a high-speed replay. Specific events were observed in detail by taking advantage of the stop feature of the tape replay. Micrographs were obtained directly from the monitor screen.

Immunofluorescence staining

A procedure adapted from that of Brinkley et al [21] was followed. After electric treatment and incubation, the cells were first fixed with formaldehyde 3% (Merck, F.R.G.) in phosphate-saline buffer (PBS) (Prolabo, France) during 20 min and then permeabilized for 1 min in cold acetone (−20°C) (Prolabo, France). To reduce non-specific antibody binding, the samples were first treated with 100 μl of goat antiserum (Nordic, The Netherlands) in PBS (5%). Cells were then incubated with specific monoclonal antibodies, respectively, anti-tubulin (N 357) and anti-actin (N350) (Amersham, U.K.) in PBS for 1 h at 37°C. After extensive rinsing in PBS, the samples were stained during 1 h with Texas red conjugated sheep antimouse IgG (N1031, Amersham, U.K.). After one further wash with PBS, the coverslips were mounted on slides in PBS/glycerol/*n*-propylgallate (50 : 25 : 2) (Prolabo, France).

Fluorescence was monitored using an epifluorescence microscope (Leitz Ortnolux, F.R.G.).

Results

1. Effect of sample temperature during the field pulse on fusion yield

The temperature of the sample at the time of application of the field could be altered by incubating the cells at various temperatures prior to the electric treatment. The incubation temperature after the electric pulse treatment was kept constant (37°C). The polynucleation index was found to be unaffected by temperature before and during the pulsation (4°C, 21°C, 37°C) (data not shown), and in subsequent experiments, the cells were pulsed at 21°C.

Fusion occurs preferentially along the electric field lines. This is clearly observable in Fig. 1, where particularly elongated polykarionic cells are present. A statistical analysis of the fused cells shows that nuclei are present as linear chains roughly parallel to the electric field lines which are perpendicular to the electrodes (histogram not shown). This is due to the vectorial effect of the field on the cell. Electropermeabilized and as such fusogenic areas on the cell membrane are those facing the electrodes [22].

2. Effect of post-pulse incubation time on fusion

The direct video-monitoring of the post-pulse fusion process showed that several sequential events are occurring. They are summarized in Fig. 2. During the first 10 min after the pulse, no morphological alteration can be detected even if permeabilization is present as detected by the free entry of dyes such as Trypan blue [23]. At time 20 min, the refringence of the cell membrane is altered and the phase-contrast observation of the cell culture is difficult; some fused cells, where the number of nuclei per cell is still low, are present. At time 30

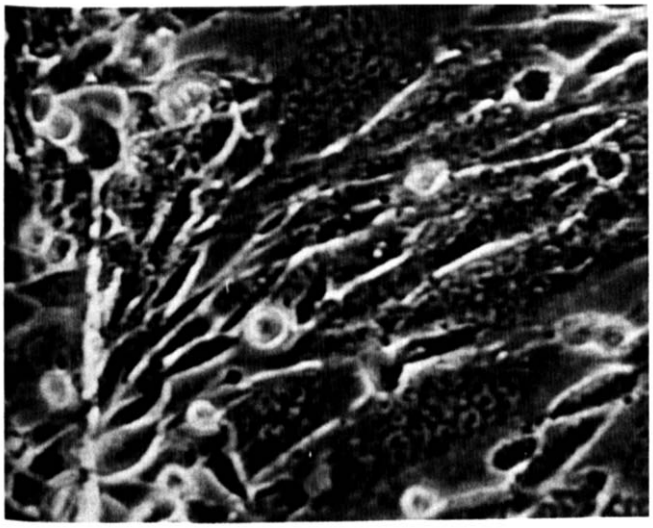

Fig. 1. Micrograph of a culture of electrofused cells. The white line on the left of the picture is the trace of the electrode. Cells on the left of the line are control cells which were not affected by the pulse. Cells on the right were electrofused. Their shape is very elongated indicating that fusion occurred preferentially in the areas facing the electrodes.

min, a high number of polykaryonic cells are present; the number of nuclei per cell is now high. At time 40 min, a high percentage of the cells is polykaryonic and nuclei are clustered in the middle of the newly formed cell. No further change can be detected with a longer incubation.

Electropulsed cells were observed after various incubation periods at 37°C. The change in the index of fusion is shown in Fig. 3. A 10 min lag was observed where no fusion was detected. For longer incubation times, the polynucleotion index increased sharply, and then leveled off between 40 and 60 min. The index remained constant for longer incubation times (up to 3 h) (data not shown) indicating that a stable configuration of the population was reached.

3. Effect of post-pulse incubation temperature on fusion

As described above, a stable level of polynucleation was observed after 40 min incubation at 37°C. With incubation at 21°C, the polynucleation index increased very slowly with time (Fig. 4). However, if the pulsed cells were incubated for 1 h at ambient temperature (21°C) and then warmed to 37°C, a stable, high polynucleation index was observed after a further period of 60 min. The index was of the same order of magnitude as that observed after incubation at 37°C immediately after pulsation. By comparing the kinetics of the increase in polynucleation index at 37°C and at 21°C, we calculated an activation energy of 75 kJ/mol.

If the pulsed cells were incubated on ice (4°C), no fusion was detected after 1 h but the cells remained permeabilized (data not shown). Nevertheless, fusion with the same high polynucleation index could still be obtained after a further 2 h incubation at 37°C.

4. Effect of drugs affecting cytoskeletal organization

Pre-pulse incubation of the cells with the drugs under the concentrations which were used are known to be sufficient for inducing the cytoplasmic effects. Colchicine induces depolymerization of microtubules, while taxol has the opposite effect, and enhances microtubule stability. Cytochalasin B is known to disorganize actin microfilaments [24].

Cells were treated with these drugs under various conditions. The effects on electrofusion are shown in Table I. Electrofusion conditions were: pulsing temperature, 21°C, 1 h post-pulse incubation at 37°C. The concentrations and duration of drug treatment employed had no effect on the cell cycle, which is thought to play a major role in the PEG-induced formation of hybridomas [25].

The most interesting result was obtained with colchicine. After pre-treatment with colchicine, we observed a 20% relative increase in the final polynucleation index. A slight inhibition of electric field-induced fusion was observed after post-pulse incubation with colchicine.

A lytic effect of post-pulse incubation in a cytochalasin-containing medium was evident. This was attributed to pulse-induced cell permeabilization, en-

TABLE I

Effect of drugs on the polynucleation index observed after electric pulsation

Cells were pulsed five times (intensity: 1.5 kV/cm, duration: 100 μs) at 21°C and then incubated at 37°C. The drug treatments were performed on different days (different cultures) for the different drugs but on the same day (same culture) for the same drug. This explains the difference in the fusion index observed in the control experiments (pulses, no drug). Fluctuations in the fusion index are observed from one culture to another, but are not observed (except the indicated S.E.) within the same culture (for the same drug and the same pulsing conditions).

Drug	Polynucleation index *I* (%)		
	Colchicine (2 μM)	Cytochalasin B (30 μg/ml)	Taxol (20 μg/ml)
Treatment			
Incubation 30 min before the pulses	61 (± 2)	63 (± 4)	51 (± 4)
Present only during the pulses	47 (± 3)	50 (± 3)	56 (± 6)
Incubation 60 min after the pulses	42 (± 3)	lysis	53 (± 8)
Controls			
No pulse, no drug	12 (± 1)	13 (± 1)	9 (± 3)
No pulse, incubation 30 min with drug	13 (± 1)	12 (± 2)	8 (± 2)
Pulses, no drug	50 (± 3)	60 (± 1)	55 (± 8)

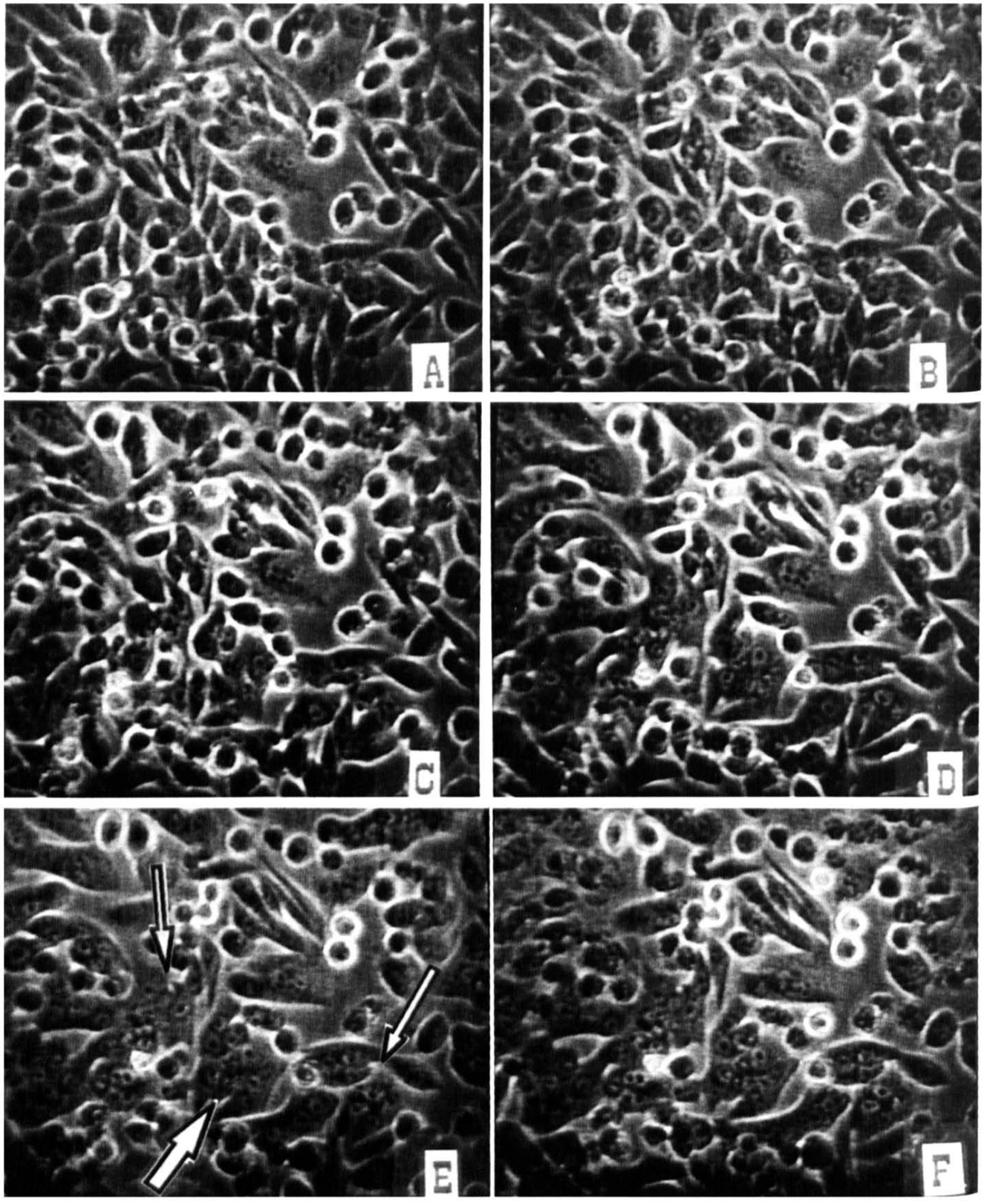

Fig. 2. Evolution of a cell culture during the post-pulse incubation. The cells were observed under the microscope after pulsing. The microscope stage was heated at 37 °C and the same place on the culture was video-monitored along the incubation. The delays following the pulses were: (A) 0, (B) 10 min, (C) 20 min, (D) 30 min, (E) 40 min and (F) 45 min. The arrows on plate E indicate fused cells which can not be observed after shorter incubations.

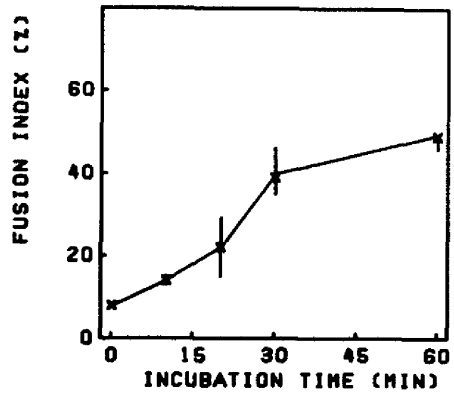

Fig. 3. Change in fusion index during the post-pulse incubation at 37°C. CHO cells were pulsed (five pulses of 1.6 kV/cm with a duration of 100 μs) at 21°C and then incubated at 37°C. The extent of fusion, quantitated by the degree of polynucleation, was determined after increasing durations of incubation.

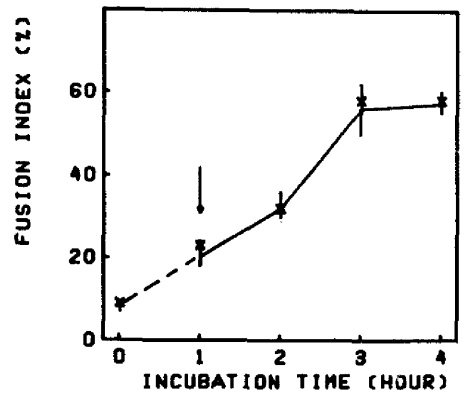

Fig. 4. Change in fusion index during the post-pulse incubation at 21°C followed by an incubation at 37°C. CHO cells were pulsed as in Fig. 1 and then incubated at 21°C (dotted line). After one hour (arrow), the incubation temperature was raised to 37°C (continuous line).

abling diffusion of the drug into the cytoplasm. Taxol appeared to have no particular effect on electrofusion yield. Drug treatment prior to the pulse treatment had no effect on the kinetics of the increase in polynucleation index during the post-pulse incubation. In all three cases, kinetics similar to those shown in Fig. 3 were observed.

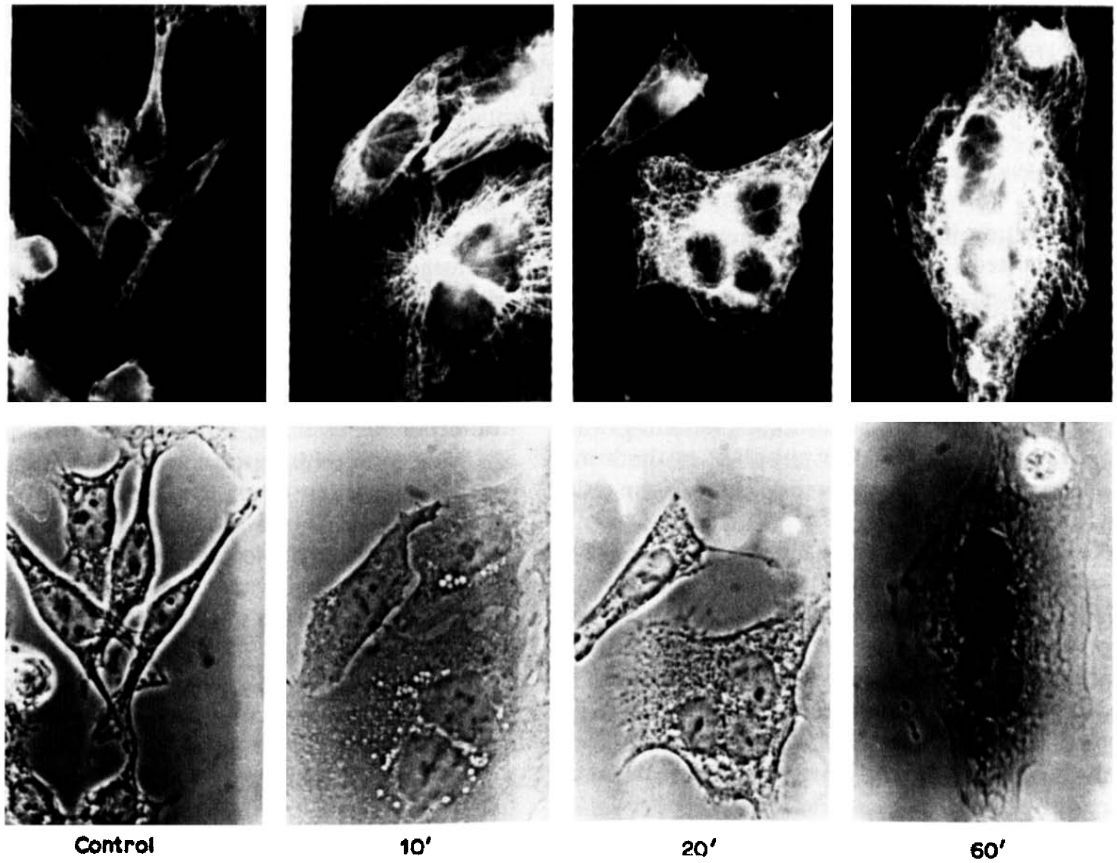

Fig. 5. Evolution of cell shape, and actin organization during the post-pulse incubation at 37°C. CHO cells were pulsed as in Fig. 1, and then incubated at 37°C. Bottom, phase contrast microphotographic pictures show the progressive formation of the polykaryons. Top, immunofluorescent staining of the actin network shows that it is not significantly altered during the incubation.

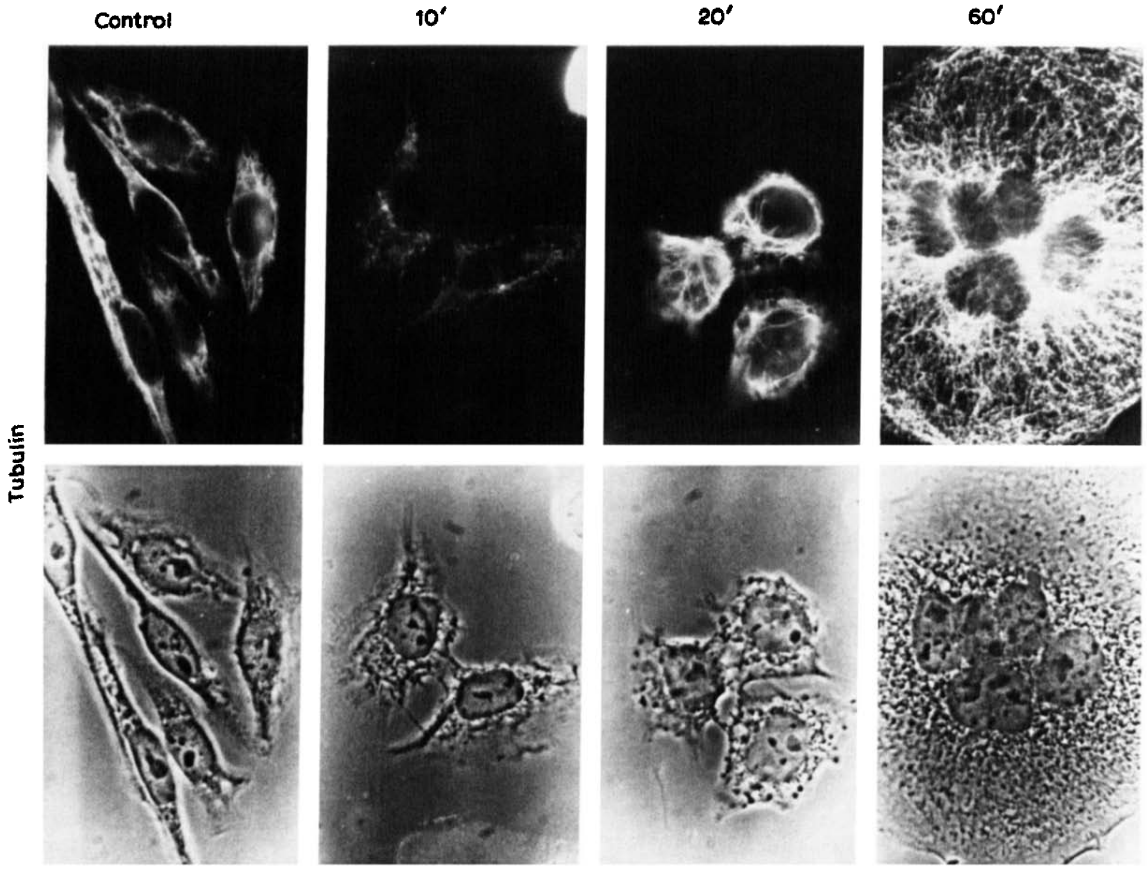

Fig. 6. Evolution of cell shape, and tubulin organization during the post-pulse incubation at 37°C. CHO cells were pulsed as in Fig. 1, and then incubated at 37°C. Bottom, the formation of the polykaryons is observed by phase contrast microscopy. Top, the tubulin network is visualized by immunofluorescent staining.

5. Changes in cytoskeletal morphology during the post-pulse incubation

A previous study had shown that the cellular morphology was altered drastically by the electrofusion process [19]. Polynucleated cells were routinely observed with a cluster of nuclei in the middle of the fused cells.

Structural reorganization of the proteinous network was studied after immunofluorescence staining with monoclonal anti-cytoskeleton antibodies. Staining was carried out at various times after pulsation of the sample. Phase contrast and fluorescence pictures of the cells were then compared.

Cytoskeleton organization in CHO was shown to be very dependent on the strain. In the case of the parent strain CHO-K1, this organization was shown to be strongly affected by external agents such as db-cAMP or forskolin [26]. Our study shows that the WTT strain displayed a peculiar actin network organization (Fig. 5) when compared to the K1 strain [26]. This is in agreement with the well known differences between the two strains: morphology, contact inhibition and aptitude to grow in suspension [27]. The absence of the long and thick actin sheaths (stress fibers) which are present in most cells is in agreement with other observations [27]. The CHO strain we used is not anchorage-dependent for its growth. The existence of actin-containing sheaths is thought to be strictly related to anchorage-dependence [27]. As shown in Fig. 5, no particular change in the organization of actin microfilaments was observed during post-pulse incubation at 37°C. A diffuse actin matrix was always present.

The organization of microtubules was markedly altered during the post-pulse incubation at 37°C (Fig. 6). Immediately after the pulse (10 min) the fluorescence labeling was only detectable around the nuclei. With longer incubation times, the fluorescence became detectable throughout the cytoplasm. After one hour, the network was morphologically similar to that of controls.

It should be noted that the nuclei were always found to be clustered in the middle of the fused cell after one hour's incubation at 37°C. This observation was most striking in highly polynucleated cells, where, in phase contrast, a cluster of nuclei was observed in the middle of a huge cell (Figs. 5 and 6).

Discussion

Cell fusion is the set of events enabling formation of viable hybrids. Different steps can be defined rather arbitrarily: (1) cell contact; (2) membrane fusion; (3)

cytoplasmic reorganization; (4) selection of genetic material. In the present study step 1 was produced by the culture conditions. At the density we were using (800 cells per mm^2), numerous cell contacts were established spontaneously on the culture dish. In this study we were only concerned with homofusion, and so only the processes in steps 2 and 3 were involved in these experiments. Membrane fusion appears as a direct consequence of electrically induced permeabilization. This artificially triggered modification of membrane structure is induced in less than one microsecond [8], but may persist for several hours after the electric-pulse treatment [15]. The effect of temperature on the kinetics of fusion is confirmative of recent observations showing that electropermeabilization induces a fusogenic state of the plasma membrane [12,13]. In the present work, when kept at 4°C, pulsed CHO cells remain permeabilized but are unable to form polykaryons. If then they are brought to 37°C, fusion occurs showing that the ability to fuse was kept by the permeabilized cells.

Both the direct video-monitoring of the culture during the post-pulse incubation and the precise evaluation of the fusion index change along the incubation showed that a dramatic change occurred between 20 and 30 min leading to a strong increase in the fusion index and to the presence of polykaryonic cells. These huge cells were in fact obtained by the fusion of already fused cells but where a low number of nuclei was present (Figs. 2 C and D). These observations are indicative that the cellular fusogenicity was a long-lived phenomena and was still present when the electric field-induced permeability could not be detected anymore. Other processes than those involved in the permeabilization are present in the cell fusion even if it is clear from the choice in the electrical parameters that permeabilization must be present for fusion to occur [13,23]. Recently published results on the electrofusion of red blood cell ghosts have shown that permeabilization was not enough to induce fusion even when the cell-cell contact was present by use of dielectrophoresis [28,29].

If the electrically induced permeability has a rather long lifetime in cells, however, in phospholipid vesicles, impermeability is restored within 15 s after treatment [10]. Consideration of the interconnections between membrane and cytoskeleton [30,31], suggested that the proteinous network might play a role in electropermeabilization. The cytoplasmic reorganization (step 3) was reflected by a clustering of nuclei in the fused cells. This indicated that the proteinous array was transiently disorganized during cell fusion. For example, membrane proteins have been observed to have high diffusion constants in the electric field-induced fusion of proteolytic enzyme-treated red blood cells [32]. An alteration in the interactions between plasma membrane and cytoskeleton was proposed to account for these observations [32]. However, the evidence is circumstantial, and it should be born in mind that the organization of the erythrocyte cytoskeleton is unusual.

The results of the present study, both on thermodynamic considerations and from the more direct biochemical and immunological observations also lend support to the idea that there is an alteration of the cytoskeleton during electrofusion.

Our results, based on the polynucleation index, showed that electrically induced fusion was a slow process, in agreement with the study of Ohno-Shosaku et al. [15] on the electrofusion of pronase-treated lymphoma cells in suspension, where the phenomena was quantitated by the rounding process, i.e., the set of events leading from two cells to the final shape of the fused cell. Compared to the movement of cellular components, it cannot readily be related to diffusion of membrane lipid components [33]. From consideration of the size of a CHO cell and the time required at 21°C to obtain 50% of the final polynucleation (when 50% of the cellular reorganization has taken place), we calculated that the diffusion coefficient of the molecules potentially involved in cell fusion by evaluating the mean time needed to move by diffusion on one quarter of the cell perimeter. This give us a value for the diffusion coefficient in the range of 10^{-10} cm^2/s. Comparison of this figure with data on lateral diffusion in biological membranes [30], would suggest that rate-limiting steps in fusion may involve cytoskeletal proteins. Such an effect was suggested in a thermodynamical analysis of cell electropermeabilization [34]. This is supported, in the present work, by the strong temperature dependence of the cellular reorganization (activation energy of 75 kJ/mol). This is in partial agreement with the work of Ohno-Shosaku et al. [15] on lymphoma cells in suspension; Ohno-Shosaku et al. reported that fusion yield fell as the sample temperature was reduced. The existence of an energy-dependent process, as proposed here, has also been observed in electrofusion of plant protoplasts where the kinetics of the rounding process were found to depend on ATP content [17].

Our experimental data provide little insight on the exact molecular processes involved, but this conclusion on the mechanism is supported by the results on the effects of drugs (Table I). It should be emphasized that the treatment with colchicine was too short to synchronize the culture, which is thought to play a beneficial role in PEG-induced hybridoma formation [25]. Cytochalasin and taxol had no effect on polynucleation kinetics, apart from the cytotoxic effects associated with cytochalasin. Treatment with colchicine prior to electric pulse treatment increased the level of polynucleation. This suggests that pre-depolymerization of microtubules could facilitate electrofusion, and it provides the first direct evidence of cytoskeletal involvement in the process. This result is in apparent conflict with the results of Ohno-Shosaku et al. [15]; Ohno-Shosaku et al.

did not observe any effect of colchicine on electrofusion of pronase-treated lymphoma cells. However, their experimental procedure was rather different (shorter incubation at a lower temperature).

The immunofluorescence results throw more light on the events affecting the cell during the post-pulse incubation period. As suggested by the absence of effects of cytochalasin B, the fluorescence pattern associated with the antiactin antibodies was stable throughout the incubation. Over a time span of a minute (the temporal resolution of our technique), microfilaments did not appear to be implicated in the events following the pulse. Just after the pulse, however, the microtubule fluorescence was seen to be limited to a ring around the nuclei, subsequently spreading throughout the cytoplasm. The vicinity of the 'microtubule organizing center' near the nucleus [24] also lends support to this observation of repolymerization starting from this point. The transient character of the disappearance suggests that microtubule depolymerization to form tubulin is induced just after the pulse treatment. Since cell impermeability reappears in less than 10 min at 37°C for CHO cells [23], repolymerization and impermeabilization occur at the same time. We envisage these processes as a restoration of normal cell function. Depolymerization of microtubules may have been facilitated by the penetration of Mg^{2+}, associated to the electropermeabilization. It has indeed been shown that after microinjection of $MgSO_4$ into the cytoplasm of PtK2 cells, a transient disassembly of the microtubule array was present [35]. This effect was induced in the 5 min following the injection but was spontaneously fully reversible upon further incubation. The F-actin microfilaments remained unaffected after $MgSO_4$ microinjection [35]. Nevertheless the penetration of Mg^{2+} played only a facilitating role because fusion can be obtained in a Mg^{2+} medium [20,36].

On the basis of these results, we propose the following scheme for electrofusion: (1) electropermeabilization; (2) cellular reorganization involving cytoskeletal proteins; (3) simultaneous depolymerization of microtubules may be a direct consequence of the permeabilization (loss of small metabolites?); (4) repolymerization of microtubules when the cell membrane recovers its barrier character; (5) clustering of nuclei in fused cells (this process is presumably facilitated by the microtubule depolymerization in step 3).

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