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LONG-LIVED MACROPINOCYTOSIS TAKES PLACE IN ELECTROPERMEABILIZED MAMMALIAN CELLS

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SUMMARY: Electropermeabilization is a technique which allows free access of molecules to cytosol. In the present study, we report on results dealing with the penetration of macromolecules. Under electric conditions that allow maintenance of cell viability at a high level, i.e., at low electric field intensity but long time duration (ms time range), all the pulsed cells become permeable to macromolecules. By loading ß-galactosidase the electro-transfered activity in the cells is maintained over 24 hours. Transfer mediated during the pulse occurs by free diffusion into the cytoplasm, while post pulse transfer takes place by a different pathway. When added a few minutes after application of the electric field, the enzyme enters the cell via a macropinocytosis-like process. This is a new long-term effect of the electric field pulse on the membrane.

Although electropulsation of cells is now routinely used in cell biology (gene transfer (1), cell hybridization, loading of cells with extracellular molecules (2)) and more recently in clinical applications (3), the molecular processes involved in the mechanisms of creation, stabilisation and annihilation of the transient structures responsible for permeation are still unknown. It was suggested that this was due to the punching of "pores" in the lipid matrix (4,5). But such a model was not borne out by other experiments showing long lived fusogenicity (6,7) and a change in the conformation of the phospholipid polar head regions (8). Such reversible membrane organization should be named "Transient permeated structures" (9). This lack of knowledge is now recognized by most scientists in the field (10) including those who previously suggested that "pores" were formed (11,12). The origin of these permeable structures induced by an electric field remains a puzzling problem. A lot of studies mainly performed on erythrocytes have reported on the diffusion of

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molecules with low molecular weight (ions, sucrose, erythritol,...) across the plasma membrane (13-15). Recently, the incorporation of macromolecules, other than plasmids (dextran, vegetal toxin, proteins, antibodies) into the cells by using electropulsation (16-20) has been reported on. And yet, the mechanisms of electrotransfer remain poorly understood.

In this study the ability of electric fields to allow the transfer of macromolecules across plasma membrane of Chinese Hamster Ovary cells under conditions compatible with their viability is analysed. Our results show that the property of long lived macropinocytosis is confered on cell membranes which are electropermeabilized.

MATERIALS AND METHODS

*Cell culture.*Chinese hamster ovary cells (WTT clone) were grown at 37°C in Petri dishes or in suspension under gentle agitation in Eagle's minimum essential medium (MEM0111) supplemented with glucose (3.5 g/l), tryptose phosphate (2.95 g/l), sodium bicarbonate (3.5 g/l), vitamines and 8% new-born calf serum. Antibiotics and glutamine (0.585 g/l) were added extemporally.

*Application of electrical pulses to the cells.*The cell permeabilization protocol for cells in suspension and in monolayer has been described elsewhere (21). Briefly, in the case of cells growing in Petri dishes, the culture medium was discarded and 1 ml of a low ionic saline and isotonic medium, the "pulsing-buffer" (10 mM phosphate, 250 mM sucrose, 1 mM $MgCl_2$, pH: 7.2) was added. Two thin stainless-steel parallel electrodes were dipped in the buffer and seated on the bottom of the culture dish. The electrodes were connected to a voltage generator which gave square-wave electric pulses (CNRS cell electropulser; Jouan, France). The voltage pulses applied to the cells were monitored with an oscilloscope included in the cell electropulser. Cells in suspension were centrifuged for 5 min at 350 g and resuspended in the pulsing medium at a concentration of 10^7 cells per ml. 100 µl of the suspension were placed between the electrodes. 5 pulses at 5 ms duration were then applied at various electric field strengths in order to permeabilize cells for macromolecules as described for gene transfer (12).

Determination of permeabilization. Electropermeabilization of cells was quantified by the penetration of nonpermeant dyes. Penetration of trypan blue (Sigma, T0887, 4 mg/ml in the pulsing buffer) was used to monitor permeabilization of cells for small molecules. Cells were pulsed, incubated 10 min at room temperature and then observed under an inverted light microscope. The percentage of permeabilized cells was 100 times the ratio of the number of blue-stained cells to the total number of cells. Penetration of fluorescein isothiocyanate dextran with average molecular weight of 18.9 and 71.2 kD (FD20, FD70) (Sigma, USA) was performed under the same protocol except that their concentration in the pulsing buffer was equal to 2 mg/ml. After incubation, cells were washed twice with pulsing buffer and observed under an inverted epifluorescence microscope. The percentage of permeabilized cells was determined as 100 times the ratio of the number of fluorescent cells to the total number of cells. Percentage of fluorescent cells as a quantification of the associated fluorescence was also performed by flow cytometry. Analysis was done with a Becton-Dickinson FACScan and an argon laser at 488 nm line at excitation. Wavelength at emission was above 530 nm.

To visualize the intracellular location of the probes, cells were observed using a 63x immersion lens under a confocal microscope (excitation 488nm; emission 530nm; LSM 410 invert laser scan, Zeiss, Germany). Computer-generated images of 1.1 µm optical sections were taken by repeated sectioning.

Penetration of ß-galactosidase (Gibco BRL, USA) was performed using a similar protocol with a final concentration in the pulsing buffer of 0.25 mg/ml. 10 minutes following pulsation, cells were washed and incubated in culture medium at 37°C in an air/CO_2 incubator for 24 hours before revealing the activity. In some experiments, revelation was performed immediately after pulsing. Cells were washed twice with PBS and fixed with formaldehyde 2% and glutaraldehyde 0.2% in PBS 5 min at 21°C. After washing twice with PBS, they were incubated for one hour at 37°C in a solution containing 1 mg/ml of 5-bromo-4-chloro-3indolyl-ß-D-galactopyranoside (X-Gal), 4 mM potassium ferricyanide, 4 mM potassium ferrocyanide, 1 mM $MgCl_2$ in a PBS buffer pH 7.3. Cells expressing the electrotransfered ß-gal activity appeared stained blue green within an hour. On the contrary, control cells remained colorless. Percentage of permeabilization was 100 times the ratio of the number of stained cells to the total number of cells.

Determination of the lifetime of permeability. Cells in suspension or on petri dishes, were pulsed in the pulsing buffer in absence of any dye but under the same conditions as for the permeabilization assay. They were kept at room temperature before adding the selected molecule diluted in the pulsing buffer. They were observed 5 minutes later or grown on Petri dishes with 2 ml of culture medium and observed 48 h later (about 2 generations).

Determination of electropulsed cell viability. Cells, in suspension or on petri dishes, were pulsed in the pulsing buffer in absence of any dye but under the same conditions as for the permeabilization assay. They were kept 5 min at room temperature and grown in Petri dishes with 2 ml of culture medium. Viability was measured by counting cells after 48 h (about 2 generations) under an inverted phase-contrast light microscope.

RESULTS

Electroloading of macromolecules.

When pulsing a CHO cell / macromolecule (FD20, FD70) suspension under long pulse (5 ms) conditions, permeabilization and loading were observed only for electric field intensities over than 0.4 kV/cm when 5 repeated pulses were applied at a frequency of 1 Hz. This critical intensity is the same needed to trigger permeabilization for small molecules such as trypan blue. When the field intensity was increased up to 1.0 kV/cm, all other parameters remaining unchanged, all cells were observed to be permeable to FD20, while a 1.6 kV/cm intensity was required to load all pulsed cells with FD70 (Fig. 1). Fluorescence was homogeneous in the cell suggesting that electropulsation gave free access to the diffusion of macromolecules as it did for small molecules.

Flow cytometry showed that above the threshold value of 0.4 kV/cm, increasing the electric field strength resulted in an increase of fluorescence intensity associated with each permeabilized cell up to 1.3 kV/cm (data not shown).

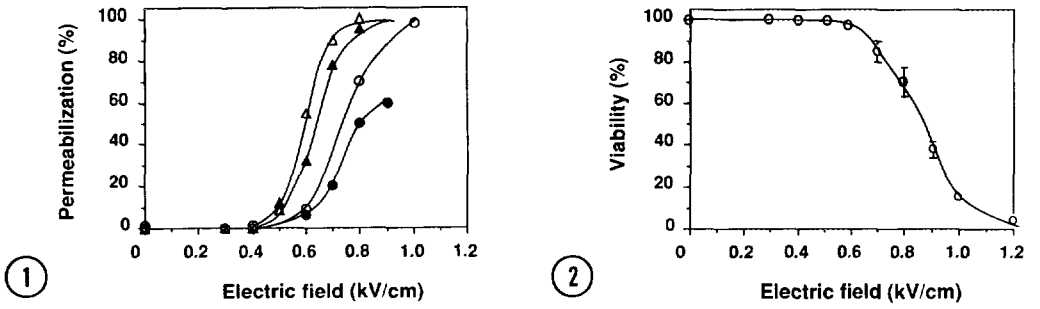


Figure 1. Effect of electric field strength on cell permeabilization.
Cells were pulsed 5 times at 5-ms duration with field of increasing strength in the presence of macromolecules.The level of permeabilization (i.e., the percentage of stained cells) is plotted in relation to the electric field strength. ▲: trypan blue; Δ: β-Galactosidase; ○: FITC-Dextran,20 kDa; ●: FITC-Dextran,70 kDa.

Figure 2. Effect of electric field strength on cell viability.
Cells were pulsed 5 times at 5-ms duration at various electric field strengths. The viability was measured as described in Materials and Methods by their ability to divide over 48 hours.

It was observed that 24 h after pulsation, the percentage of fluorescent cells did not change. Long pulse duration with low electric field intensity which is what we used, allowed penetration of macromolecules while slightly affecting the viability of the cells (Fig. 2).

Electroloaded enzymes remain active.

The detection of fluorescence in the cell cytoplasm is just evidence that transfer takes place but cannot tell us if the macromolecule is still intact. An enzyme, the ß-galactosidase, was therefore used to improve analysis of the phenomenon of electropermeabilization of cells for macromolecules. Its activity can easily be detected by giving cells a blue green coloration. The active form of the enzyme is made of 4 subunits, which must be intact after transfer to the cell. As for FITC labeled macromolecule experiments, cells were pulsed with electric field parameters that only slightly affected their viability: 5 pulses, 5 ms. They were grown plated on Petri dishes or in suspension. Cells grown on Petri dishes were pulsed at 0.8 kV/cm intensity in pulsing buffer containing the protein and incubated for various times before testing the electro-transfered activity. The percentage of cells revealing activity did not change significantly with time, at least not during 48 hours, i.e. about 2 generations, indicating that the activity was maintained in the cells during division (data not shown). As shown in figure 1, permeabilization of cells in suspension was again detected only for electric field intensities above a threshold value close to 0.4 kV/cm. Increasing the electric field strength therefore results in an increase in the number of cells in which the activity is detected. Results are given in Table I and figure 3. Enzymatic activity could be detected in all the cell population when the cells

Table I

Effect of time, t, between pulsation and addition of ß-galactosidase on the expression of the electrotransfered activity into CHO cells

time (min):	-5		1		5		10		60	
pulse duration (ms):	1	5	1	5	1	5	1	5	1	5
plated cells:										
Expression (%)	85	100	85	90	80	95	80	100	nd	nd
Viability (%)	80	80	80	80	80	80	80	80	nd	nd
cells in suspension:										
Expression(%)	60	100 70*	40	100 1*	30	100 2*	15	100 1*	25	100
Viability (%)	95	85	90	75	95	70	95	65	90	70

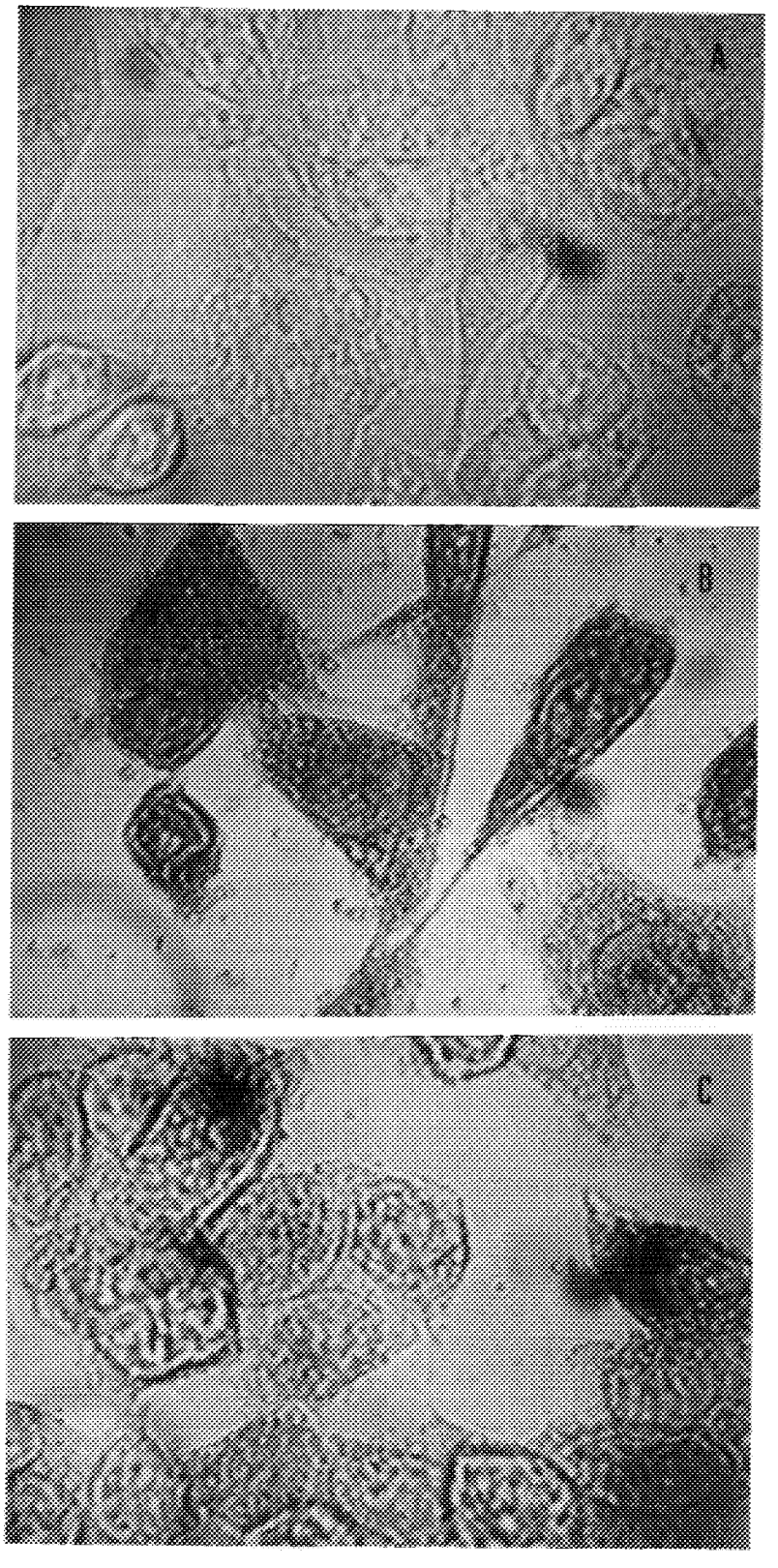
CHO cells were pulsed 5 times at 0.8 kV/cm in pulsing buffer in presence or in absence of ß-galactosidase at 0.25 mg/ml concentration. They were either plated on Petri dishes or in suspension. When the macromolecules were present during pulsation, they were added 5 min before pulsation (t=-5). When absent, they were added 1,5,10 or 60 min after pulsation (t=1, 5, 10 or 60). Electrotransfered ß-galactosidase activity and cell viability were measured 24 h after the electric treatment. *: Under these conditions, cells in suspension were pre-treated with colchicine prior to pulsation.

were pulsed 5 times, 0.8 kV/cm intensity and 5 ms duration. The viability of plated cells as well as of cells in suspension was highly preserved, being close to 70%.

Electroloading takes place in vesicles if the macromolecules are added after the pulse.

Taking the well known observation that electropermeabilization for small molecules is long lived into account (9), experiments were performed when the enzyme was absent during the pulses but added at increasing delays after them (up to 60 min). Experiments were performed at 21°C, revelation of enzymatic activity was performed 24 hours later. Results are shown in Table I. Transfer of enzyme into cells was always present when ß-galactosidase was added after pulsation, even after a long period, i.e. more than 60 minutes, when penetration of trypan blue is no longer

Figure 3. Micrographs of electropulsed CHO cells.
Cells were pulsed, or not, in pulsing buffer containing β–galactosidase, the presence of which was detected 24 hours after by adding X-Gal as a substrate.
A: CHO control cells in suspension.
B: CHO cells in suspension pulsed in the presence of the enzyme.
C: CHO cells in suspension pulsed in the absence of the enzyme which was added 10 minutes after pulsation.


A
B
C

present. Interestingly, the observation of cells under a microscope revealed a great difference in the coloration pattern. When ß-galactosidase was present during pulsation, the color was uniform in the cell cytoplasm, while it was localized in large vesicles when the enzyme was added after pulsation (figure 3). Such specific intracellular localization of the dye added several minutes after electropulsation was also observed in the case of fluorescent high molecular weight dextrans (FD70). This intracellular localization was confirmed by sections of cells (nominal thickness 1.1 µm) by confocal microscopy, in the minutes following electropulsation, while a classical homogeneous label throughout the cytoplasm was observed in the case where FD70 was present during pulsation (figure 4).

Correlation between macropinocytosic vesicles and the electropermeabilized part of the cell surface.

The origin of these "macropinocytotic" vesicles should clearly be the plasma membrane of the pulsed cell. It is known that only a very limited part of the cell surface is affected by cell electropermeabilization (15). If Es is the critical field strength which triggers cell electropermeabilization, then when a cell is pulsed with a field intensity E, the part of the cell surface, Ap, which is affected by electropermeabilization is (22):

$$Ap = 1/2 \times 4\pi r^2 (1 - Es/E)$$

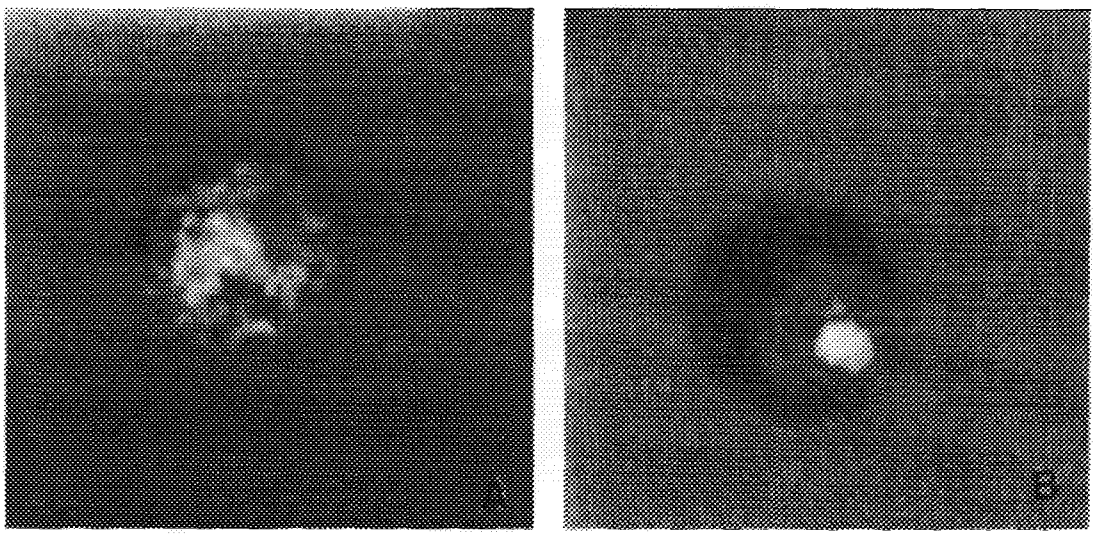


Figure 4 . Confocal micrographs of electropulsed CHO cells.
A: CHO cell pulsed in the presence of FD70
B: CHO cell pulsed in the absence of FD70 which was added 30 minutes after pulsation.
Micrographs were obtained from confocal microscope and printed on monochrome XLI Bitmap SuperDriver on LPT1 on Laser Jet printer.

r being the radius of the cell (6 μm for CHO cells in suspension). The parameter 1/2 is linked to the polarized character of electropermeabilization and to the shift associated with the resting potential difference (23). Under our conditions (Es = 0.4 and E = 0.8 kV/cm), Ap = 110 μm^2, the average size of macropinocytic vesicles is 3 μm in diameter, i.e. their surface is less than $4\pi (1.5)^2$, i.e. 27 μm^2. This supports the hypothesis that the macropinocytotic vesicle buds from the electropermeabilized part of the cell surface. As the cell surface area is:

$$At = 4\pi r^2 = 450\ \mu m^2$$

the vesicle surface is a 5% loss of the cell surface, i.e. only a 2.5 % loss in diameter. We were not able to detect such a small change.

Colchicine inhibits the formation of these vesicles.

Macropinocytosis is known to be inhibited by treating cells with cytochalasin or colchicine (24,25,26). We have recently show that the lifetime of the membrane's electropermeabilized state was strongly reduced by pretreating the cells with colchicine (27,28). The effect of colchicine pretreatment was checked on by incubating the cells with colchicine (6.3 μM, 30 min, 37°C) in order to depolymerise the microtubule network (27). The percentage of cells expressing activity was unchanged if the βgal/ CHO cell mixture was pulsed, but it dramatically decreased when the enzyme was added after pulsing the cells (Table I).

DISCUSSION

When applying long pulse duration (ms time range) the permeabilization of cells for macromolecules under non lethal conditions can be obtained. Under such conditions, all pulsed cells can be electropermeabilized for macromolecules and more than 80% of them remain viable after the electric treatment.

However, the molecular mechanisms underlying this phenomenon in membranes have not yet been elucidated. The fact that i) macromolecules added after the application of the pulses are able to enter the cells more than one hour after pulsation while trypan blue penetration under the same conditions is no longer detected in the cells when added 15 minutes after pulsation, and that ii) their localization is not homogeneous in the cytoplasm but only present in a large vesicle strongly supported the hypothesis that electropermeabilization could induce a long term macropinocytosis like effect in cells. It was previously observed that when added to electropermeabilized cells macromolecules were sequestred in small vesicles (28). This process was prevented when the cells were pretreated with cytochalasin (29). No check was made to see if the activity of the transfered molecules was preserved. Nevertheless, these observations bear out our conclusion that macropinocytosis is associated with electropermeabilization. Its sensitivity to

both cytochalasin and colchicine, the size of the induced vesicles suggests that this electrically induced incorporation of molecules is mediated by macropinocytosis and not by a clathrin dependent process or by the formation of 95 nm non clathrin coated vesicles (26,30). Moreover, under our experimental conditions leading to partial depletion of K^+ in cells due to K^+ release from pulsed cells, any endocytotic clathrin dependent process should be blocked (26).

The origin of these "macropinocytosis-like" vesicles is not clear, but their size supports the hypothesis that they come from the cell membrane area which has been electropermeabilized. Moreover, we noted a significant increase in the density of microvilli at the cell surface following the electropermeabilization of cells (31). Another major consequence of membrane electropermeabilization is the induction of a fusogenic state (6,7). One could therefore imagine that such post pulsation phenomena leading to the formation of potentially fusogenic membrane ruffling areas could be due to this "macropinocytosis-like" process, macropinosomes having been shown to form in membrane areas where ruffling activity is maximum (30).

So, as a conclusion, when using electric fields to permeabilize cells one has to take into account the fact that effects over a long time range may be induced in the cells even when membrane selective permeability has been restored again.

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