Modulation of Electrically Induced Permeabilization and Fusion of Chinese Hamster Ovary Cells by Osmotic Pressure

Marie-Pierre Rols and Justin Teissié*

Centre de Recherche de Biochimie et de Génétique Cellulaires du CNRS, 118 route de Narbonne, 31062 Toulouse Cédex, France

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ABSTRACT: Cells can be transiently permeabilized by application of high electric pulses of short duration. A direct consequence of this treatment is to induce a fusogenic state in the pulsed membrane. The molecular events underlying these phenomena remain to be explained. During our work, we investigated the effects of pulsing buffer osmotic pressure on both electric field induced permeabilization and fusion of Chinese hamster ovary cells growing either in monolayers or in suspension. Osmotic pressure has no effect on the induction step of permeabilization, but its increase was shown to inhibit the expansion step and to decrease the efficiency of the resealing phase. Fusion efficiency was greatly affected by the osmotic pressure and by the physiological state of the cells. When cells were grown plated and when intercellular contacts were spontaneous and present during pulsation, increasing the osmotic pressure resulted in an increase in the fusion index. The opposite effect was observed for cells growing in suspension and brought into contact after pulsation. These results were tentatively explained in terms of the effect of the osmotic pressure on the membrane organization and cell–cell contact quality.

The cell plasma membrane is a highly impermeable barrier to the free exchange of molecules between the cytoplasm and the external medium. Transfer of exogenous material into the cytosol appears as a limiting step in cell biology and biotechnology. However, it has recently been shown that application of electric fields to cells can make their plasma membrane permeable (Neumann & Rosenheck, 1972). Under suitable conditions, electro-induced permeabilization can only be transient and does not affect the viability of the cells. The lifetime of the membrane-permeated state can be controlled by physical and chemical parameters of the technique such as pulse intensity, number and duration, temperature, and pulsing buffer composition (Kinosita & Tsong, 1977; Rols & Teissié, 1989). Furthermore, another property of electrotreated cells is their ability to fuse. Cells brought into contact before (Senda et al., 1979) or after (Sowers, 1986; Teissié & Rols, 1986) application of the electric pulses can fuse and lead to the formation of viable polycaryons. The driving force involved in the permeabilization is thought to be linked to an increase in the transmembrane potential (Bernhard & Pauly, 1973) which, when larger than a threshold value of about 200–300 mV (Teissié & Tsong, 1981), triggers a change in the permeability properties of the membrane. The molecular events involved in these phenomena are still unknown.

There are only a few experimental observations of the events affecting the membrane. Quick-freezing electron microscopy of Pronase-treated erythrocytes showed short-lived (1 s) morphological alterations of membrane during their electrofusion (Stenger & Hui, 1986). Scanning electron microscopy showed that the density of microvilli on the surface of Chinese hamster ovary cells (CHO cells)[1] increased immediately after application of the field (Escande-Geraud et al., 1988). ^{31}P NMR studies on CHO cells pointed out alteration of phospholipid organization during the permeated state of the membrane caused by reorientation of polar heads (Lopez et al., 1988). As the organization of the polar head groups is responsible for the hydration forces present when cells are brought into close contact, these observations would explain the fusogenic property of electropermeabilized membranes, because these repulsive forces prevent spontaneous cell fusion. Part of the energy required to permeabilize cells is then used to modify structural organizations inducing the order of the interfacial water molecules (Gruen et al., 1981). Electropermeabilization should reflect a change in membrane organization. All physical parameters able to alter the interfacial order by altering the membrane organization should be involved in the phenomena of electropermeabilization and in its consequent electrofusion.

It has been shown that hydrostatic pressure induces modification in membrane organization, specially in hydrocarbon order parameter and hydrogen bonding between head groups (Mc Donald, 1984). Moreover, the presence of an appropriate osmotic gradient is sufficient to promote fusion of lipid bilayer and phospholipid vesicles (Fisher & Parker, 1984; Akabas et al., 1984). As theoretical models describing the membrane as a homogeneous solid or a viscoelastic fluid predict that electropermeabilization should be a consequence of turgor pressure and electrocompressive stresses (Crowley, 1973; Zimmermann et al., 1977; Dimitrov, 1984), the osmolarity of buffers should play a regulating role in electropermeabilization and electrofusion of mammalian cells. Such a prediction is not valid when one considers that electropermeabilization is the consequence of the growth of structural defects (Abidor et al., 1979).

In this study, we analyze the effect of osmotic pressure on both electropermeabilization and electrofusion of mammalian cells. Electrofusion is a direct consequence of permeabilization but requires at least two distinct conditions: (i) closed contact

* To whom correspondence should be addressed.

[1] Abbreviations: CHO, Chinese hamster ovary; dP/dE, slope of curve at 50% permeabilization; E_o, threshold value for permeabilization; E_s, apparent threshold value for permeabilization; NMR, nuclear magnetic resonance; TPS, transient permeated structure(s); PEG, poly(ethylene glycol).

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between apposed cell membranes; (ii) suitable electric field pulses inducing a reorganization in the membrane leading to a fusogenic state. For cells in a monolayer, natural contacts are present during electric pulsation, whereas for cells in suspension they can be created after the electric treatment (Sowers, 1986; Teissié & Rols, 1986). It is then possible to investigate the effect of osmotic pressure on these two states of cells and to study the respective influence of osmotic pressure on both the permeabilization step of the fusion process and the quality of intercellular contact. Results are discussed in terms of membrane reorganization leading to changes in the extent of electropermeabilization and alterations in intercellular contacts. Chinese hamster ovary cells were chosen for these studies because of their ability to grow either in suspension or in monolayers and for the considerable amount of information we have already gathered about their electropermeabilization.

Materials and Methods

Materials

Chinese hamster ovary cells [CHO cells, clone WTT; see Gottesmann (1985)] are donated by Prof. Zalta (this institute). This strain differs from the parent CHO-K1 in not being strictly anchorage-dependent. It grows at 37 °C plated in monolayer in petri dishes (35 mm in diameter, Nunc, Denmark) but has been adapted for suspension culture under gentle agitation (100 rpm) in Eagle's minimum essential medium (MEM 0111, Eurobio, France) supplemented with 8% newborn calf serum (Boehringer, FRG), penicillin (100 units/mL), streptomycin (100 mg/mL) and L-glutamine (1.16 mg/mL). Cells are maintained in exponential growth phase (4×10^5 to 10^6 cells/mL) by daily dilution of the suspension. Cells grown in suspension can be replated easily.

Sample Preparation for Permeabilization and Fusion Tests with Plated Cells. Monolayers of cells are obtained by plating cells in suspension on petri dishes, and keeping them at 37 °C in a 5% CO_2 atmosphere. The cell density is 200 cells/mm^2 for the permeabilization assays and 600 cells/mm^2 for the fusion experiments. Monolayers of cells grown on a sterile coverslip are obtained by plating cells in suspension of petri dishes on which a coverslip was placed before adding the cell suspension.

Sample Preparation for Permeabilization and Fusion Assays with Cells in Suspension. Cells in suspension are centrifuged for 5 min at 350*g* (1000 rpm; Jouan C500 centrifuge, France) and then resuspended in the pulsation medium which is a low ionic strength pulsing buffer (10 mM phosphate, 1 mM $MgCl_2$, and 250 mM sucrose, pH 7.4) (Blangero & Teissié, 1983). Its osmolarity can be changed and is determined by its sucrose amount: iso-, hypo-, or hyperosmolarities are obtained with 250, 100, and 400 mM sucrose which correspond respectively to 270, 107, and 450 mOsm. Sucrose, being an unpermeant molecule, induces an osmotic stress on the membrane when the buffer is not isotonic.

Methods

The methods were adapted from those previously described (Teissié & Rols, 1988).

Electropulsation was operated by use of a CNRS cell electropulsator (marketed by Jouan, France). It gave square-wave electric pulses. Two thin stainless-steel parallel electrodes, connected to the voltage generator, gave a uniform electric field. In this way, the pulse field intensity and duration could be kept constant, and were not dependent on the pulsing buffer composition. The voltage pulse applied to the cell suspension was monitored with an oscilloscope incorporated in the cell pulsator. The field intensities were those observed on the oscilloscope.

Penetration of trypan blue (Sigma T0887, 4 mg/mL in the pulsing buffer) was used to monitor permeabilization. Just before application of the pulses, the culture medium was replaced with 1 mL of the dye solution. After 5-min incubation following pulsing, the cells were washed with pulsing buffer. Microscopic examination could easily distinguish the permeated cells whose cytoplasmic components were stained blue.

For cells in suspension, a similar protocol was used: cells were washed by centrifugation, and the cell pellet was resuspended at 5×10^5 cells/mL in trypan blue containing pulsation medium. One hundred microliters of cell suspension was placed between the electrodes on a culture dish. Voltage pulses were then applied (10 pulses, 100-μs duration). After 5-min incubation, the percentage of electropermeabilized cells was determined by microscopic observation.

In some experiments, ATP release was used to monitor permeabilization. ATP leakage from electropulsed cells was followed with a luciferin–luciferase assay (Teissié & Rols, 1988).

The size limit of molecules able to cross the electropermeabilized membrane was estimated by a competition technique as described in Schwister and Deuticke (1985) and Scherrer and Gerhardt (1971). Poly(ethylene glycol) (Merck, 5% w/v, a concentration which did not create an osmotic stress on the cells) was added to the pulsing buffer in the presence of trypan blue. Increasing the molecular weight of PEG hinders incorporation of trypan blue into the electropulsed cells. From the molecule size of the PEG which inhibits the penetration of trypan blue, the TPS sieving size limit is obtained (Schwister & Deuticke, 1985).

Addition of 2.5 mM calcium chloride to the pulsing buffer induced cell lysis when cells were electropermeabilized due the cytoplasmic calcium imbalance (Rols & Teissié, 1989). It was then impossible to restore the natural cell impermeability under conditions of pulsing parameters known otherwise to leave cell viability unaffected. The calcium-induced lysis was detected by the trypan blue penetration.

Reversibility of electropermeabilization was assayed by measuring the ability of cells to accept trypan blue after being electropermeabilized in the pulsing buffer in the absence of the dye (10 pulses, 100-μs duration, 1.5 kV/cm). After the indicated time lapse, pulsing buffer was replaced by the one containing trypan blue. Cells were incubated for 5 min at room temperature before the percentage of blue cells corresponding to cells still permeable to trypan blue was counted.

CHO cell diameter was obtained by directly measuring the size of cells on the monitor of a video camera (JVC, Japan) connected to an inverted microscope (Leitz, FRG). More then 200 cells were assayed per condition.

The cell fusion protocol for plated cells where contacts were present prior to pulsing has been described previously (Blangero & Teissié, 1983). Briefly, just before pulsation, the culture medium was replaced by 2 mL of the pulsing buffer, and voltage pulses were applied. After 2-h incubation at 37 °C, the fusion yield was then determined from the ratio of the number of nuclei from polynucleated cells to the total number of nuclei. In CHO cells, a background of polynucleation of about 10% was always present.

The cell fusion protocol for cells in suspension where the contact was created after pulsing was described elsewhere (Teissié & Rols, 1986). The cell suspension was washed by centrifugation at 100*g* for 5 min (800 rpm, C500 Jouan centrifuge) at room temperature. The cell pellet was then re-

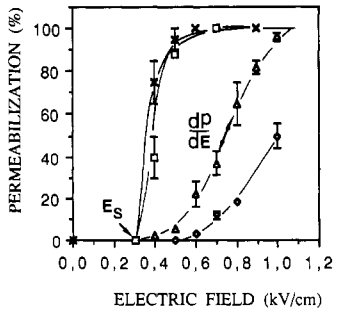

FIGURE 1: Effect of electric field intensity on plated CHO cell permeabilization. Permeabilization was observed by trypan blue penetration and counting the percentage of stained cells. Ten pulses of 100-μs duration were applied in pulsing buffers (10 mM phosphate/1 mM $MgCl_2$, pH 7.4) with different sucrose concentrations: (□) 100 mM sucrose (hypoosmolar buffer); (△) 250 mM sucrose (isoosmolar buffer); (◇) 400 mM sucrose (hyperosmolar buffer); (×) pulsing buffers containing the three different sucrose concentrations and 3 mM $CaCl_2$.

suspended in the pulsation medium at 5×10^5 cells/mL. Two hundred microliters of the cell suspension was placed between the electrodes on a culture dish. Voltage pulses were then applied (10 pulses, 1.6 kV/cm, 100 μs), and the permeabilized cells were centrifuged immediately in an Eppendorf tube at 45g for 10 min (700 rpm, C500 Jouan centrifuge) in order to pellet the cells and create contacts for their fusion. In some experiments, 1 mL of standard isotonic pulsing buffer was present in the tube during the centrifugation step. They were then incubated at 37 °C for 15 min. The sample was then dispersed in 2 mL of culture medium, and the resulting cell suspension was poured into a culture dish and incubated for 2 h in an air/CO_2 atmosphere at 37 °C. The plated cells were observed, and the fusion index was determined as described above.

All experiments were repeated at least 3 times at 2- or 3-day intervals in order to avoid fluctuations of results within a given set of experiments due to the physiological state of the cells. Relative changes induced by the osmotic pressure were always reproducible even if the absolute extent of permeabilization was changed (see Figures 1–3).

Results

Effect of Osmotic Pressure on Electropermeabilization. The effect of an electric field on cell electropermeabilization is proportional to the size of the cells (Bernhardt & Pauly, 1973). As tonicity changes of the suspension medium can induce modifications in cell size (Schmitt & Zimmermann, 1989), the size distribution of CHO cells, growing in petri dishes and in suspension, was measured under different osmotic pressure conditions. An average diameter of 14 μm (±1) was found for cells in suspension; cells in monolayers were shown to have size of 20-μm width for 40-μm length. These values did not change significantly with the osmotic agent concentration of pulsing buffer (100, 250, and 400 mM sucrose) during the 30 min following the change in buffer. Minor differences in cell shape appeared, however, in the case of cells in suspension. As previously described by Sarkadi (Sarkadi et al., 1984), a decrease in natural surface heterogeneities due to membrane evaginations occurred for CHO cells suspended at low sucrose content as observed under an inverted phase-contrast microscope (data not shown). An increase in the size of membrane folds was observed at high sucrose content. In contrast to lipid vesicles (Needham & Hochmuth, 1989) or myeloma cells (Schmitt & Zimmermann, 1989), CHO cells do not behave as osmometers but are able to maintain their volumes in a definite range of osmolarities. This may be due

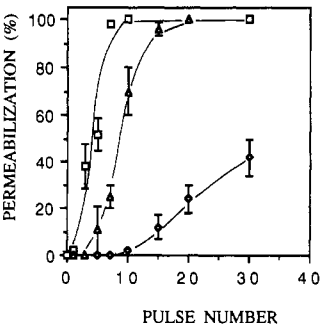

FIGURE 2: Effect of pulse number on the extent of CHO permeabilization. Pulses of 100-μs duration and 0.7 kV/cm were applied in pulsing buffers containing different sucrose concentrations: (□) 100 mM (hypoosmolar buffer); (△) 250 mM (isoosmolar buffer); (◇) 400 mM (hyperosmolar buffer).

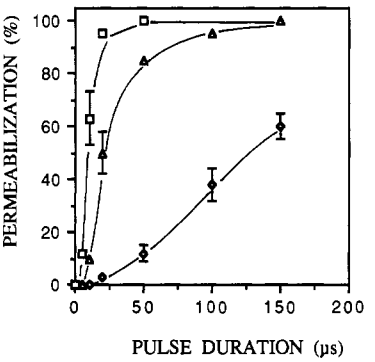

FIGURE 3: Effect of pulse duration on the extent of CHO permeabilization. Ten pulses of 0.7 kV/cm were applied in pulsing buffers containing different sucrose concentrations: (□) 100 mM (hypoosmolar buffer); (△) 250 mM (isoosmolar buffer); (◇) 400 mM (hyperosomolar buffer).

to the interaction between the membrane and the cytoskeleton as described for erythrocytes (Heubush et al., 1985). Hydrodynamic pressure is then applied to the membrane of the CHO cells.

Permeabilization of cells was performed as described under Materials and Methods, by following the incorporation of trypan blue into electropulsed cells. As already described (Rols & Teissié, 1989) and shown in Figure 1, permeabilization of CHO cells, in petri dishes, appeared only at electric field intensities higher than an apparent threshold value (E_s) which depends on the buffer osmolarity. This value was higher for cells electropulsed at high sucrose content. As previously reported (Rols & Teissié, 1989), E_s depends on the number of pulses and on the pulse duration. For a pulse number larger than 10 and a pulse duration longer than 100 μs, it reached a threshold value (E_o) of 0.3 kV/cm which does not depend on osmolarity. Increasing the field intensity above E_s resulted in an increase in the percentage of stained cells; 100% permeabilization was obtained with weaker fields at low rather than high osmotic pressure. The efficiency of permeabilization (described by the slope of the curve dP/dE at 50% permeabilization) was greatly enhanced by reducing the sucrose content of the pulsing medium. As reported above, electropermeabilization was strongly affected by the pulse number (Figure 2) and the pulse duration (Figure 3), but in both cases, the effect of the two parameters was more pronounced when the sucrose amount of the pulsing buffer was low.

Similar results were obtained with CHO cells growing in suspension; it was only when the field intensity became higher than the threshold E_o of 0.7 kV/cm that permeabilization occurred. This threshold was not dependent on the pulsing buffer tonicity, but again the efficiency of permeabilization

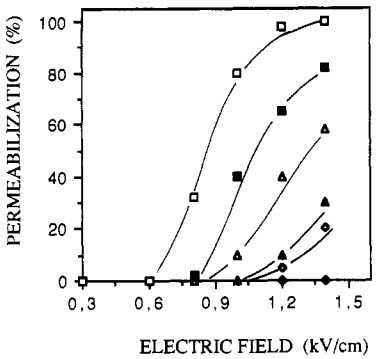

FIGURE 4: Effect of electric field intensity on the extent of CHO permeabilization in the presence of poly(ethylene glycol). Ten pulses of 100 μs were applied in pulsing buffer containing different amounts of sucrose: (□) 100 mM; (△) 250 mM; (◇) 400 mM sucrose in the presence of PEG of increasing molecular weight (open symbol, PEG 1450; closed symbol, PEG 6000).

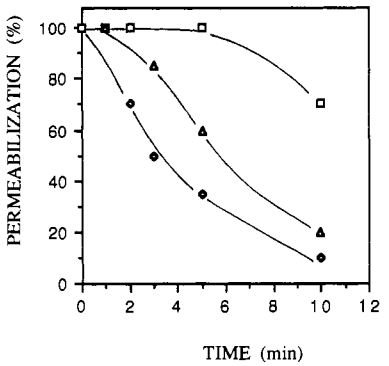

FIGURE 5: Effect of osmotic pressure on the resealing rate of CHO cells at 21 °C. Cells were pulsed 10 times at 100 μs and 1.5 kV/cm intensity in pulsing buffers containing different sucrose concentrations: (□) 100 mM (hypoosmolar buffer); (△) 250 mM (isoosmolar buffer); (◇) 400 mM (hyperosmolar buffer). Trypan blue was added at various intervals after the pulses. The level of permeabilization was taken as the percentage of stained cells.

was improved by decreasing osmotic pressure (data not shown). Since the osmotic pressure of the pulsing buffer strongly determined the efficiency of permeabilization, the effect of osmotic pressure during the 5-min incubation delay following the pulses was investigated by changing the buffer immediately after pulsing. That did not affect permeabilization which was only dependent on osmotic pressure during the electric treatment (data not shown). This shows a synergy only between the application of the field and the osmotic pressure, not with postpulse events.

The nature of the osmoticum was found to have no effect. Similar results were obtained with sucrose, sorbitol, and trehalose (data not shown).

By adding calcium in pulsing buffers, it is possible to detect permeabilization of cells for very small entities (calcium ion) by counting the percentage of lysed cells as explained under Materials and Methods. This confirms our previous observations that permeabilization was specific to the diffusion species (Escande-Geraud et al., 1988). As shown Figure 1, similar permeation curves to Ca^{2+} in the three different pulsing buffers were obtained. They were displaced to lower fields as compared to the one for trypan blue and were close to that then obtained with hypotonic buffer, the threshold field value E_o remaining unchanged.

Poly(ethylene glycol) molecules of different molecular weights were added to the three different osmotic pressure media in order to follow the effect of the sucrose content on sieving of the transient permeated structures (TPS). As shown in Figure 4, osmotic pressure greatly affected sieving of the TPS. In the same electric conditions of pulse intensity, duration, and number, larger PEG molecules were required to block trypan blue penetration in conditions of decreased osmotic pressure.

(A) Effect of Osmotic Pressure on the Resealing Process of Transient Permeated Structures. As described under Materials and Methods, osmotic pressure was kept the same during pulsing and postpulse incubation but brought back to isotonicity when trypan blue was present. As shown in Figure 5, resealing depended on the sucrose content of the pulsing buffer. Increasing the osmotic pressure of the buffer, apparently, made recovery of the original impermeability of the cells easier. It should be emphasized that comparing resealing processes in different experimental conditions should be done with pulsing parameters giving the same extent of permeabilization. Such a correction was previously described and proved to be needed for an accurate description of the phenomena (Rols & Teissié, 1989). In the case of high sucrose content buffer, the conditions in the resealing experiment (10 pulses of 100 μs, 1.5 kV/cm intensity) give a permeabilization of 80% which is obtained in the isotonic buffer for 0.9 kV/cm. We previously described that the rate of natural selective permeability of membrane recovery depended on electric field strength (Rols & Teissié, 1989), 50% resealing in the isotonic buffer occurs in less than 1 min for a 0.9 kV/cm intensity which is faster than the 3 min required in hypertonic buffer at 1.5 kV/cm. It can then be concluded that the resealing for cells pulsed in isotonic buffer is faster than those obtained in a hypertonic one. Using the same approach, we concluded that the resealing for plated cells in hypotonic buffer is faster than that obtained in isotonic condition.

Table I: ATP Leakage from Electropulsed CHO Cells[a]

sucrose concn of pulsing buffer (mM)	intensity of signal[b]	rate of ATP leakage[b]	cell state
100	2.0 (±0.2)	200	suspension
250	1.8 (±0.1)	135 (±5)	
400	0.9 (±0.2)	125	
100	0.8 (±0.1)	120 (±10)	monolayers
250	0.6 (±0.2)	75	
400	0.2 (±0.1)	17 (±2)	

[a] CHO cells either in petri dishes or in suspension were electropermeabilized (10 pulses, 100-μs duration, 0.8 and 1.2 kV/cm, respectively) under different osmotic pressure conditions. [b] Arbitrary units.

(B) ATP Leakage. ATP leakage from electropulsed CHO cells growing either in suspension or in petri dishes was measured as described under Materials and Methods, and was another indication of electropermeabilization of cells. Applying electric pulses, in suitable conditions, resulted in a very fast increase in signal; the thresholds E_o, needed to induce permeabilization, kept values of 0.3 kV/cm for cells in petri dishes and 0.7 kV/cm for cells in suspension, as obtained with the dye trypan blue (data not shown). However, as described in Table I, ATP leakage depended on osmotic pressure: maximal luminescence signal intensity and the initial rate of ATP leakage were greatly reduced in the presence of large sucrose amounts. This observation is in complete agreement with our data using trypan blue.

Effect of Osmotic Pressure on Electrofusion. Cells growing in monolayers in petri dishes present natural contacts and were used to study the impact of osmotic pressure on electrofusion for cells having natural intercellular contacts during electropulsation. As shown in Figure 6, electrofusion performed by pulsing the cells with a 100-μs duration occurred for field intensity greater than a threshold E_f of 0.6 kV/cm, which is

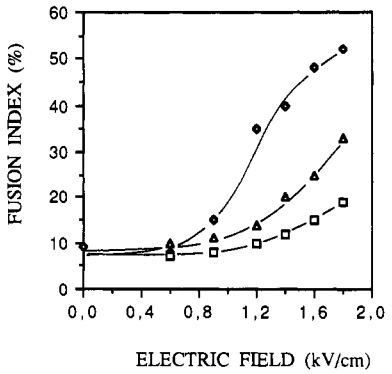

FIGURE 6: Effect of electric field intensity on plated CHO cell fusion. A pulse of 100 μs was applied to the cells in pulsing buffers containing different sucrose concentrations: (□) 100 mM (hypoosmolar buffer); (△) 250 mM (isoosmolar buffer); (◇) 400 mM (hyperosmolar buffer). Pulsing buffers were then replaced by culture medium and cells incubated for 1 h at 37 °C. The fusion index was determined.

the same for the three buffers. Above this value, increasing pulse intensity resulted in an increase in fusion efficiency. However, at a given electric field, the fusion index was enhanced by increasing the osmotic pressure associated with the pulsing buffer. The maximal fusion index was, however, the same whatever the osmolarity of the pulsing buffer.

In the case of cells in suspension, contacts were created after application of pulses by simple centrifugation (Teissié & Rols, 1986). The influence of osmotic pressure is then on the permeabilization step of the fusion process. Cells in suspension in pulsing buffer with different sucrose contents were pulsed under the same electrical parameters of pulse duration, number, and intensity and were then put into contact. The fusion index was observed to decrease from 25% (±3) in 100 mM sucrose down to 7% (±1) in 400 mM sucrose, a basal level of 5% being present and a 14% (±4) index being detected in 250 mM sucrose. These fusion indexes were not affected by the osmotic pressure of the buffer during the centrifugation (data not shown).

Discussion

Application of a suitable electric field to cells makes the plasma membrane become transiently permeable (Neumann & Rosenheck, 1972). Induction of transient permeated structures (TPS) occurs when the field intensity becomes higher than a threshold value depending on the size of the cells. These values, 0.3 kV/cm for CHO cells in monolayers and 0.7 kV/cm for cells in suspension, are independent of the osmotic pressure associated with the pulsing buffer (Figure 1). The efficiency of electropermeabilization to trypan blue (i.e., the percentage of blue stained cells) is nevertheless enhanced by decreasing the osmolarity of the pulsing medium, in similar pulsing conditions (Figures 2 and 3), with field intensities higher than these thresholds.

All these results, obtained either with plated cells or with cells in suspension, are in agreement with a two-step mechanism of TPS formation proposed some years ago (Kinosita & Tsong, 1979; Chernomordik et al., 1983) and observed recently with CHO cells (Rols & Teissié, 1989), a step in TPS induction, as soon as the field intensity becomes higher than a threshold value E_o, and a step in increase in TPS sieving and number by increasing the field intensity, the number, and the duration of pulses. Our results show that the induction phase is not dependent on the osmotic pressure (E_o being constant) but the expansion appears to be strongly affected by it. The TPS resealing process is also dependent on osmotic pressure and is shown to be faster when performed at low sucrose content (Figure 5).

The major conclusion of this study is that the induction of permeabilization is triggered for the same field intensity, E_o, whatever the osmotic pressure applied to the cell membrane, E_o being the thermodynamic threshold not the apparent one, E_s. The fact that E_o is a constant appears to contrast with the report on lipid vesicles (Needham & Hochmuth, 1989) even if one corrects the data by the size effect. Nevertheless, one should observe that these results with model systems deal with E_s, i.e., the apparent value when the pulse duration is short (60 μs), not with the real parameters, E_o, which is only observed with long pulses, as we did. The second point is that it is the expansion step, as previously suggested in the case of erythrocytes (Kinosita & Tsong, 1979), which is affected by the osmotic pressure.

This effect of osmotic pressure on electropermeabilization is confirmed by the results on the induced leak of ATP. Decreasing osmotic pressure facilitates the expansion of TPS and/or increases their number. As the expansion of TPS will increase their sieving, our observation is that decreasing the sucrose content of the buffer facilitates the penetration of large molecules such as PEG (Figure 4); we can conclude that the expansion of TPS is increased by a decrease in osmotic pressure. The penetration of small species such as Ca^{2+} is not affected. One should consider that due to their small size, their influx is not linked to the sieving of the TPS but to the density of TPS. The number of TPS is then not affected, and osmotic pressure plays a role only on the extension step of the TPS.

According to models developed to explain electropermeabilization, conditions for membrane rupture leading to membrane permeability should derive from the balance between electrostatic and elastic restoring forces. Applying a pressure should result in an increase in membrane tension and then in a decrease in the membrane voltage needed for rupture (Dimitrov, 1984). This was reported in plant protoplasts (Zhelev et al., 1986) and algae (Zimmermann et al., 1977). However, as in both cases no study of pressure effect on cell size was performed, these results cannot be considered as conclusive due to the fact that only animal cells exposed to anisotropic medium are reported to regulate their volume (Sarkadi et al., 1984). Our observations suggest that as E_o is not dependent on osmotic pressure, the model of the electrocompressive force inducing the "breakdown" (Crowley, 1973; Zimmermann et al., 1979, Needham & Hochmuth, 1989) is not valid in the case of CHO cells. Among different explanations, it is clear that the existence of microheterogeneities and of the cytoskeletal network rule out the thermodynamic description of the membrane as a homogeneous solid, which is postulated in these models. Another thermodynamic description of electropermeabilization relies upon the existence of small structural defects in the membrane which grow in size under the action of the transmembranous electric field (Abidor et al., 1979; Weaver et al., 1984). In this model, no prediction concerning the impact of pressure on electropermeabilization is given except that the expansion steps appear critical in these explanations.

We must emphasize the consequence of our observations that CHO size is not affected by osmotic pressure. The applied pressure on the membrane keeps the same magnitude in the hypoosmotic and in the hyperosmotic buffers. It is only the direction of application of the pressure which is inverted. As a consequence, it is difficult to explain our observations by an effect on the stretching properties of the membrane as is done by others with lipid vesicles (Needham & Hochmuth, 1989).

One should take into account one of the striking consequences of electropermeabilization, the induced fusogenicity

(Sowers, 1986; Teissié & Rols, 1986). The molecular reorganization associated with electropermeabilization gives a decrease in the intermembrane repulsive forces. It has been shown that due to the lack of surface tension in bilayers, membranes present large fluctuations, which are responsible for enhancement of hydration repulsive forces when cells are in close proximity (Ostrowsky & Sornette, 1985; Sornette & Ostrowsky, 1984; Evans & Parsegian, 1986). More energy must then be given to the system of the two interacting membranes in order to bring them to a fusogenic organization. Decreasing the osmotic pressure of the pulsing medium results in a decrease in cell surface evaginations, as observed by phase-contrast microscopy in CHO cells in suspension (whose membrane becomes smoother in hypoosmotic buffer) and by a decrease in membrane undulations. This can explain our observations about the effect of osmolarity on electropermeabilization of CHO cells in line with our previous work dealing with ionic effects (Rols & Teissié, 1989). The step of TPS induction is independent of osmotic pressure (E_0 being the same whatever the buffer). In contrast, the step of TPS extension, which is observed to be dependent on osmotic pressure, is enhanced by lowering the sucrose content. This is confirmed by the results with cells in hypertonic medium: the observed increase in membrane undulations, and then in membrane structures leading to hydration forces, can explain the more difficult permeabilization. As described for the effect of bulk hydrostatic pressure (MacDonald, 1984), the stress associated with osmotic pressure will change the packing of the lipids and as such the interaction between lipids and proteins. The induced volume change would affect the hydration properties of the interface and as a consequence the energy required to dehydrate it.

Fusion of plated CHO cells was observed for field intensities greater than 0.6 kV/cm. Above this value, index fusion was enhanced and even more easily by increasing the sucrose amount in pulsing buffer (Figure 6), the same final index being present. As we showed in this case, permeabilization of membrane is more limited, this suggests that a compensating effect was present in cell–cell contacts. Facilitation of cell contact at high pressure can be explained by a flattening effect of pressure on cells inducing a more favorable quality in contact. Fusion is triggered under smoother conditions at high pressure, but the number of cells which are able to be fused remains the same. This can be explained by the cell–cell contact orientation relative to the fields (Teissié & Blangero, 1984). An opposite observation was obtained with cells in suspension where contacts were created after pulsing which showed an increase in the fusion index at low osmotic pressure, i.e., in conditions of a highly permeabilized state. In that case, fusion efficiency was only under the control of permeabilization efficiency.

Results on osmotically swollen cells indicate that fusion was facilitated but dramatic alterations of the cytoplasmic organization were present as these cells behave as osmometers (Schmitt & Zimmermann, 1989). It was shown that electrofusion of Pronase-treated human erythrocytes and myeloma cells, aligned by dielectrophoresis and submitted to electric field, was osmotic pressure dependent (Ahkong & Lucy, 1986; Brown et al., 1986). Cells in 150 mM erythritol fused into long chains, while fusion of erythrocytes was negligible in 400 mM erythritol. This agrees with our results on cells electropermeabilized in suspension and then brought into contact. It was then suggested that osmotic swelling may provide a driving force for the membrane fusion reaction as in the case of vesicle and planar bilayers (Akabas et al., 1984) and erythrocytes (Ahkong & Lucy, 1986). These speculations are not supported by our experimental observations on CHO cells. The membrane fusion step of cells in suspension is not directly altered by osmotic pressure, in agreement with experiments on exocytosis in mast cells (Zimmerberg et al., 1987). Its effect is to modulate the extent of electropermeabilization by affecting the TPS sieving, and as such, it alters the fusogenic character of the permeabilized membrane.

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References

Abidor, I. G., Arakelyan, V. B., Chernomordick, L. V., Chizmadhev, T. A., Pastushenko, V. F., & Tarasevich, M. R. (1979) *Bioelectrochem. Bioenerg. 104*, 37–52.

Ahkong, Q. F., & Lucy, J. A. (1986) *Biochim. Biophys. Acta 858*, 206–216.

Akabas, M. H., Cohen, F. S., & Finkelstein, A. (1984) *J. Cell Biol. 98*, 1063–1071.

Bernhardt, J., & Pauly, H. (1973) *Biophysik (Berlin) 10*, 89–98.

Blangero, C., & Teissié, J. (1983) *Biochem. Biophys. Res. Commun. 114*, 663–669.

Blangero, C., & Teissié, J. (1985) *J. Membr. Biol. 86*, 247–253.

Brown, S. M., Ahkong, Q. F., & Lucy, J. A. (1986) *Biochem. Soc. Trans. 14*, 1129–1130.

Chernomordik, L. V., Sukharev, S. I., Abidor, I. G., & Chizmazhev, Y. A. (1983) *Biochim. Biophys. Acta 736*, 360–373.

Crowley, J. (1973) *Biophys. J. 13*, 711–724.

Dimitrov, D. S. (1984) *J. Membr. Biol. 78*, 53–60.

Escande-Geraud, M. L., Rols, M. P., Dupont, M. A., Gas, N., & Teissié, J. (1988) *Biochim. Biophys. Acta 939*, 247–259.

Evans, E. A., & Parsegian, V. A. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83* 7132–7136.

Fischer, L. R., & Parker, N. S. (1984) *Biophys. J. 46*, 253–258.

Gottesman, M. M. (1985) in *Molecular Cell Genetics*, Wiley-Interscience, New York.

Gruen, D. W. R., Marcelja, S., & Pailthrope, B. A. (1981) *Chem. Phys. Lett. 82*, 315–320.

Heubusch, P., Jung, C. Y., & Grenn, F. (1985) *J. Cell. Physiol. 122*, 266–272.

Kinosita, K., & Tsong, T. Y. (1977) *Biochim. Biophys. Acta 471*, 227–242.

Kinosita, K., & Tsong, T. Y. (1979) *Biochim. Biophys. Acta 554*, 479–497.

Lopez, A., Rols, M. P., & Teissié, J. (1988) *Biochemistry 27*, 1222–1228.

MacDonald, A. G. (1984) *Philos. Trans. R. Soc. London, B 304*, 47–68.

Needham, D., & Hochmuth, R. M. (1989) *Biophys. J. 55*, 1001–1009.

Neumann, E., & Rosenheck, J. (1972) *J. Membr. Biol. 10*, 279–290.

Ostrowsky, N., & Sornette, D. (1985) *Chim. Spectra 25*, 108–111.

Rols, M. P., & Teissié, J. (1989) *Eur. J. Biochem. 179*, 109–115.

Sarkadi, B., Attisans, L., Grinstein, S., Buchwald, M., & Rothstein, A. (1984) *Biochim. Biophys. Acta 774*, 159–168.

Scherrer, R., & Gerhardt, P. (1971) *J. Bacteriol. 107*, 718–735.
Schmitt, J. J., & Zimmermann, U. (1989) *Biochim. Biophys. Acta 983*, 42–50.
Schwister, K., & Deuticke, D. (1985) *Biochim. Biophys. Acta 816*, 332–348.
Senda, M., Takeda, J., Abe, S., & Nakamura, T. (1979) *Plant Cell Physiol. 20*, 1441–1445.
Stenger, D. A., & Hui, S. W. (1986) *J. Membr. Biol. 93*, 43–53.
Sornette, D., & Ostrowsky, N. (1984) *J. Phys. (Les Ulis, Fr.) 45*, 265–271.
Sowers, A. E. (1986) *J. Cell Biol. 102*, 1358–1362.
Teissié, J., & Tsong, T. Y. (1981) *Biochemistry 20*, 1548–1554.
Teissié, J., & Rols, M. P. (1986) *Biochem. Biophys. Res. Commun. 140*, 258–266.
Teissié, J., & Rols, M. P. (1988) in *Dynamics of membrane proteins and cellular energetics* (Latruffe, N., Gaudemer, Y., Vignais, P., & Azzi, A., Eds.) pp 251–268, Springer-Verlag, Berlin and Heidelberg.
Weaver, J. C., Powell, K. T., & Mintzer, R. A. (1984) *Bioelectrochem. Bioenerg. 12*, 393–404.
Zhelev, D. V., Dimitrov, D. S., & Tsoneva, I. (1988) *Bioelectrochem. Bioenerg. 19*, 217–225.
Zimmerberg, J., Curran, M., Cohen, F., & Brodwick, M. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 1585–1589.
Zimmerman, U., Beckers, F., & Coster, H. G. L. (1977) *Biochim. Biophys. Acta 464*, 399–416.

Estimation of Disk Membrane Lateral Pressure and Molecular Area of Rhodopsin by the Measurement of Its Orientation at the Nitrogen–Water Interface from an Ellipsometric Study†

Christian Salesse, Daniel Ducharme, Roger M. Leblanc,* and François Boucher
Centre de Recherche en Photobiophysique, Université du Québec à Trois-Rivières, 3351 Boulevard des Forges, CP 500, Trois-Rivières, Québec, Canada G9A 5H7
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ABSTRACT: The internal lateral pressure of a bilayer has been estimated by numerous investigators. Most of these measurements were made by using the monolayer technique. In our approach, the disk membrane lateral pressure was estimated by assuming that this value is equal to the surface pressure necessary to maintain the transmembrane orientation of rhodopsin. The orientation of rhodopsin at the nitrogen–water interface was determined by using ellipsometry, which can measure the thickness of the film. By examining surface pressure and ellipsometric isotherms of intact and partially hydrolyzed rhodopsin, we have determined that a lateral pressure of 38 mN/m is necessary to give rhodopsin its natural transmembrane orientation and that surface pressures exceeding 45 mN/m lead to the formation of multilayers in the disk membrane film. At 38 mN/m, pure rhodopsin is found to have a molecular area of 2300 Å^2.

A number of studies have evaluated the lateral pressure in a lipid bilayer by using model systems. Blume (1979) and Albrecht et al. (1978), who compared phase transitions of phospholipids in monolayers and bilayers, reported values of 30 and 12 mN/m, respectively. Hui et al. (1975) found, from electron diffraction studies, that the molecular packing of a bilayer corresponds to that of the precursor monolayer at a surface pressure of 47 mN/m. Demel et al. (1975), who compared the action of various phospholipases on erythrocyte membranes and on phospholipid monolayers, concluded that the lipids of the outer monolayer of the erythrocyte membrane experience a lateral surface pressure between 31 and 34.8 mN/m. Nagle (1980) and Gruen and Wolfe (1982) reported a theoretical value of 50 mN/m. In their calculations of a membrane lateral pressure, Georgallas et al. (1984) considered the interaction between lipid chains in opposite halves of the bilayer and found a value of 30.5 mN/m.

In the disk membrane of rod visual cells, rhodopsin comprises at least 95% of the intrinsic proteins (Krebs & Kühn, 1977; Amis et al., 1981). In these membranes, the bilayer structure maintains the transmembrane orientation of rhodopsin. In order to estimate the lateral pressure needed to preserve this orientation, we have measured rhodopsin orientation in monolayers at the nitrogen–water interface. Analysis of these data gives the possible orientations rhodopsin can take under different conditions, and the minimum lateral pressure required to maintain its natural transmembrane orientation.

Monolayer methodology has the great advantage, as a model membrane system, that packing pressure can be varied at will. Moreover, because of its high sensitivity in the submonolayer region (Bootsma & Meyer, 1969), ellipsometry has already been shown to be a powerful and well-proven method in deriving information regarding the orientation of molecules in monolayers at the air–water interface (Den Engelsen & De Koning, 1974a,b; Ducharme et al., 1985, 1987, 1990). Accordingly, ellipsometry, along with the monolayer technique, was used to investigate the orientation of intact rhodopsin, partially hydrolyzed rhodopsin, and rhodopsin in intact disk

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* To whom correspondence should be addressed.

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