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Reversible plasma membrane ultrastructural changes correlated with electropermeabilization in Chinese hamster ovary cells

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Chinese hamster ovary cells (CHO) grown in monolayers were permeabilized to molecules with molecular weight up to 1000 by high intensity 100 μs square wave electric field pulses. This permeability was transient and the cell viability was not affected. It was not possible for molecules with a molecular weight larger than 1500 to penetrate inside the cytoplasm if lytic pulsing conditions were not used. In order to investigate the ultrastructural changes associated with this transient and limited permeabilization, cells were chemically fixed a few seconds after their pulsation and observed by electron microscopy. By scanning electron microscopy, numerous microvilli and blebs were observed almost immediately after application of the field. No other membrane changes were observed. Permeabilization of the membrane was visualized at the electron microscopic level by penetration of Ruthenium red. The appearance of osmotic pressure-dependent 'blebs' was indicative of local weakening of the plasma membrane. Most of these effects were fully reversible and disappeared within 30 min at 37 °C with the formation of huge polykaryons when cells were in contact before pulsing.

Introduction

The plasma membrane is a highly impermeable barrier to penetration of exogenous molecules into the cytoplasm. Transfer only takes place via the action of specific 'carrier' proteins which actively transport molecules across the cell membrane with a concomitant expenditure of energy. Experimental alteration of the cytoplasmic content can therefore only be achieved by drastic procedures of a physical (microinjection) or chemical (poly(ethylene glycol), virus) nature [1]. Recently, a convenient technique using direct application of an external electric field to a suspension of cells has been developed. If the field intensity is above a certain threshold, which depends on the nature of the cell, the plasma membrane becomes transiently permeable and exogenous molecules can pass directly into the cytoplasm. Under controlled conditions, this 'permeability' is fully reversible. If the cells are in close contact, cell fusion is observed [2]. Cell viability can be unaffected by the electric field.

Electropermeabilization is now used routinely for gene transfer [3–5], and in cell hybridization experiments [6–8]. It has been proposed as a method for the introduction into cells of small molecules such as sucrose [9] or even ions such as calcium [10]. However, in this latter application,

Abbreviation: CHO cells, Chinese hamster ovary cells.

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cell integrity must be maintained, and there are few data on the longer term effects of this treatment on cell structure and function. Experiments on the transfer of Ca^{2+} ions into sea urchin eggs showed dramatic alterations of the membrane with high field pulses [10] although damage could be considerably reduced using more moderate field strengths [11]. 'Blebbing' has been detected in pulsed hepatocytes, which was attributed to the method of isolation [9]. No nuclear alterations were observed at low intensities, although at high field intensities marked alterations were seen [12]. Vacuolisation of the endoplasmic reticulum was observed in islets of Langerhans after their irreversible electropermeabilization [13].

We have investigated the molecular events associated to the electropermeabilization in Chinese hamster ovary cells (CHO). We have employed repetitive electronically controlled microsecond square wave pulses applied directly to cells growing on the culture dish. This method appears to produce better results than the discharge method (exponentially decaying field), since it avoids the initial high intensity pulse and does not require the cells to be brought into suspension by treatment with trypsin. Under our experimental conditions, no cytotoxic effects were observed.

The aim of this study was to characterize the ultrastructural changes occurring within a few minutes following the pulse and which are responsible for (a) the non-specific permeabilization of the membrane to small molecules [9,10,16,17] and (b) the induction of a fusogenic state of the cell [2,6,7].

Electron microscopy is well suited for the direct observation of the morphological changes in fused cells, and cultured CHO cells are readily observed by electron microscopy. Fixation, embedding and oriented thin sectioning can be performed without disturbing the 'in situ' anchorage of the cells. Artefacts of classical sample preparation such as scraping and centrifugation are thus avoided.

Materials and Methods

CHO cells were grown in monolayers on 18 × 18 mm glass coverslips at 37 °C in a 5% CO_2/95% air atmosphere in Eagle's minimum medium (MEM 0111, Eurobio), supplemented with 8% newborn calf serum, penicillin (100 U/ml), streptomycin (11 μg/ml) and L-glutamine (1.16 mg/ml).

Application of electrical pulses to the cells

Before the treatment, the culture medium was discarded and a low ionic content saline buffer: the 'pulsing buffer' (250 mM sucrose, 1 mM $MgCl_2$ in 10 mM phosphate buffer, pH 7.7) was added. The electrodes (width 18 mm, length 18 mm) were dipped in the buffer as previously described [2] and five pulses (600 volts/cm) with a 100 μs duration (1 s delay) were applied to the electrodes, the field magnitude being monitored on line with an oscilloscope. All the cells growing on the coverslip were submitted to electrical pulses. The pulsing buffer was then removed and culture medium was added. The cells were incubated for various times at 37 °C (0–60 min).

Control cells were treated in a similar way (except that the field intensity was zero). Incubation in the 'pulsing buffer' at 21 °C during periods as long as 30 min was observed not to affect the morphology of the CHO cells (data not shown).

Determination of the permeabilization

Electropermeabilization of plated CHO cells was quantitated by two different methods (1) penetration of Ca^{2+}, leading to cell lysis and (2) penetration of Trypan blue ($M_r = 960$) giving a blue staining of the nuclei.

In case (1), cells were pulsed 10-times with a duration of 100 μs in a pulsing buffer containing 3 mM $CaCl_2$ and Trypan blue (0.4% w/v). They were incubated 5 min after the pulses and the number of blue-stained cells was counted in the population.

In case (2), cells were pulsed ten times with a duration of 100 μs in a pulsing buffer to which Trypan blue (0.4% w/v) was added. They were incubated 5 min after the pulses and the percentage of blue-stained cells was evaluated.

Reversibility of the electropermeabilization was assayed by the Trypan blue test. The cells were pulsed in the 'pulsing' buffer which was substituted by the Trypan blue-containing buffer after the indicated delay. Cells were incubated 5 min thereafter.

Trypan blue penetration was used to monitor the induced 'permeabilization' of the membrane

and not to assess the cell viability. Cell viability was checked by observing the growth of cells 24 h after pulsation. Up to 2 kV/cm no cell lysis was detected, except of course when Ca^{2+} was present in the pulsing buffer.

Treatment of cells

For light microscopy, cells were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 30 min at room temperature and examined in a Leitz Ortholux II microscope at different times after the electric pulse.

For scanning electron microscopy, glutaraldehyde-fixed cells were post-fixed in 1% osmium tetroxide (1 h at room temperature) and exposed to 1% tannic acid (1 h at room temperature). After several washes, the cells were treated with 2% uranyl acetate in 70% acetone for several hours [14]. After complete dehydration, the cells were critical point dried, gold-coated and observed in a JEOL JSM 840 microscope.

For transmission electron microscopy, cells were immersed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer at 37°C for 30 min and post-fixed in 1% sodium cacodylate-buffered osmium tetroxide for 1 h at room temperature. Embedding of cells was performed 'in situ' in Epon 812. Ultrathin sections were cut perpendicular to the culture plane, collected on pioloform covered slot copper grids and contrasted with uranyl acetate and lead citrate.

Ruthenium red was added to the washing and post-fixative solutions (0.5 mg/ml) according to Luft [15].

Being an electron dense marker, Ruthenium red is a convenient indicator of the integrity of the plasma membrane, and allows direct monitoring of the kinetics of the membrane resealing.

Results

Electropermeabilization of plated CHO cells

Cells were treated with electric fields of increasing intensity. Permeabilization was assayed as indicated in 'Materials and Methods' either by the Ca^{2+}-induced lysis (monitored by the associated staining by Trypan blue) or by the direct penetration of Trypan blue. Permeabilization was observed with the two approaches only when the field intensity was larger than 0.3 kV/cm (Fig. 1) whatever the number of applied pulses (data not shown). Clearly, the increase of permeabilization with the increase in field intensity was stronger when the Ca^{2+} assay was used. With a field intensity of 0.6 kV/cm, all cells were stained when Ca^{2+} was present but a strength of 1 kV/cm was needed for the direct staining.

The reversibility of electropermeabilization was observed by adding the dye at different times following the pulses. As shown in Fig. 2 at room temperature, the process is very fast and a 50% decrease in the number of permeabilized cells is observed in less than 15 min. After 1 h, less than 10% of the cells are still permeabilized. If the experiment was run at 37°C, the permeabilized state was even more shortlived (Fig. 2). After less than 10 min, no cells were permeable to Trypan blue any more.

The size of the molecules which can permeate was previously shown to be restricted [16,17] in the case of erythrocytes. We assayed the penetra-

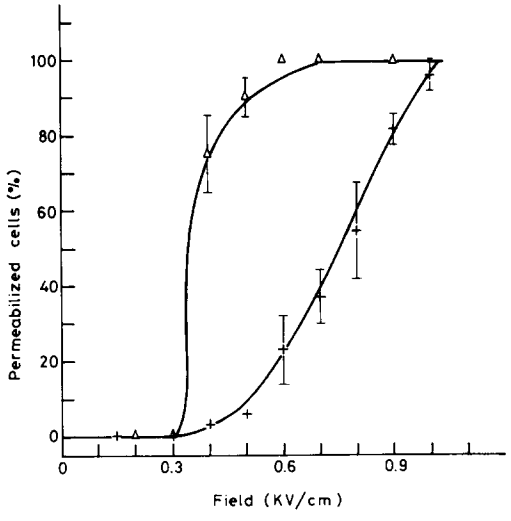


Fig. 1. Modulation of the permeabilization by the electric field intensity CHO cells were treated by ten successive square wave pulses (duration: 100 μs, delay: 1 s) with different intensities. The pulsing buffer was complemented by $CaCl_2$ (3 mM) and Trypan blue (0.4% w/v) (△) or by Trypan blue (0.4% w/v) (+). The percentage of blue-stained cells was then computed from the observation of the cells under the microscope. Permeabilized cells were stained in blue. Error bars refer to standard errors. The variations were observed by repeating the experiments on different cell batches.

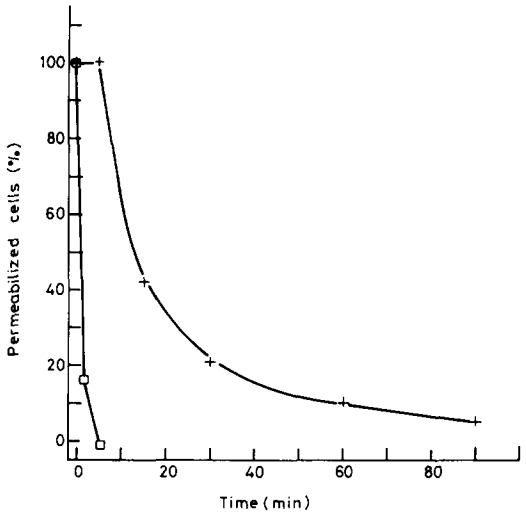


Fig. 2. Reversibility of the electropermeabilization. CHO cells were pulsed ten times (duration: 100 μs, delay: 1 s, field intensity: 1 kV/cm). After the indicated durations following the pulses, the pulsing buffer was substituted by a pulsing buffer containing Trypan blue (0.4% w/v). The percentage of blue-stained cells was determined thereafter. Pulsation and incubation temperatures were room temperature (+) or 37 °C (□).

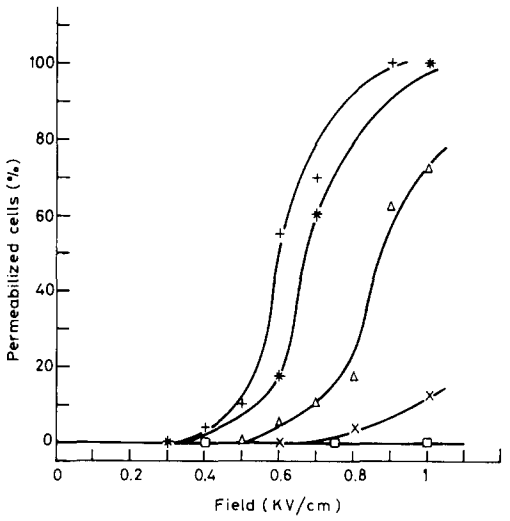


Fig. 3. Modulation of electropermeabilization by the size of external molecules. CHO cells were pulsed ten times (duration: 100 μs, delay: 1 s) with increasing field intensity. The pulsing buffer containing Trypan blue (0.4% w/v) (+) was complemented by Dextran 1500 (30 mM) (*), by PEG 1000 (7% w/v) (△), by PEG 1450 (7% w/v) (×), by PEG 3350 (7% w/v) (□) or Dextran 4100 (30 mM) (□). The percentage of blue-stained cells gave the percentage of permeabilized cells.

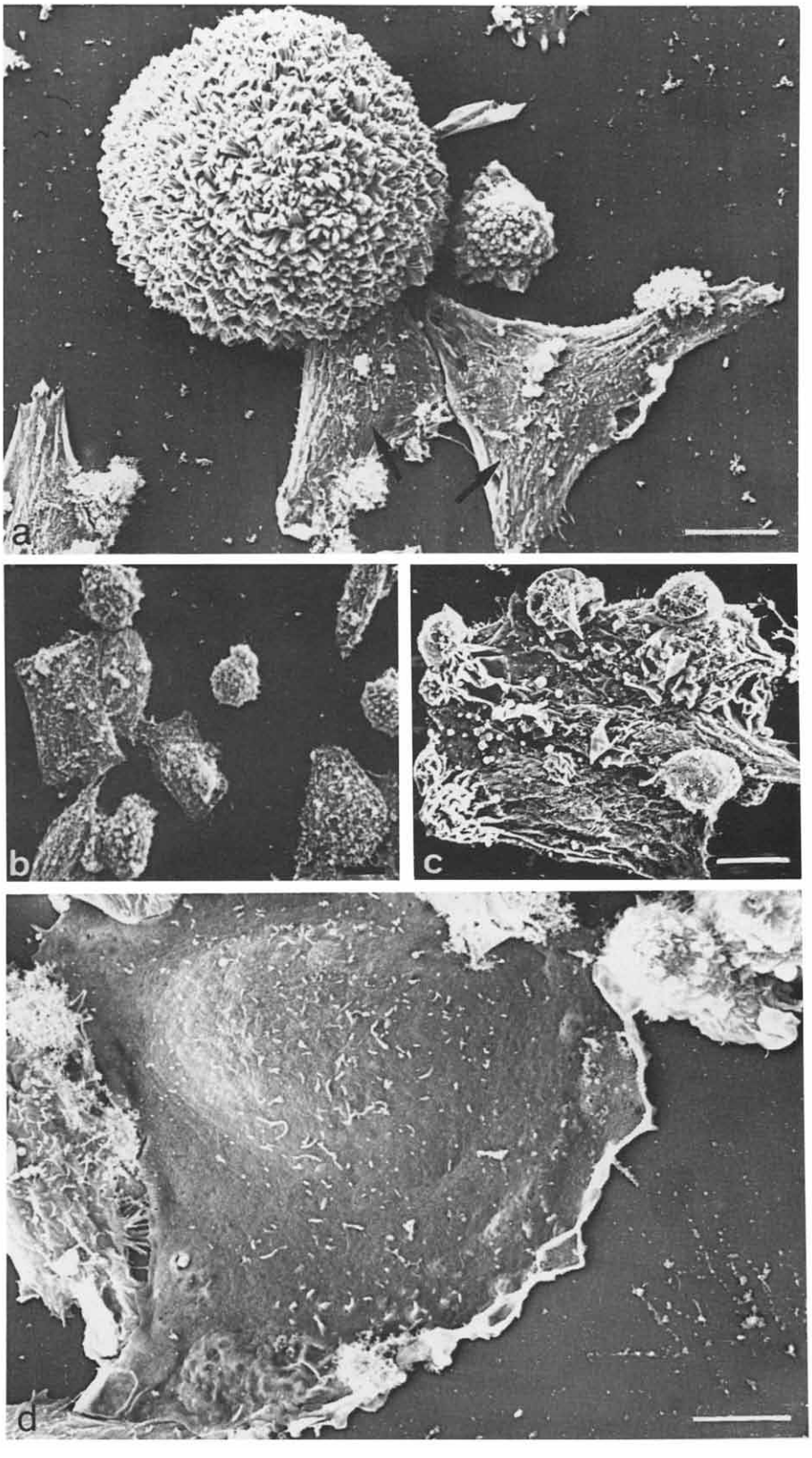
tion of Trypan blue by adding to the pulsing buffer different molecules with increasing molecular weights. As shown in Fig. 3, large molecules (M_r larger than 1500) inhibited the penetration of Trypan blue and medium-sized molecules (M_r of the order of 1500) reduced it. On the other hand, molecules of the same chemical composition but with a molecular weight close to the one of Trypan blue (PEG 1000, Dextran 1500) did not affect the penetration of the dye. This observation showed that the effect on the penetration was not due to a chemical alteration of the membrane induced by the added molecule but to a size effect. As the experiments were run at a constant mass concentration, i.e., with decreasing molar concentration when the molecular weight of the added compound was increased, this effect cannot be explained by events linked to the osmotic pressure. The osmotic pressure is linearly linked to the molar concentration and as such is reduced with the high molecular compounds.

The electric charge which is carried by the permeant species does not play a decisive role in the permeation – ions or molecules positively charged (Ca^{2+}, ethidium bromide (data not shown)) or negatively charged (Trypan blue) can penetrate into the cytoplasm after electropermeabilization.

In agreement with results on erythrocytes, the molecular weight of molecules able to permeate electrotreated cell membrane is limited to M_r 1500. The electric field-induced transformation can then not be described by a direct transfer of the supercoiled plasmid across a pore [3].

The organization of the permeant structure in the cell membrane is then an open question. Furthermore, it is known to be associated to a fusogenic state of the membrane [18]. An ultrastruct-

Fig. 4. Morphological changes induced by the electric pulses (scanning electron microscopy). (a) Morphology of the monolayer of control cells: interphase (arrows) and round metaphase cell (bar = 5 μm). (b and c) Just after the electric pulses (bar: 10 μm). (d) 20 min after the pulses, big, round, flattened cell with few microvilli. No transient blebs were observed (bar = 5 μm).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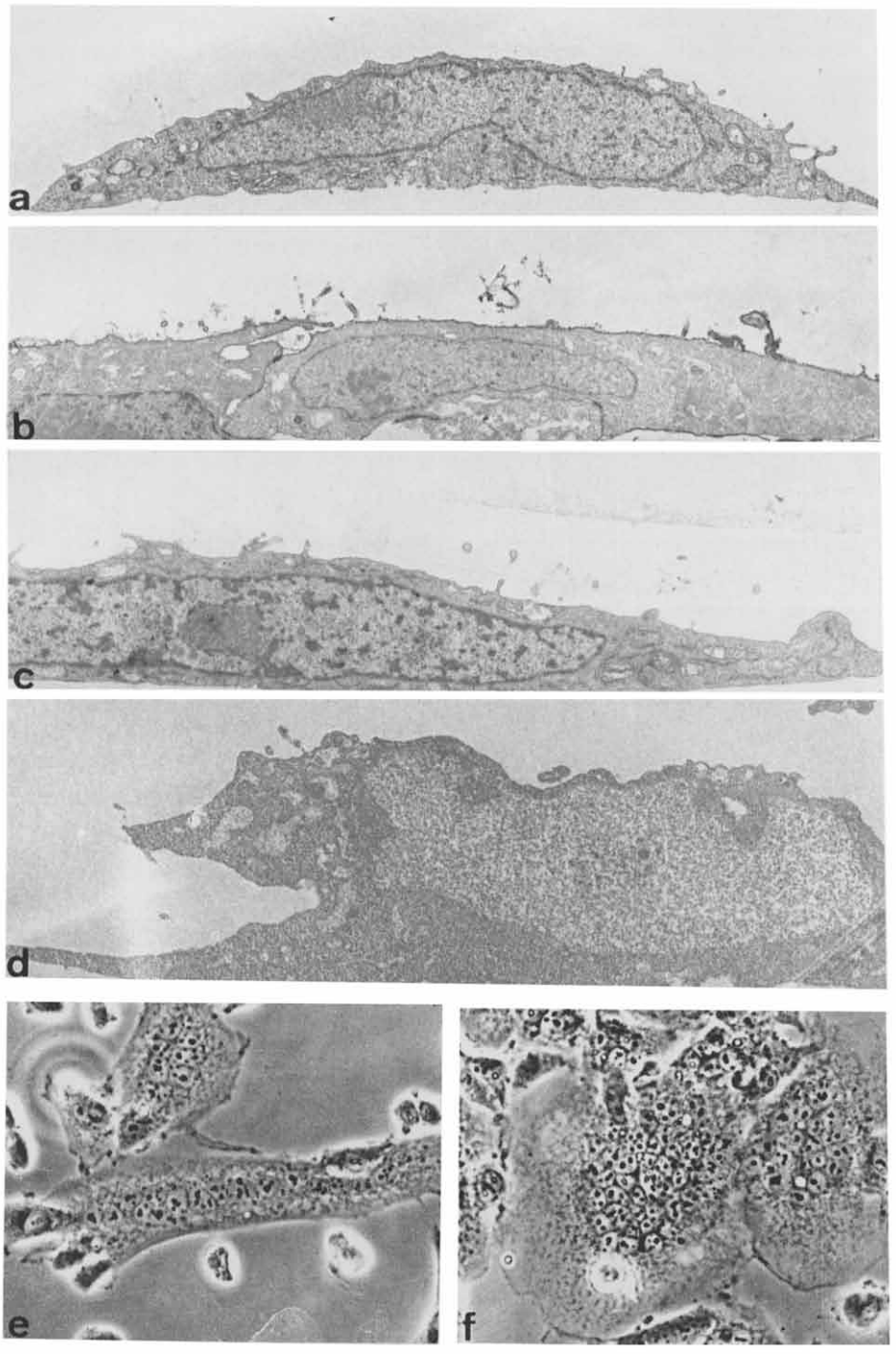

Fig. 5. (a–d) Ultrastructure of CHO cells 'in situ' (transmission electron microscopy). (a) Control cell (×6000); (b) control cell exposed to Ruthenium red (×6000); (c) cell fixed just after the electric pulses (×7000); (d) cell fixed just after the electric pulses then exposed to Ruthenium red (×7500). (e and f) 20 min after the pulses, big, elongated cells (e) or round, flattened cells (f) were polynucleated (phase contrast×500).

ural study of pulsed cells was attempted to obtain more informations on this 'transient' membrane organization.

Characterization of changes in cell morphology

Using a field intensity of 0.6 kV/cm (5-times with a duration of 100 μs) as described in the Materials and Methods induced the permeabilization of 100% of the CHO population (Fig. 1) and was high enough to trigger the fusion [2]. This condition did not affect the cell viability. For all these reasons, it was very well suited for the ultrastructural analysis of reversibly electropermeabilized cells.

CHO cells, in non-synchronized monolayers, are morphologically heterogeneous. According to other studies on the same cell line [19], it is difficult to correlate cell morphology to progress through the cycle. The shapes of cell range from relatively flat and smooth with few microvilli and blebs (predominantly observed in interphase cells) to round with many microvilli (mitotic cells). The topography of the normal monolayer is illustrated in Fig. 4a.

In order to determine the morphological changes produced by permeabilization-inducing pulses, the fixatives were added on cells just after pipetting the pulsing buffer. The operation only takes a few seconds, and short-lived effects of the electrical pulses on the cells may be observed (time scale in the second range). At this time, numerous microvilli were visible on all cells (Figs. 4b and 4c). In addition, numerous cells showed big spherical blebs and other blebs, crushed by the critical point dehydration (Fig. 4c). These alterations were observed over the first 10 min after application of the electrical pulses. No crushed blebs were present when cells were fixed after longer periods of recovery in the culture medium at 37°C (Fig. 4d). Ruffles which were visible at the edge of the giant cells could be compared to those observed in normal interphase CHO cells (Fig. 4a). Thin sections showed that these ruffled membranes displayed the same organization as that observed in the control cells.

20 min after the pulse, some cells had long pseudopodia which were often linked with neighbouring cells. Bridges, connecting fusing cells, were observed when cells were aligned which resulted in the formation of a polynucleated cell with many aligned nuclei (Fig. 5e). When the cells were in close contact on the coverslip, a big flat polykaryon was observed due to membrane fusion (Fig. 5f).

TABLE I

ELECTRICAL FIELD-INDUCED MORPHOLOGICAL MODIFICATIONS IN PLATED CHO CELLS

The analysis was operated on 36 different cells. A square of 25 μm^2 was scrutinized for the determination of the density in microvilli. Control samples are cells which are washed in the pulsing buffer but which are not pulsed; pulsed samples are treated by five pulses of kV/cm with a duration of 100 μs. Cells are fixed just after the pulses.

	Control sample	Pulsed sample
Cell surface (in μm^2)		
Mean	980	797
Standard deviation	534	336
Density in microvilli (per μm^2)		
Mean	12.4	17.6
Standard deviation	2.5	3
Number of fillipodia per cell		
Mean	9	14.9
Standard deviation	5	7.6

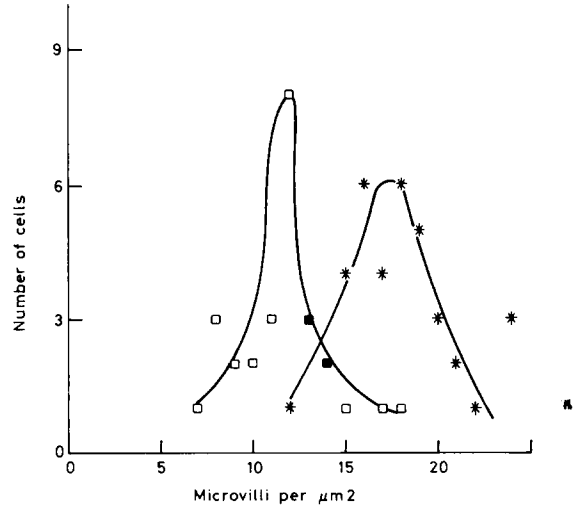

Fig. 6. Histogram of microvilli density in control and electropulsed cells. □, The results with control cells; *, with the pulsed samples, but fixed in the second following the pulses (0.6 kV/cm, five times, 100 μs).

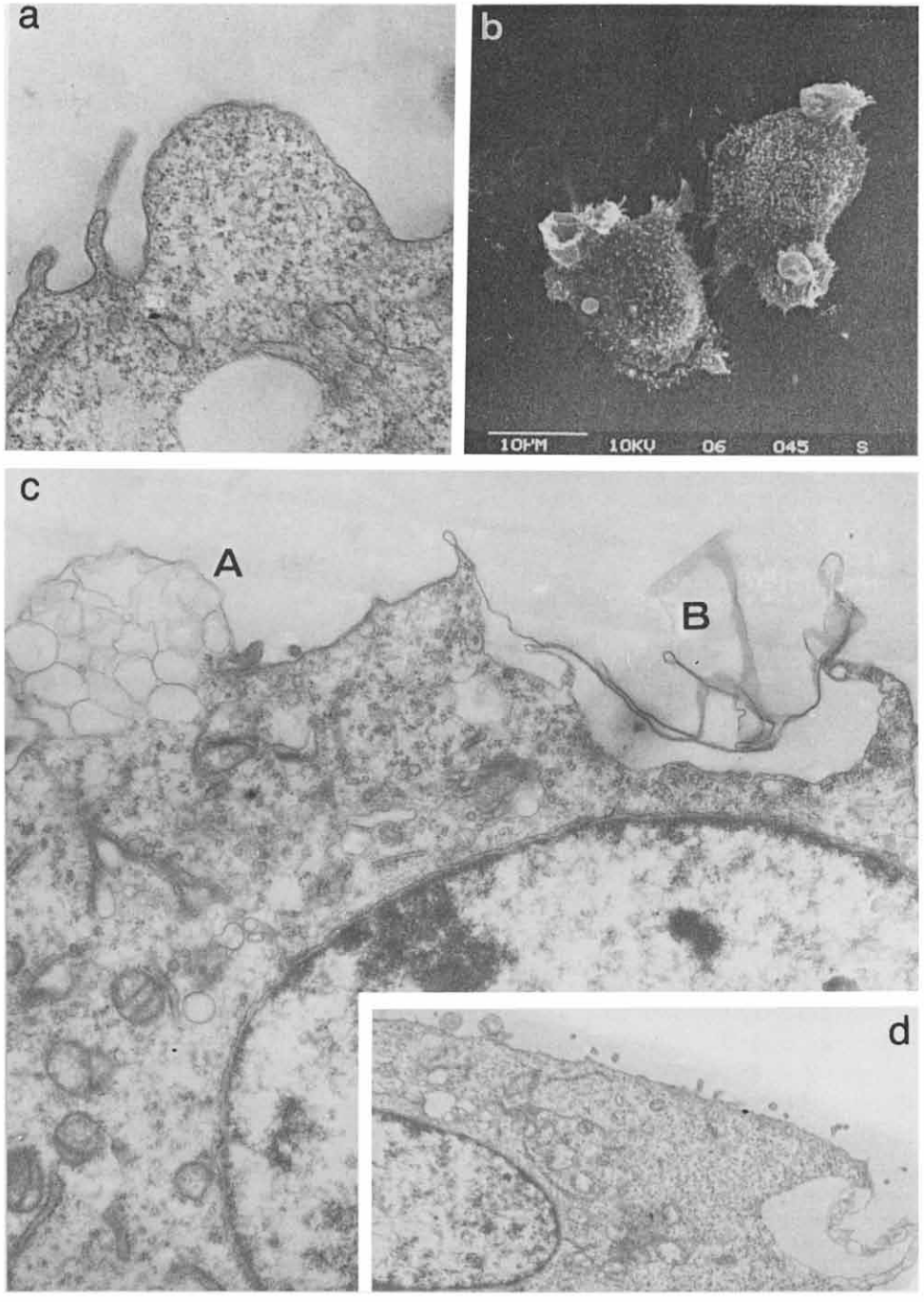

Fig. 7. (a) Ultrastructure of blebs in CHO control cell (×20000). (b–d) Just after electric pulses. (b) Large spherical blebs are present on the cell surface (scanning electron microscopy). (c) The transient blebs contain membrane material: some blebs contained numerous vesicles 'spherical blebs' (A), others were non-vesicular membranous elements 'crushed blebs' (B) (×20000); (d) these blebs can be localised on the edge of the cells (×5600).

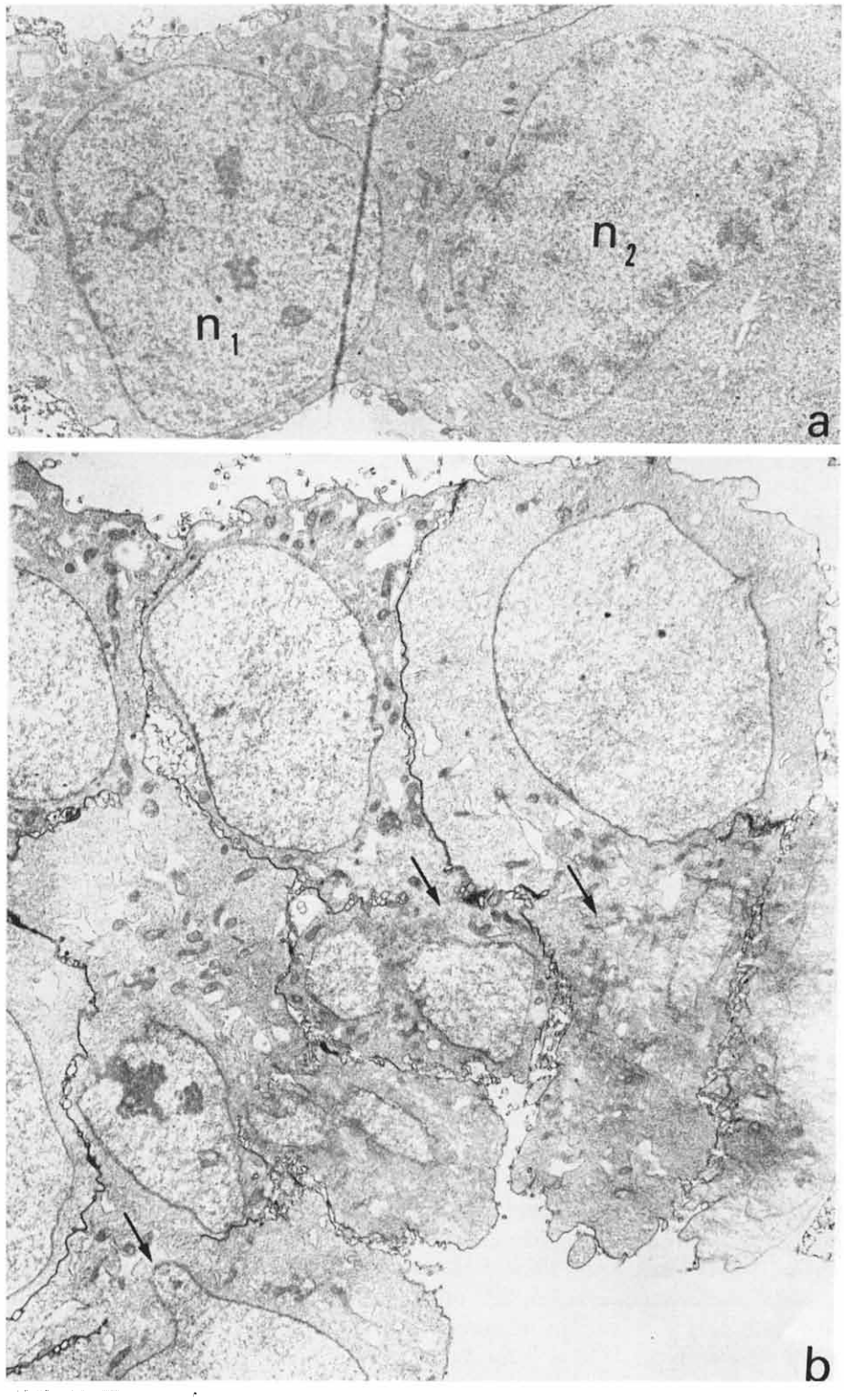

Fig. 8. (a and b) Cells incubated in the 37 °C culture medium following the electric pulses (5 min) and treated with Ruthenium red during fixation. (a) Interphase nucleus (n_1), prophase nucleus (n_2) (×6500); (b) several bridges between neighbouring cells were observed (arrow) (×6000).

From these observations we can conclude that the major effect which was induced during or in the very first seconds following the pulse was the eruption of numerous microvilli. This increase in the density of villi was transient and the same density as before pulsation was observed after the 20 min post-field incubation at 37 °C where fusion was taking place.

Table I shows the statistical analysis fo the morphological parameters of CHO cells under the control conditions (i.e., with short incubation in the pulsing buffer with no electric pulse) and just after pulsing. The means of the size of the cell surface and of the number of filopodia are not affected by the pulsation in a statistical significant way. The density of microvilli increased by 40%. This increase is statistically meaningful (difference in the means equal to the sum of the standard deviation) (Fig. 6).

Normal CHO cell ultrastructure and changes following application of electrical pulses.

Interphase CHO cells appeared to form a monolayer of flattened cells at the surface of the petri dish (Fig. 5a). The nucleus presented a reticulated nucleoli and small masses of condensed chromatin in close contact with the nuclear periphery. Ruthenium red did not penetrate the plasma membrane of normal cells and the top sides of the cells were intensely contrasted (Fig. 5b).

When the cells were fixed immediately after application of the pulses, their ultrastructure was relatively little changed. In the nucleus, more condensed chromatin was visible along the nuclear envelope (Fig. 5c), and some cytoplasmic vacuoles appeared. Frequently, thin sections of the spherical blebs were observed: these transient blebs described in scanning electron microscopy (Fig. 7b) appeared different in sections from the blebs seen in control cells which contained cytoplasmic components (compare Figs. 7a and 7c). Transient blebs were only formed by an assembly of numerous small vesicles (Fig. 7c, A). Frequently, these 'vesicular profiles' were observed on the upper side of the plasma membrane and also near the marginal zone of the membrane (Fig. 7d). Other deformations could correspond to the crushed bleb observed in scanning electron microscopy (Fig. 7b): they correspond on thin sections to non-vesicular membranous protrusions (Fig. 7c).

Cells fixed immediately after application of the electric pulses became permeable to Ruthenium red (Fig. 5d) but after a 5 min incubation in the culture medium (37 °C) following the electrical pulses, Ruthenium red did not penetrate into the cells and only the membranes which were in contact with the extracellular space were stained. This experimental procedure can detect membrane fusion between neighbouring cells, where groups of cells are in close contact (Figs. 8a and 8b). Numerous bridges were observed between interphase cells, and also between interphase and prophase cells (Fig. 8a).

When the cells were incubated for at least 20 min after the pulses, numerous polynucleated cells were observed.

By comparing the results obtained with Ruthenium red and those by scanning electron microscopy, it was clear that membrane permeabilization (detected by the permeability to Ruthenium red) occurred long before the formation of polynucleated cells.

Discussion

Square wave electrical field pulses induced the reversible permeabilization of the plasma membrane of plated CHO cells. The viability of the pulsed cells was not affected at all. Pulsed cells were checked to grow as control cells in the days following their pulsation (data not shown). In the present work, the permeabilization is a well-controlled process which is triggered only when the field is larger than a threshold (0.3 kV/cm). This threshold is not dependent on the kind of molecules or ions which permeate (Ca^{2+}, ethidium bromide, Trypan blue, PEG 1000, Dextran 1500). Our observation that the percentage of permeabilized cells is a function of both the intensity of the field (if larger than 0.3 kV/cm) and on the nature of the permeant species, is in agreement with the results on erythrocytes [17]. The size of the permeant structure is a function of the field intensity and of the pulse duration (which is kept constant in our study). If the permeant species is small (Ca^{2+}), the electropermeabilization is an all-or-not process which is triggered as soon as the field is larger than the threshold (Fig. 1). If the molecule

is larger (Trypan blue), then the field intensity should be stronger in order to increase the size of the defects (Fig. 1). The electrically induced permeant structures are not sensitive to the electric charge of the molecule which diffuses across the membrane. Positively charged (ethidium bromide) or negatively charged (Trypan blue) molecules can penetrate into the cytoplasm of electropermeabilized cells.

The electropermeabilization is thus linked to the occurrence of short-lived unspecific structures. But under conditions where the cell viability is not affected, the molecular weight of molecules which are able to cross the plasma membrane is limited. This is a puzzling problem in the understanding of the mechanisms underlying gene transfer which is obtained under similar field conditions [3–5].

The ultrastructural experiments were performed in line with this conclusion in order to provide more informations on the events. A previous study on erythrocyte ghosts showed very transient membrane changes whose life times (second time range) were not correlated with the one of the permeabilization state (minute time range) [16,17,31]. Furthermore, ghosts could not be considered as representative of the behaviour of viable mammalian cells. The time resolution of their study was very fast (ms time range) [26] and cannot be compared with the one in the present work. In our experiments, the time limit was in fact linked to the fixation step of the sample. However, it should be emphasized that glutaraldehyde fixation is relatively fast (about 15 s) which allows the observation of early events affecting the membrane. The present study showed that blebs and microvilli are induced within this time span.

Previous studies on CHO K1 cells (a transformed strain) have shown that the morphology of the cell is highly sensitive to its position in the cell cycle and to the culture density [20–22]. As a consequence, the cell morphology is not clearly defined. A more marked alteration was observed during 'reverse transformation' induced by treating CHO K1 cells with dibutyryl cyclic AMP [20,23,24]. The ribosome-filled knobs disappeared after treatment, and the cell surface became smooth with an absence of microvilli. These changes are not observed in pulsed cells, although other alterations are detectable, such as the eruption of numerous microvilli and penetration of Ruthenium red.

In order to obtain significant information on the processes linked to the pulse, we carried out a statistical analysis of the changes in the morphological parameters of the cell surface (Table I). The only significant alteration, we concluded, is the increase in the density of microvilli (Fig. 6). This observation was not present in poly(ethylene glycol)-treated cells [25] and appeared as specific to the electropermeabilization.

Since the time span for fixation is so short (less than 15 s), the considerable increase in plasma membrane surface area associated with the formation of microvilli is probably due to rearrangement of the membrane bilayer rather than to synthesis of new membrane components. This rearrangement can be explained by a change in the packing of the lipid matrix as a consequence of the alteration of the electrical properties of the cell, or by a shrinkage of the cell leading of a reduction of its volume, its surface staying constant.

These molecular rearrangements in the plasma membrane are not detected after chemical fixation and resin embedding. More sophisticated methods, using cryomicroscopy or cryofracture, will probably be required for observation of such rearrangements [25]. However, the penetration of Ruthenium red would suggest that the membrane barrier function had effectively been suppressed. These alterations were reversible, and the electron-dense tracer was unable to penetrate cells after 5 min of recuperation at 37 °C. This observation was in agreement with experiments previously reported using Trypan blue for example. After such a short interval (5 min), few polynucleated cells were detected on phase contrast microscopy, although at the ultrastructural level numerous bridges could be seen where the cells were in close contact (high density culture). Membrane fusion is the first step in the process leading to cell fusion. Formation of a polynucleated cell with clustered nuclei [2] requires considerable intracellular reorganization, particularly of the cytoskeleton. This process takes longer and fusioned polynucleated cells can only be observed on phase contrast after more than 20 min at 37 °C.

Immediately after the pulse application, a layer of condensed chromatin in close contact with the nuclear envelope was visible on ultrathin sections. The presence of Mg^{2+} ions in the pulsing buffer can explain this change [26]. A field intensity sufficient to allow permeation and fusion of CHO cells did not damage the cells, and relatively moderate alterations were observed. The transient disruption of the plasma membrane enabled exchange of ions and molecules between the intra- and extracellular spaces.

The appearance of numerous microvilli was too fast to be due to synthesis of proteins such as actin. Furthermore, it is known that the amount of membrane-bound actin in CHO cells remains constant during the cell cycle [27]. However, microfilament polymerization could have occurred in the time available (a few seconds). The molecular basis of such a polymerization could be due to leakage of small molecules such as cAMP, ATP or GTP. cAMP is thought to play a critical role in the organization of microtubules and microfilaments [28]. Application of electric pulses to CHO cells in suspension induces release into the medium of substances absorbing around 260 nm (data not shown).

Blebs are a common feature of electrically pulsed cells, as already described for pulsed hepatocytes [9]. But we wonder if they are not associated with glutaraldehyde fixation. Glutaraldehyde is known to cross-link proteins specifically, leaving lipids unaffected [29,30]. Using this fixative agent, blebs have also been observed in corneal cells [30]. These blebs are thought to stem from 'defects' in the structure of the membrane [29]. This idea is supported by our observations that the size of the 'transient blebs' is increased if fixation is carried out in hypotonic medium where the cytoplasmic internal pressure will tend to induce ballooning (data not shown). The existence of these transient blebs is directly correlated with the presence of the permeabilized state of the plasma membrane. These transient blebs could indicate weakening of the membrane either by reorganization of lipids of higher mobility, or by localized destruction of the cytoskeleton, leaving intrinsic proteins free to move within the bilayer. It is difficult to prove that there is a direct contact between cytoplasm and external medium. Such a direct link would make the formation of blebs impossible. However, the nature of 'permeant' structures which allow the passage of Ruthenium red or macromolecules, such as plasmids, requires explanation. We feel that the weakening of the membrane which induced the blebs represents a permeant structure where this type of molecular exchange can take place.

Acknowledgements

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