Biochimica et Biophysica Acta, 1111 (1992) 45–50

BBAMEM 75773

Experimental evidence for the involvement of the cytoskeleton in mammalian cell electropermeabilization

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(Received 21 May 1992)

Key words: Electropermeabilization; Cytoskeleton; (CHO cell)

Chinese hamster ovary (CHO) cells and human erythrocytes were pulsed by using square-wave electric-field pulses. This treatment induced their permeabilization. This phenomenon appears to be a three-step process of creation, expansion and annihilation of permeated structures. Altering the cell cytoskeleton, either with drugs, such as colchicine, known to depolymerise the microtubules in CHO cells, or by high temperature shock to affect the spectrin-actin network in erythrocytes, induced no modification on the first two steps of the electropermeabilization process, but was associated with a dramatic decrease in the stability of the electro-induced permeated structures. These experimental observations support the hypothesis of an implication of cytoskeleton in electropermeabilization in agreement with thermodynamic conclusions.

Introduction

The cell plasma membrane acts as a highly impermeable barrier to the diffusion of molecules between cytoplasm and external medium. Only a limited number of compounds can, in fact, enter the cell through the existence of specific transport systems, with concomitant expenditure of energy. The transfer of exogenous material into cells has remained a puzzling problem in cell biology for many years.

To gain access to the cytoplasm in intact cells, several methods have been developed including treatment with viruses, erythrocyte- and liposome-mediated transfer, poly(ethylene glycol)- and saponine shock and, more recently, microinjection and electropulsation [1]. Among them, electropermeabilization appears to be a convenient technique, keeping the cell viability rather unaffected. It presents the advantages of high reproducibility. It is simple, highly efficient and does not contaminate the cells by chemical additives. It is obtained by applying external electric-field pulses onto the cells which, when higher than a threshold value, cause membranes to become permeable [2]. This permeabilization can only be transient under controlled electric-field conditions and, as such, does not affect cell viability. Electropulsation is now routinely used in cell biology and biotechnology to introduce plasmids into the host genome for genetic manipulation, a phenomenon called electrotransformation [3,4]. Another property of the electropulsed membrane is its associated fusogenicity; cells put into contact before or after application of the electric pulses can fuse, a phenomenon called electrofusion [5–7].

Despite the increasing use of the electropulsing phenomenon, molecular mechanisms involved in the process are still to be elucidated. Several theoretical models have been proposed to explain the permeabilization through the formation of pores in the membrane [8,9]. Some experimental data have been obtained on model membrane systems. However, these theoretical models cannot explain experimental observations in the case of viable cells, in particular the high stability of the permeated state of cells. The lifetime of permeabilization in pure phospholipid vesicles is shorter than 1 s [10,11]. It can reach hours in case of mammalian cells [12,13] and plant protoplasts [14]. The same discrepancy exists in other field-associated processes. Although the electric field induced fusion between liposomes is a very fast process (time-range of a few seconds [15]), the electrofusion of plant protoplasts [16] and mammalian cells [17] requires hours. Moreover, the fact that permeabilization only occurs at a well-defined position on the cell surface and that permeated areas are not laterally mobile [18], suggests that compounds other than phospholipids are involved in the phenomenon. Indeed, from results on erythrocyte ghosts fusion [18] and mammalian cells [17] it can

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be calculated that the lateral diffusion coefficient of the components inside the permeated structures is close to 10^{-12} cm^2/s, a value that corresponds to membrane structures linked to the cytoskeleton [19].

The aim of the present study was to investigate the role of the cytoskeleton in the phenomenon of electropermeabilization. For this purpose, we used Chinese hamster ovary cells because of our knowledge on their electropermeabilization [20–22] and human erythrocytes which have a particular cytoskeleton and whose electropermeabilization is well-documentated too [12,23]. It has been detected by following the release of hemoglobin after pulsation and has been shown to be a long-lived phenomenon which can persist up to several hours after pulsation and is controlled by the temperature and the composition of the incubating medium [24]. Here, we report on the effect of altering the cytoskeleton either by chemical treatment (colchicine in the case of CHO cells) or by thermal treatment (in the case of erythrocytes) on electropermeabilization. Permeabilization was followed both by the penetration of exogenous molecules (Trypan blue in the case of CHO cells) and by the release of endogenous molecules (hemoglobin in the case of erythrocytes).

A preliminary report of this work was presented at the 47th International Meeting of Physical Chemistry: 'The Cell In Its Four Dimensions' in Gif-sur-Yvette, France, 24–28 September 1990.

Materials and Methods

Cell culture. Chinese hamster ovary cells (CHO cells) have been adopted in a large number of somatic cell-genetics laboratories (see Gottesman [25] for a review). We selected the wild-type CHO cell line (WTT clone), which differs from the parent CHO-K1 in being not strictly anchor-dependent. It grows plated in a monolayer (generation time 16–18 h) but has been adapted for suspension culture at 37°C under gentle agitation (100 rpm) in spinner flasks (generation time 18–20 h). Cells grown in suspension can be replated very easily. They are cultured in Eagle's minimum essential medium (MEM 0111; Eurobio, France) supplemented with glucose (3.5 g/l), tryptose phosphate (2.95 g/l), sodium bicarbonate (3.5 g/l), vitamines and 6% new born calf serum (Boehringer-Mannheim, Germany). Penicillin (100 units/ml), streptomycin (100 μg/ml) and L-glutamine (0.584 mg/ml) were added extemporally. Cells were maintained in exponential growth phase ($(4–10)\cdot 10^5$ cells/ml) by daily dilution of the suspension. For some experiments, cells were replated readily on Petri dishes (35-mm diameter, Nunc, Denmark) and kept at 37°C in a 5% CO_2 incubator (Jouan, France).

Electropermeabilization. The cell-permeabilization protocol for cells in monolayer or in suspension has been described elsewhere [26]. Briefly, in the case of CHO cells plated in petri dishes, the culture medium was discarded and 1 ml of a low ionic-content saline buffer, the 'pulsing buffer' (10 mM phosphate buffer, 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 traces of the electrodes on the dish allowed to separate pulsed cells from the control ones. The electrodes were connected to a voltage generator which gave square-wave electric pulses (CNRS electropulser; Jouan, France). The pulses applied to the cells were monitored with an oscilloscope incorporated into the cell electropulser.

Determination of the permeabilization. Electropermeabilization of CHO cells was quantified by penetration of unpermeant dyes, such as Trypan blue (T0887, 4 mg/ml in pulsing buffer, Sigma, USA). Cells were pulsed, incubated 5 min at room temperature and then observed under an inverted light microscope (Leitz, Germany). Cells were considered as permeabilized if their cytoplasm was stained in blue. The percentage of permeabilized CHO cells was 100 times the ratio of the number of blue-stained cells to the total number of cells.

Electropermeabilization of red blood cells from healthy donors, was quantified by the release of intracellular compounds, such as hemoglobin [12]. Red blood cells were washed by centrifugation (5 min, $11000\times g$, Hettich centrifuge, Germany) and resuspended into the pulsing buffer at a concentration of $5\cdot 10^7$ cells/ml. They were then pulsed and incubated for 1 h at 4°C before washing. The supernatant was removed and its optical density measured at 413 nm, the wavelength of maximal absorption for hemoglobin.

Determination of reversibility. This was assayed by the Trypan blue test. Cells were pulsed in the pulsing buffer which was replaced by the dye-containing buffer after the indicated time-span. Cells were incubated 5 min thereafter. Dye penetration was used to monitor the induced permeabilization of the membrane and not to assay the cell viability. Penetration of dye in still permeable cells was used in the case of CHO cells; permeabilization was again considered as positive if the cell cytoplasm was stained blue.

In the case of red blood cells, the release of hemoglobin after the electric pulses was measured in relation to the post pulse incubation time and was indicative of the presence of permeated structures in the membrane [12].

Determination of electropulsed cell viability. Plated CHO cells were pulsed in the pulsing buffer in absence of dye under the same conditions as for permeabilization assays. They were kept for 5 min at room temperature and the pulsing buffer was discarded and replaced by 2 ml of culture medium. Viability was measured by

following the growth of cells over 48 h (i.e., about 2 generations).

Cell treatments. CHO cells treatments affecting the ATP level were performed according to literature. Cells were either incubated for 3 h in pulsing medium before electric treatment, or were treated with 10 mM sodium azide (Sigma, USA) and 10 mM 2-deoxyglucose (Sigma, USA) after pulsation [27].

CHO cell treatments affecting the cytoskeleton organization were performed by using either nucleotides or drugs. Nucleotides (ATP and GTP) were used as polymerising agents of cytoskeleton [28]. Cells were incubated 30 min at 37°C in their culture medium with 1 mM of ATP or GTP. Then, they were pulsed according to the standard protocol. Drugs (colchicine and cytochalasin) were used as depolymerisating agents of the cytoskeleton [29]. Colchicine (Sigma, USA) was prepared as a stock solution in DMSO at 2 mg/ml and kept at 4°C. It was diluted 10-times in water when used and aliquot fractions were taken off from this last solution. Cytochalasin B (Sigma, USA) was prepared as a stock solution in DMSO (1 mg/ml) and kept at −20°C. It was diluted in distilled water when used for experiments at 3 different concentrations (2, 20 and 60 μM). Red blood cell cytoskeletons were affected according to a protocol described by Heubush [30]. They were incubated for 1 h at 50°C before use, to alter the spectrin-actin network and were observed to change their shape which became spherical.

Immunofluorescence staining was performed according to literature [17,31].

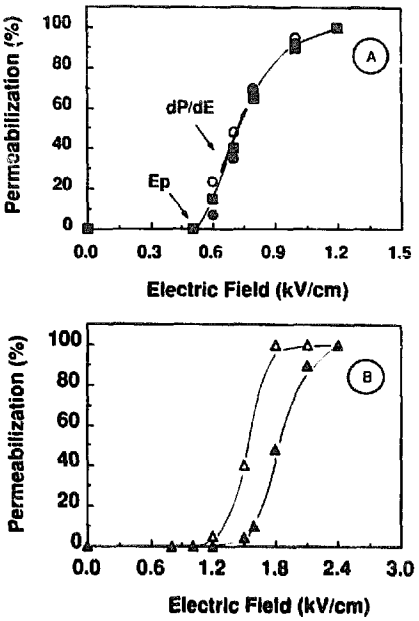

Fig. 1. Effect of electric field intensity on permeabilization of cells. (A), CHO; (B), erythrocytes. CHO cells were pulsed 10 times, 100 μs duration, with different intensities in pulsing buffer containing Trypan blue. The percentage of blue-stained cells, determined after a 5-min incubation delay, gave the percentage of permeabilization (○, control cells; ●, cells treated with 6.3 μM colchicine prior to pulsation; ■, cells treated with cytochalasin B 2–60 μM). Red blood cells were pulsed 10 times, 100 μs duration, with different intensities in pulsing buffer. After 1 h incubation at 4°C, they were centrifuged and the optical density of supernatant measured. Its value was related to the percentage of permeabilization (100% being obtained by a hemolytic shock of cells); △, control cells; ▲, cells incubated 30 min at 50°C prior to pulsation.

Results

Electropermeabilization of CHO cells

Cells were treated with electric field of increasing intensity at constant pulse number and duration (10 pulses, 100 μs duration). Permeabilization, assayed by the penetration of Trypan blue, was only detected for electric-field intensities greater than a threshold value E_p of 0.5 kV/cm. Increasing electric field intensity above this value resulted in an increase of the percentage of permeabilized cells (Fig. 1A).

This permeated state of the electro-treated cell membranes was only transient and could disappear with time if pulsing conditions were not too drastic. When cells were pulsed at 1.8 kV/cm, a value which allowed to permeabilize all the cell population, half of the cells became impermeable to Trypan blue in less than 6 min. After a 30 min incubation time, all the cells found their original impermeability again (Fig. 2A). ATP-depleted CHO cells pulsed under the same electric field conditions stayed permeable to Trypan blue, even 30 min following pulsation and were unable to grow on petri dishes (data not shown). On the contrary, CHO cells in mitosis, obtained by mitotic sheaking, became impermeable to Trypan blue in less than 1 min, indicating that in such cell membranes the transient permeability had a very short lifetime (data not shown).

Electropermeabilization of red blood cells

Human erythrocytes could be easily permeabilized by application of electric field pulses as previously reported [12,23]. Using square wave electric pulses, we detected the release of hemoglobin (i.e., the induction of a permeated state of the red blood cell membranes) for electric field intensities over a threshold value of 1.0 kV/cm at 10 pulses of 100 μs duration. As for CHO cells, increasing the intensity of the pulses resulted in an increase of the permeabilization efficiency, as indicated by the increase in hemoglobin leakage (Fig. 1B). This permeabilization was only transient and its lifetime depended on several parameters, such as the temperature or the composition of the pulsing buffer [12]. Under our experimental conditions, i.e., electric field pulses intensity of 2.4 kV/cm, 10 times, 100 μs duration, it appeared that hemoglobin was able to flow out of the cells during about 1 h after pulsation, which means that their membranes were still perme-

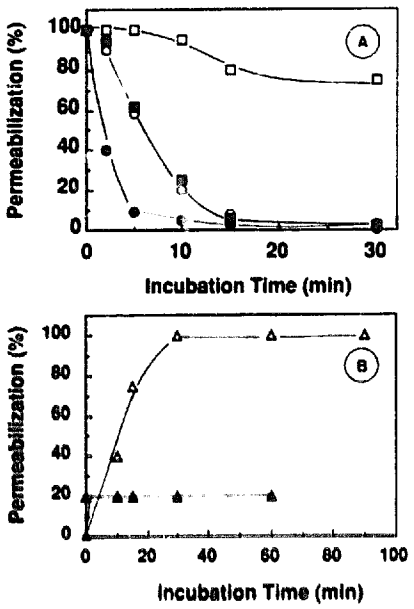

Fig. 2. Effect of the incubation time on the resealing process of electropermeabilized cells at 21°C. (A), CHO cells; (B), erythrocytes. CHO cells were pulsed 10 times, 100 μs duration, 1.8 kV/cm intensity in pulsing buffer. Trypan-blue-containing pulsing buffer was added to the cells after the indicated delay. Level of permeabilization was taken as the percentage of stained cells. (○, control cells; ●, cells treated with colchicine 6.3 mM prior pulsation; ■,□, cells treated with cytochalasin B respectively 2 and 20 or 60 μM prior pulsation). Red blood cells were pulsed 10 times, 100 μs duration at 2.4 kV/cm intensity in pulsing buffer. They were centrifuged after the indicated delay and the optical density of supernatant was measured. It determined the percentage of permeabilization (△, control cells; ▲, cells incubated 30 min at 50°C before pulsation).

able to large molecules after 1 h when cells were incubated at room temperature after electric-field treatment (Fig. 2B).

Effect of treatments affecting the cytoskeleton on electropermeabilization

CHO cells. Application of electric-field pulses on CHO cells allows both the penetration of exogeneous unpermeant molecules, such as Trypan blue (see Fig. 1A) and the release of cytoplasmic compounds, such as ATP and GTP [22,32]. As the polymerisation of cytoskeleton, mainly of its two major components (microtubules and microfilaments), is controlled by these nucleotides [28,29] we checked whether the field-induced depletion of ATP and GTP had a role. Addition of 1 mM GTP and ATP in the pulsing buffer, added to maintain their concentration in the cytoplasm at a constant level, did not result in any change in the permeabilization curve, nor in the resealing process (data not shown).

Two drugs, colchicine and cytochalasin B, known to inhibit respectively microtubule and microfilament polymerisation were used [31,33]. Incubation of cells 30 min at 37°C in culture medium with the drugs resulted in morphological alterations of the cells. Colchicine, used at a concentration of 6.3 μM, induced depolymerisation of the microtubules which were then only present around the microtubule organizing centers, as observed after immunofluorescence staining (data not shown). The cell diameter did not change after this treatment although several small pseudopods appeared around the cells. Cytochalasin, used at 3 different concentrations of 2, 20 and 60 μM, induced a decrease in the size of the cells, of their adherence to the petri dishes and was associated with a dramatic decrease in cell viability (up to 90%) for the two higher concentrations.

As shown in Fig. 1A, neither the incubation of the CHO cells with colchicine nor with cytochalasin B induced any change in the permeabilization curve: the threshold E_p and the slope of the curve, dP/dE ($P = 50\%$), were not affected. But, the resealing rate was greatly affected when cells were treated with colchicine: half of the cell population became unpermeable to Trypan blue in less than 2 min (Fig. 2A), with 6 min being the required time in the case of control cells as previously described. In the case of cytochalasin treatment, no difference was observed at the lower concentration. But at 20 μM and above, most cells were still dye-permeable after 1 h incubation at room temperature. But as we reported above, the drug has a lethal effect on its own.

Red blood cells. The alteration of the two-dimensional cytoskeleton of erythrocytes was performed by incubating the cells for 1 h at 50°C [30]. This treatment induces a change in the conformation of spectrin [34] and then an alteration in the spectrin-actin complex. After this thermal treatment, we observed a morphological alteration of erythrocytes which became spherical (diameter about 6 to 7 μm) loosing their original cup-like shape (diameter 7 to 8 μm). This was a direct indication of the spectrin-actin network alteration.

As shown in Fig. 1B, this treatment resulted in a slight but significant increase in the threshold value of electric field needed to permeabilize the cells from 1.0 to 1.2 kV/cm. As in the case of CHO cells, the resealing rate was greatly affected by this cytoskeleton alteration. Hemoglobin leakage out of the pulsed cells was only detected immediately following the electric-pulse treatment (i.e., in less than 1 min, the time required to do the experiment). The amount of hemoglobin in pulsing buffer did not increase with time as for untreated cells and kept the same value throughout the incubation time showing the dramatic increase in the resealing rate of heat treated cells (Fig. 2B).

Discussion

As previously reported by Kinosita and Tsong on red blood cells [12] and more recently confirmed by us

on CHO cells (20–22,35), the electropermeabilization phenomenon can be described by a three step process of (i), induction of transient permeated structures for electric field intensities greater than a threshold value E_p; (ii), expansion of these permeated structures which is related to the slope dP/dE of the permeabilization curve and then (iii), resealing of the permeabilization, the step that occurs after application of the electric pulses.

The experimental results reported here are consistent with such a description of the electropermeabilization phenomenon. The threshold value for permeabilization, E_p, is found to be smaller for CHO cells than for erythrocytes, an observation which agrees with what is known about the dependence of the threshold on the size of the cells [36,37]. E_p increases slightly when the erythrocytes have been heated, a treatment which induces a decrease in the red blood cell size. The first two steps of the process do not depend on the cytoskeleton state of the cells, as shown by the lack of effect of its depolymerisation when treating the cells with drugs or by heat. The third step of resealing appears on the contrary to be dramatically affected. On both systems of cells, CHO cells treated by colchicine and erythrocytes treated by high temperature, the resealing is speeded up. Moreover, preliminar experiments using synchronized CHO cells reported here show that cells in mitosis have a shorter-lived permeated state, mitosis being the phase of the cell cycle where microtubules are no longer present on the plasma membrane level.

We previously developed a quantitative analysis of electropermeabilization in terms of flow F of molecules m diffusing across the electropermeabilized cell membrane [22]. F is given by:

$$F_{(m,t)} = P_m \cdot \Delta m_{(t)} \cdot A \cdot X_{(N,T)} \cdot (1 - E_p/E) \cdot e^{-Kt} \qquad (1)$$

where P_m is the permeability coefficient of m accross the electropermeabilized membrane, Δm the concentration difference of m between cell interior and cell exterior, A the cell membrane surface and $X_{(N,T)}$ is the probability that permeabilization indeed occurs at a given point in the critical cap of the cell surface and is dependent on the pulse N and pulse duration T. E is the applied electric field intensity, E_p the threshold value for permeabilization and t the post-pulse time.

Permeabilization is detected only when the amount of accumulated dye during loading is larger than a critical value. As this value depends on F, F is controlling the detection of permeabilization. If F is smaller than an experimental threshold, the cell cannot be considered as being permeabilized. But, if F is larger than the experimental threshold, the cell is considered as being permeabilized. Resealing curves are then observed to be composed by 2 regions: a lag region which, when present, is indicative of the fact that the extent of recovery was not large enough to prevent an inflow of dyes large enough to be detected, and a curve which fits first-order kinetics well. The resealing rate constant K does not depend on the electric field intensity E. Its value can be calculated according to Eqn. 1 [22]. It is increased from 0.170 ($\pm$0.003) min^{-1} for control cells to 0.452 ($\pm$0.004) min^{-1} for colchicine-treated cells.

Morphological alterations of CHO cells have been detected in the few seconds following application of the electric field [38]. By scanning electron microscopy we observed that the density of microvilli on the cell surface was increased after pulsing. This alteration was present over the first min following the application of the pulses and then disappeared when the cells recovered their original selective permeability. A generation of cell processes was observed by using high-frequency fields where apparently only a deformation of the plasma membrane was observed [39,40]. However, in our experiments, microfilament polymerisation could be due to leakage of intracellular molecules such as ATP, GTP, cAMP [28,29] or penetration of Ca^{2+} (external concentration about 4 μM under our experimental conditions). These second metabolites are know to play a role in the organization of the cytoskeleton. Then, the cytoskeleton appears to be affected as a consequence of electropermeabilization not by a direct effect of the field, in agreement with experimental observations reported by others. Reversible electro-induced blebs, as well as cell protusions, have been observed in cells submitted to an electric field [40,41].

In a previous paper on CHO cell electrofusion, a phenomenon which can occur when cells in contact are pulsed [5,42,43], we reported direct biochemical and immunological observations, showing that the cytoskeleton has an active role and alters electrofusion [17]. Electrofusion appeared to be affected by colchicine treatment: depolymerisation of microtubules appeared to facilitate the fusion process. Taxol and cytochalasin B treatments, however, had no effect.

So, at this stage of our knowledge on electropermeabilization and electrofusion phenomena, the cytoskeleton and mainly the microtubules appear to be altered as a consequence of membrane permeabilization and to play a regulatory- and key role in the stabilization process of electropermeabilized cell membranes, presumably due to their interconnection with the plasma membrane [19,31].

For cells treated with drugs affecting the cytoskeleton, the resealing step, and as a consequence the associated activation energy of the process, are shown here to be affected. This is the reflection of an alteration in the interaction between the lipid-membrane protein and lipid-cytoskeleton complex. The energy for resealing depends on the temperature and on the energy pool of the cells. It can be given either by thermal

motion or by an enzymatic process (in agreement with the temperature-dependence of the resealing process, activation energy of 75 kJ/mol [17]). The hypothesis of the energy-dependence of resealing is strongly supported by the fact that resealing does not occur when starved CHO cells are used. This energy barrier could be greatly increased if interconnections between membrane components and cytoskeleton proteins existed, mainly for microtubules. Such a change in the activation energy must affect the kinetics of resealing by a decrease of the rate constant. This induces an increase in the lifetime of permeabilization. This increase in stabilization of permeated structures could be explained by the low lateral mobility of membrane proteins which are linked to cytoskeleton [33].

All these experimental observations strongly suggest that the cytoskeleton is involved in the electropermeabilization phenomenon. Firstly, it appears to be altered as a consequence of permeabilization at a morphological level and secondly, it seems to play a regulatory role in the stabilizating process of electropermeabilization. Molecular mechanisms involved in such a process are now to be elucidated.

Acknowledgements

M.P. Rols was partly supported by the Association pour la Recherche sur le Cancer. Thanks are due to J. Zalta for help with the cell culturing, for Dr. Robb for re-reading the manuscript and to J. Maurel for typing the manuscript.

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