ELSEVIER

Biochimica et Biophysica Acta 1240 (1995) 229–236

BBA Biochimica et Biophysica Acta

Electric field mediated loading of macromolecules in intact yeast cells is critically controlled at the wall level

V. Ganeva [a,b], B. Galutzov [b], J. Teissié [a,*]

[a] *Dept. 3, Glycoconjugués et Biomembranes, LPTF CNRS 118 Route de Narbonne, 31062 Toulouse cédex, France*
[b] *Department of Biophysics, University of Sofia, Sofia 1000, Bulgaria*

Received 20 April 1995; accepted 3 August 1995

Abstract

The mechanism of electric field mediated macromolecule transfer inside an intact yeast cell was investigated by observing, under a microscope, the fluorescence associated to cells after pulsation in a buffer containing two different hydrophilic fluorescent dyes. In the case of a small probe such as propidium iodide, a long lived permeabilized state was induced by the field as classically observed on wall free systems. Penetration of a 70 kDa FITC dextran was obtained only by using drastic conditions and only a very limited number of yeast cells which took up macromolecules remained viable. Most dextrans were trapped in the wall. A dramatic improvement in transfer of dextrans was observed when the cells were treated by dithiothreitol before pulsation. A cytoplasmic protein leakage was detected after the electric treatment suggesting that an irreversible damage took place in the walls of many pulsed cells. Electroloading of macromolecules in intact yeast cells appears to be controlled by a field induced short lived alteration of the envelope organization.

Keywords: Macromolecule transfer; Electric field mediation; Electroloading; Cell wall; (Yeast)

1. Introduction

Cell membranes are made permeable to ions and molecules when cells are submitted to electric field pulses with an intensity larger than a critical value, specific of the pulsed cell. This phenomenon is called 'electropermeabilization' and proved to be reversible or irreversible dependent on the degree of membrane organizational changes. Whatever the cell species treated, this process shows common features [1–5]. The number of permeabilized cells and the flow of molecules across the cell membrane increase with an increase in the field strength. The flow of molecules across the cell membrane increases with an increase in pulse duration and number of applied pulses. This was explained by a voltage driven membrane reorganization. A side effect is nevertheless present leading to a loss of cell viability. Gene transfer can be observed upon electropermeabilization, this is called electrotransformation. This is obtained with walled as well as wall-less systems. The field must then affect the organization of the wall such as to make it permeable to macromolecules. The fungal wall is a structure which is not present in mammalian cells but plays a key role in yeasts. Very few data are available for the mechanism of transfer of macromolecules mediated by the electric field in the case of walled systems except in the case of *Escherichia coli* [5–11]. In the case of yeasts, Weaver et al. [12,13] have studied the permeabilization and cell viability after electropulsation in the case of intact *Saccharomyces cerevisiae* and *S. pombe* yeast cells. The penetration of fluorescent molecules was detected trough the fluorescence associated to cells by fluorescence activated cell sorting. As with other cell systems, the number of fluorescent (so called permeabilized) cells increased with the field strength in the case of *S. pombe* where the staining of a large fraction of the population was observed. In the case of *S. cerevisiae*, the number of fluorescent cells first increased with the field intensity but then decreased under stringent conditions [14]. One should mention that in their approach, a cell was considered as permeabilized when a positive fluorescence signal was observed. No direct assessment of penetration in the cytoplasm was given. Another problem was that the number of fluorescent cells was the same as the number of cells which lost their viability. As observed in the case of bacteria, transformation is obtained by electropulsing the yeast-plasmid mix-

* Corresponding author. Fax: +33 61 335860; e-mail: justin@lptf.biotoul.fr.

SSDI 0005-2736(95)00181-6

ture [15]. A pretreatment with thiol compounds, known to break disulfide bridges, gave a strong increase in yeast cell transfection [16,17].

Taking into account the role which is played by yeasts in the production of genetically engineered proteins, a better understanding of yeast electrotransfection is of prime importance. In the present study, the transfer of macromolecules inside the cell cytoplasm is studied in the case of *S. cerevisiae*. Fluorescent labelled dextran inflow and cytoplasmic protein leakage induced by electropulsation were analyzed down to the cellular level and compared to the permeabilization of a low molecular dye penetrating freely through the wall. It appears that the wall plays a key role in the control of the transfer of macromolecules.

2. Materials and methods

2.1. Cells

All experiments were carried out with *S. cerevisiae*, strain AH-215. Cells were grown aerobically at 30°C with moderate shaking (Biolaffite, France) in YPD medium (1% w/v yeast extract, 2% w/v bactopeptone, 2% w/v glucose), up to the mid-log phase.

Before electropulsation, they were washed twice with PBY, i.e., Tris-tricine buffer 10 mM, $CaCl_2$ 0.1 mM, $MgCl_2$ 0.1 mM, pH 7.5 and resuspended in the same buffer at a final concentration of 10^8 cells/ml.

2.2. Electropulsation

Electropulsation was run with a CNRS Electropulsator (Jouan, France) which generated square wave voltage pulses. Two parallel stainless steel flat electrodes at a distance of 0.3 cm and with a width of 0.5 cm were used. They were brought into contact to a Petri dish to build an open chamber. 50 μl of yeast suspension ($5 \cdot 10^6$ cells) were poured between the electrodes at room temperature (21°C) (RT) and pulses were applied. The Joule heating was limited due to the low ionic content of PBY. Pulse wave shapes were monitored on line with an oscilloscope (Enertec, France). Cells were subjected to one single pulse with different field strengths. Pulse durations were 10, 15 or 20 ms, conditions previously observed to give a high transfection yield [17].

Sterile operating conditions were obtained by working under a laminar flow hood (ESI, France). Electrodes were sterilized by rinsing with ethanol 70%. Buffers were filtered on a Sterivex filter (Millipore, France). Pipetman cones (Gilson, France) were autoclaved.

2.3. Electropermeabilization assay

Membrane permeabilization was assayed by the influx of either fluoresceinated dextran (FD), a macromolecule, or propidium iodide (PI), a low molecular weight dye, into cells.

FD 70 (M_r = 71 200) bears one fluorescein moiety per 100 glucose groups (Sigma, USA). As the fluorescent group is negatively charged at neutral pH, as it is the case in PBY, FD 70 bears a charge of the same sign. Excitation and Emission maxima are respectively 492 and 525 nm. FD 70 was added at a final concentration of 0.25 mM to cells at the above concentration of 10^8/ml in PBY. After pulsation, yeast cells were incubated during 5 min at RT before being washed four times in PBY. It was checked that no fluorescence was detected in the final supernatant.

PI is strongly hydrophilic (M_r = 660) (Sigma, USA). It is widely used in fluorescence cell analysis to check cell membrane integrity. Excitation and emission maxima are respectively 493 and 639 nm [18]. A strong increase in the fluorescence quantum yield is detected when the dye is bound to double strand nucleic acids. A small increase is present when it is bound to membranes. PI was added to the yeast suspension in PBY at a final concentration of 80 μM. After pulsation, yeast cells were incubated during 5 min at RT. They were observed without any washing by taking advantage of the very low fluorescence of the free dye.

The number of fluorescent cells was counted under an inverted fluorescent microscope (Leitz, Germany) (HBO Arc lamp, H3 or N2 block filters) connected to a video monitor (RCA, USA) through a light intensifying camera (Lhesa, France) to improve the sensitivity. Permeabilization is then the relative percentage of fluorescent cells in the population.

2.4. Determination of the permeabilized state lifetime

This was checked for permeabilization to small molecules. PI (final concentration: 80 μM) was added to the yeast suspension at different times after electropulsation (post pulse delay). Permeabilization was assayed as described previously.

2.5. Viability assay

This was observed through the growth of colonies on nutrient medium complemented with agar (2% w/v). Cells were pulsed in dye free PBY to avoid toxic effects due to PI or FD 70.

2.6. Turbidity changes

This was assayed at RT by measuring the OD at 660 nm of an aliquot of yeasts in PBY on a spectrophotometer (Perkin Elmer, USA).

2.7. Pretreatment with thiol compounds

500 μl of dithiothreitol (DTT) at 50 mM in Tris-tricine 10 mM were added to the same volume of yeast cells in

PBY. The mixture was incubated during 30 min at 30°C. After two washes in PBY, they were resuspended at a final concentration of 10^8/ml in PBY.

2.8. Protein determination

The quantitative measurement of proteins in the yeast cell supernatant after electropulsation was performed by use of a kit taking advantage of the Coomassie Brilliant Blue G-250 interaction with proteins (Bio-Rad, USA).

40 μl of pulsed cells ($25 \cdot 10^6$ cells in PBY) were diluted 10 times in PBY 30 s. after pulsing. After post-pulse incubation times ranging from 1 min to 1 h at RT, the suspension was centrifuged at 11 000 × g (Hettich, Germany) and the supernatant was kept on ice. 600 μl of PBY and 200 μl of the reagent kit were added to 200 μl of supernatant. The mixture was incubated at RT during 15 min. Its absorbance at 595 nm was measured on a Perkin Elmer spectrophotometer by using the treated supernatant of intact unpulsed cells as a blank. Reference curve was obtained by using bovine serum albumin.

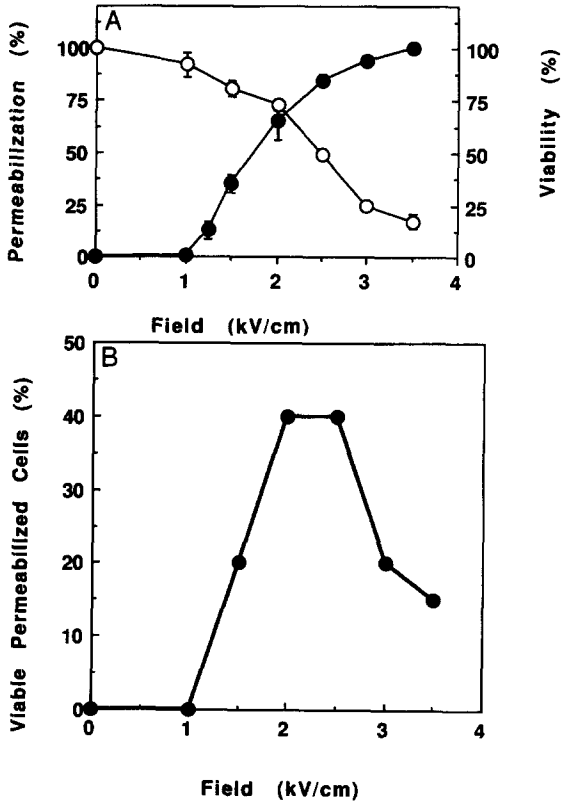

Fig. 1. Yeast electropermeabilization to propidium iodide. (A) Yeast cells were pulsed once with a pulse duration of 10 ms. Permeabilization (●) was assayed by the relative percentage of fluorescent cells in the population. Viability (○) was checked by the ability of pulsed cells to grow. (B) Quantification of the percentage of viable permeabilized yeast cells. As a loss of viability is associated to electropermeabilization, we make the assumption that cell death is due to its permeabilization. The percentage of permeabilized cells must then be corrected from the percentage of dead cells to know what is the percentage of viable permeabilized cells. The plots in A are corrected that way.

DTT treatment of cells did not give any protein leakage as shown by the Bio-Rad assay.

2.9. Alcohol dehydrogenase activity

40 μl cell suspension (10^9 cells per ml) were subjected to one single pulse (15 ms duration). After a 30 min incubation at RT and centrifuging for 1 min at 11 000 × g, 15 μl of supernatant were incubated for 5 min with 10 μl of 0.1 M NAD^+ in 10 μl ethanol (99% v/v) and 65 μl 0.1 M phosphate buffer, pH 9. The absorption at 340 nm was measured using the assay with unpulsed cells as a blank.

2.10. Protoplast formation

30 μl of cell suspension were centrifuged at 11 000 × g during 1 min, the supernatant was discarded and the pellet was resuspended in 100 μl of phosphate buffer 40 mM, pH 7.5. 100 μl of 2-mercaptoethylamine 220 mM in water were then added and the suspension was incubated during 10 min at 30°C with moderated shaking and then diluted in 1 ml of sorbitol 1.2 M. The supernatant was discarded after centrifugation and the pellet was resuspended in 80 μl of sorbitol 1.2 ml. 20 μl of a solution of lyticase (2 mg/ml, 4000–10 000 EU/mg protein) in phosphate buffer 50 mM, pH 7.5 were added to the suspension. This mixture was incubated during 90 min at 30°C with continuous shaking. The protoplasts were finally washed one time with sorbitol 1.2 M and kept in the same buffer.

3. Results

3.1. Electropermeabilization to small molecules

This was assayed through the staining of cytoplasmic nucleic acids by PI. One single pulse of 10 ms was applied to the yeast suspension in PBY (Fig. 1A). Permeabilization was triggered only for field strengths larger than 1 kV/cm. A further increase in field strength gives an enhancement in permeabilization. All cells were permeabilized if the strength of the field was larger than 3.4 kV/cm. The viability of pulsed cells was affected as soon as the permeabilization was observed (Fig. 1A).

When PI was added at different times after pulsing, a fast decrease in the number of stained cells was first observed followed by a slower decrease during 20–25 min (Fig. 2). The fluorescent cells which were then present were cells with an irreversible loss of membrane impermeability. Pulses with longer duration led to an increase of cells with such an irreversible damage (Fig. 2). The number of dead cells was larger than the number of cells irreversibly permeable to PI (Fig. 3). This is indicative that membrane resealing is not enough to preserve viability.

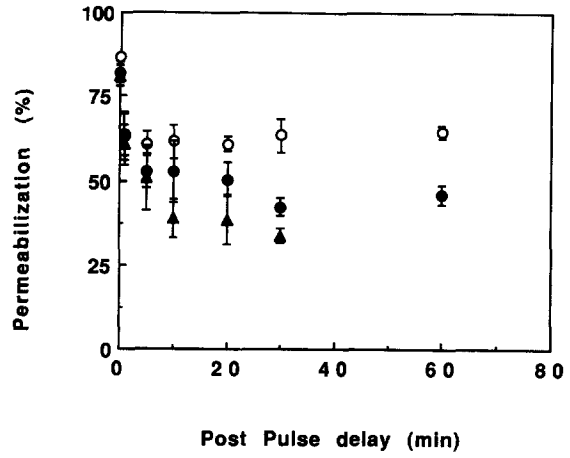

Fig. 2. Post-pulse decay in the number of permeabilized cells to PI. Yeast cells were pulsed and the dye was added at the indicated delay to the pulsed suspension. Permeabilization was assayed by the relative percentage of fluorescent cells in the population. Experimental conditions were one pulse of 2.5 kV/cm lasting 10 (▲), 15 (●) or 20 ms (○).

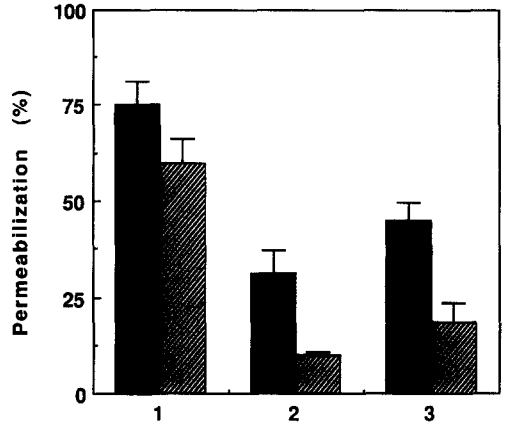

Fig. 4. Effect of a post-pulse protoplastisation on the cell associated FD70 fluorescence. Cells were pulsed in a buffer containing FD70. Fluorescence positive cells were counted as permeabilized. They were washed and observed (gray results). They were protoplastized (hatched results).

As the number of irreversibly damaged cells, i.e., (100 − survival) was always smaller than permeabilization, a fraction of the permeabilized cell population remained viable (Fig. 1B). An optimal field strength was present for PI loading while preserving viability.

3.2. Electropermeabilization to macromolecules

In intact cells, no cell associated fluorescence was observed even if the FD-70/yeast cells mixture was incubated during 3 h. Under the same pulsing conditions as with PI (1 pulse, 2.5 kV/cm, 10 ms), a fraction of the cell population was labelled by FD-70. But the cell population was less sensitive than in the case of small molecules such as PI. Only 30% was labelled as compared to 70% in the previous experiments with PI (Fig. 4). One puzzling observation was that the percentage of cells which remained able to grow after permeabilization to FD-70 was only slightly larger than 70%, i.e., 100–30. There was an optimum field strength in FD-70 labelling of cells. For a pulse duration of 10 ms, this was observed at 3 kV/cm. Higher field strengths were associated to a lower number of fluorescent cells. From the results with PI, this can not be associated to a decrease in the number of permeabilized cells. Post addition of FD-70 to electropulsed yeast cells gave a very low fluorescence even when it took place only 5 min after pulsing. The associated fluorescence was weaker if the addition was run after a longer delay.

The percentage of FD-70 labelled cells depended on the growth phase. A 40% difference was detected between cells in the exponential growth phase and the stationary state (Fig. 5). Such a dependence was not detected for the staining with the low molecular dye PI.

3.3. Effect of DTT treatment

Thiol compounds gave a reduction of disulfide bridges in cell walls which induced an increase in their porosity. Electropermeabilization to PI was not affected by a treatment with DTT (25 mM). Treatment by thiol compounds did not affect the membrane sensitivity to the field. But such a pre-treatment with DTT gave an enhancement of

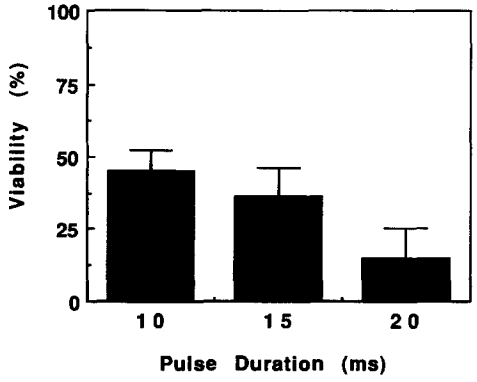

Fig. 3. Loss of viability is controlled by the pulse duration. Yeast cells were pulsed once with a field strength of 2.5 kV/cm.

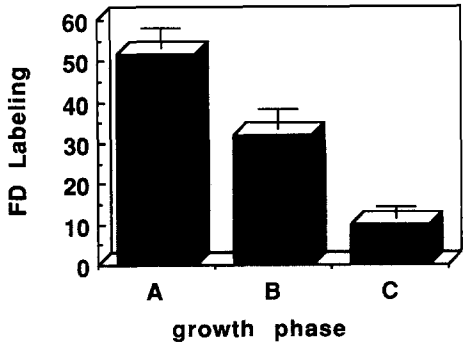

Fig. 5. Influence of growth phase on FD70 labeling. One single pulse of 2.5 kV/cm with a duration of 10 ms was applied to intact cells in different growth stages (A: early log, B: middle log, C: stationary phase).

30% in the number of cells which were labelled with FD-70. No increase in the loss of viability was associated to the DTT treatment.

3.4. Protoplastisation of pulsed cells

As the yeast wall may be stained by FD-70, this was controlled by protoplastisation of pulsed cells. The procedure was carried out 90 min after the electrical treatment. As shown in Fig. 4, the enzymatic digestion of the cell wall gave a strong decrease in the number of cells which were labelled by FD-70. But only a small decrease in the number of cells which were stained by PI was observed, reflecting the lysis of a very limited number of pulsed cells during protoplastisation.

The strong decrease in the number of FD-70 labelled cells, when preparing protoplasts from them, suggested that besides the lysis already observed in the case of PI labelling, in most cases there was no influx of the macromolecules in the cytoplasm. It was only its strong binding to the wall organization, which took place, being due to the structural organization brought by pulsing under membrane permeabilizing conditions. The reversible character of FD-70 binding was supported by our observation that the number of fluorescent cells and the intensity of fluorescence in each cell was decreased by the number of washes following the pulse (data not shown). A stable level of the two parameters was obtained only after 4 washes, condition where the supernatant was not fluorescent anymore. We observed that the fluorescence of each cell was decreased by a factor of two by increasing the number of washes from 2 to 4. This was not mentioned in previous studies [14,19].

3.5. Turbidity changes after pulsation

The optical density (OD_{660}) was measured 10 min after pulsing (Fig. 6) when the membrane is permeable (Fig. 2).

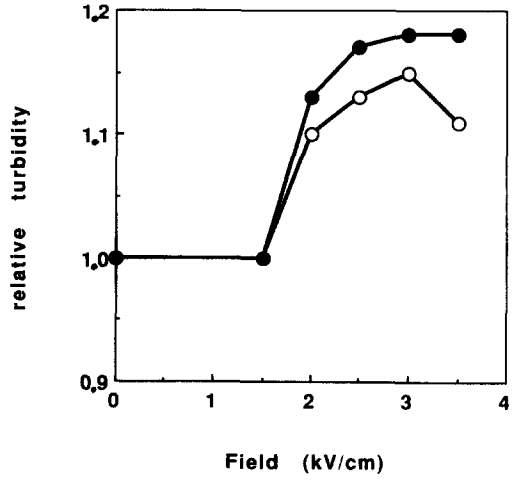

Fig. 6. Changes in cell turbidity induced by electropermeabilization. Yeast cells were pulsed once with a duration of 15 ms. Turbidity was observed through the optical density at 660 nm of the sample either 10 min (●) and 60 min (○) after pulsing.

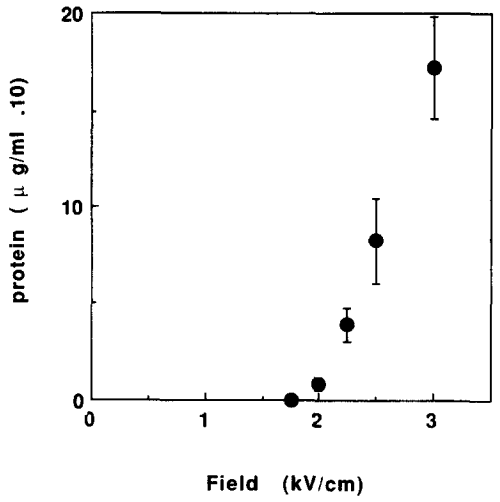

Fig. 7. Protein leakage from pulsed cells. Yeast cells were pulsed once for 15 ms at different field strengths. The amount of proteins which leaked out during the 30 min following the pulse was assayed by the Bio-Rad kit.

A small increase (up to 20%) was observed when cells were electropermeabilized at 2.5 kV/cm. No further increase was detected when using stronger fields. Slightly lower changes in OD_{660} were observed 60 min after pulsing when resealing has taken place (Fig. 2). They reflected the irreversible damages of a subpopulation of the pulsed samples. Due to the minute volume of the cumulated cytoplasmic volume of pulsed cells when compared to the observed volume, the contribution of ions and molecules, which have leaked out, was not contributing to the change in light scattering. The observed change in OD was then due only to minor changes in shape and volume among pulsed cells, no cell clumping or disruption being observed under the microscope (data not shown).

The conclusion was then that no cell rupture as observed with wall less systems took place with intact yeast cells. This was true even when cells were pulsed under stringent conditions (high intensity, long pulses). Cells were preserved as integral units even if a large portion of the population had its membrane irreversibly damaged as shown by Fig. 2.

3.6. Cytoplasmic protein leakage

Protein efflux from the pulsed cells was another assay of the field effect. Such a leakage was observed to be dependent on the field strength. A threshold value of 1.75 kV/cm (1 pulse lasting 15 ms) was observed (Fig. 7). The amount of released proteins then was larger with an increase in the intensity of the field. The leakage was increased by longer pulse duration at field strengths larger than this critical value (data not shown). The kinetics of protein efflux shows that it is a long lasting process even if a very fast leakage is detected just after the pulse. This is strongly controlled by the pulse duration (Fig. 8A).

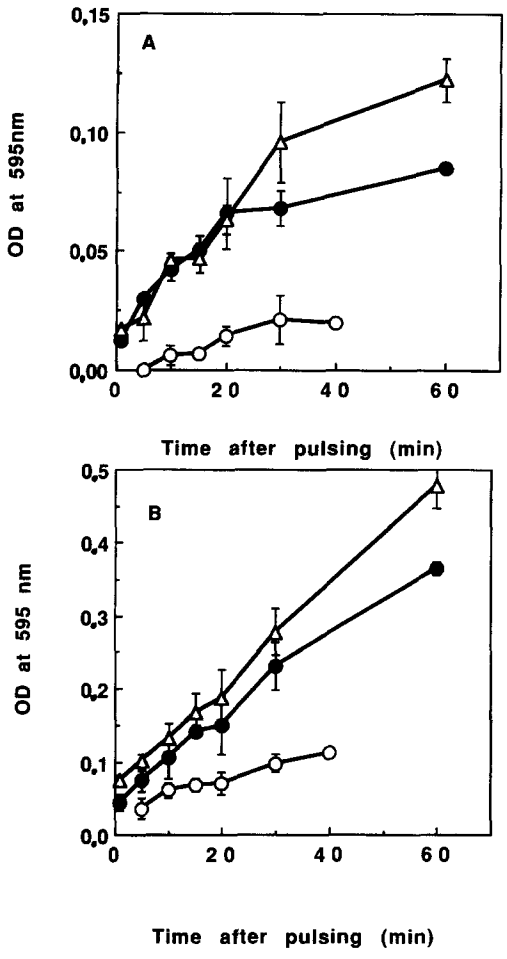

Fig. 8. Kinetic of the protein leakage after electropulsation. Cells were pulsed one time at 25 kV/cm for 10 (○), 15 (●) or 20 ms (▲). The amount of proteins in the supernatant was assayed by the optical density of the Bio-Rad reaction. (A) Intact cells. (B) DTT pretreated cells.

When cells were pretreated with DTT, the leakage of proteins was increased 7 times but remained a long lasting event (Fig. 8B). This facilitating effect of a DTT pretreatment was very important when cells were treated with short pulses, i.e., conditions where the leakage was small.

Large cytoplasmic proteins such as alcohol dehydrogenase (M_r = 140 kDa) were released by electropulsation. The DTT pretreatment increased 4 times the detected activity observed 30 min after pulsing (0.5 U/ml with 10^9 cells). This has to be compared with the 30% increase in FD-70 fluorescent cells but when the incubation lasted only 5 min. Even when cells were DTT treated, the yeast wall remained a barrier to the free diffusion of macromolecules whatever the direction of flow.

4. Discussion

Transfer of macromolecules inside the yeast cytoplasm occurs through the wall and the plasma membrane. The cell envelope is built by 3 components: the plasma membrane, the periplasmic space and the cell wall which is presumed to have a definite porosity. The wall is mainly an assembly of glucan, chitin and mannoproteins in almost equal quantities [20]. Glucan forms the microfibrilar network inside the wall and is responsible of its rigidity [21,22]. Mannoproteins, which are covalently bound to glucan, form an amorphous layer in the outermost part of the envelope and determine the wall porosity by disulfide bridges, formed between their complexes and by the side-chains of glucanase soluble mannoproteins [19,22,23]. The integrity of this layer is further stabilized by hydrogen bonds and ionic interactions [24].

PI electroloading is not affected by the presence of the envelope as predicted by the free diffusion of the dye across the wall. But electropermeabilization must affect both the wall and the membrane to provide a pathway for macromolecules. The occurrence of damages in the envelope was already reported in the case of electropulsation of yeast cells [25]. The process is reversible at the membrane

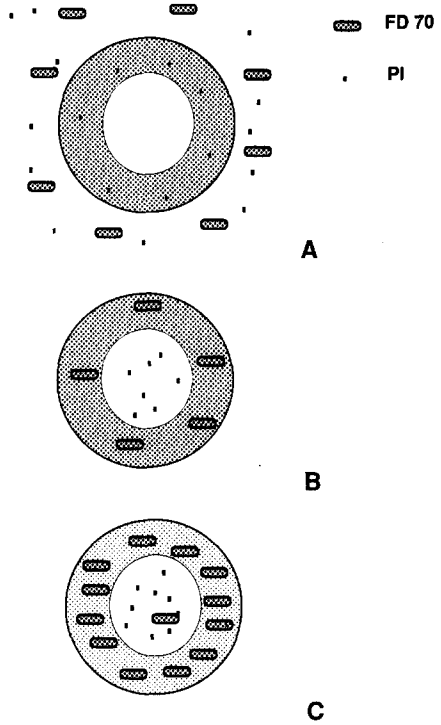

Fig. 9. Electroloading of pulsed intact yeasts. (A) Control cells. Yeast cells are pictured as spheres. The low molecular hydrophilic dye PI can freely diffuse across the wall (gray area) while the macromolecular hydrophilic FD70 remains excluded. The intact membrane prevents the loading of the cytoplasm (central part). The same pattern is obtained if cells are pre-treated by DTT. (B) Pulsed cells. Cells are washed after pulsing and resealing. PI can cross the plasma membrane and is loaded in the cytoplasm. FD70 is trapped in the cell wall but cannot cross the plasma membrane. (C) Pulsed DTT treated cells. Due to the DTT treatment, the cell wall organization is perturbed (lighter gray). Cells are washed after pulsing and resealing. PI can cross the plasma membrane and is loaded in the cytoplasm. More FD70 is trapped in the wall than in B and a weak cytoplasmic loading takes place.

level for a large portion of the permeabilized population as shown by the PI assay (Fig. 2) but this reversibility is not preserving the cell viability and may explain in part the long lived leakage of proteins (Figs. 6 and 7A,B).

Results on FD-70 loading show that when the cell remains viable, this macromolecule is not loaded in the cytoplasm but is mainly pushed inside the wall where it remains trapped and cannot be washed out. In most cases, it is only when the cell viability is irreversibly affected that the macromolecule is loaded inside the cytoplasm. This conclusion is made clear from the effect of a postpulse protoplastisation (Fig. 4). The different steps in transfer are described in Fig. 9.

It is hard to explain how the field may alter the wall organization. Taking into account the importance of electrostatic interactions in mannoprotein layer for cell wall stability and the wall flexibility [26,27], one can imagine that the external field affects the cell wall porosity. But this is observed only when the field strength is high enough to induce membrane permeabilization. One may suggest that under the action of the electric field, a displacement of counter-cations, which neutralize the negative charges of mannoproteins, may take place leading to an enhancement of their electrostatic repulsions. Another suggestion is that as FD-70 is negatively charged, it may be actively moved through the wall during the pulse by electrophoresis or rather by electroosmosis as suggested previously in the case of electropermeabilized membrane [28]. This last explanation is supported by the need to have a permeabilization of the membrane to support electroosmosis, a condition which is present when FD70 binding to the wall is detected (same critical field as for PI penetration). Other evidences of such an active role of the field are the observations, (i) of the lack of labeling with a postpulse addition of FD70 and (ii) in the case of protein leakage, that a major outflow occurs during (or just after) the pulse (Fig. 8A and B). The slow leakage which follows is indicative that an irreversible alteration of both the wall and the membrane remains present. This has to be related to the loss of viability and more precisely to the irreversible permeability to PI shown in Fig. 2.

The observation of a decreased number in FD-70 labelled cells when strong fields are used cannot be explained by a decrease in permeabilization. Nevertheless, a recent study emphasizes the heterogeneous response of yeast cells to the field [29]. This decrease may be related to irreversible damages to the yeast envelope, i.e., to wall and membrane, in such a way that it cannot prevent the leakage of entrapped FD-70 during the post-pulse washes. This is in agreement with the increased leakage of proteins. Under our experimental conditions, this does not take place with PI because the external dye was not washed out being non fluorescent.

Membrane electropermeabilization to PI is not enough to permit the loading of yeast cytoplasm with large macromolecules such as FD-70 as observed in the case of wall less cells. (i) The lack of correlation between FD-70 loading and protein leakage, and (ii) the slow protein leakage, show that the wall of electropermeabilized intact cells still prevents a free exchange across the envelope. This conclusion remains true even when the cells were treated with DTT to alter the S-S bridges. Loading of FD-70 under conditions which preserve cell viability can be evaluated, as for PI, by making the assumption that cell viability can be lost only by cells which were permeabilized. Viability after permeabilization is then the percentage of FD-70 positive cells minus the percentage of lysed cells (i.e., 100 minus the percentage of viable cells). This is very small and may explain why the level of electrotransfection of yeast cells is very low.

Acknowledgements

This work was partly supported by a fellowship of the Ministère de l'enseignement supérieur et de la recherche to V.G.

References

[1] Kinosita, K. Jr. and Tsong, T.Y. (1977) Proc. Natl. Acad. Sci. USA, 74, 1923–1927.
[2] Kinosita, K. Jr. and Tsong, T.Y. (1977) Nature 268, 438–441.
[3] Kinosita, K. Jr. and Tsong, T.Y. (1977) Biochim. Biophys. Acta 471, 227–242.
[4] Rols, M.P. and Teissié, J. (1990) Biophys. J. 58, 1089–1098.
[5] Eynard, N. (1992) These de Doctorat de l'Universite Paul Sabatier de Toulouse.
[6] Dower, W.J., Miller, J.F. and Ragsdale, C.W. (1988) Nucleic Acids Res. 16, 6127–6145.
[7] Sixou, S., Eynard, N., Escoubas, J.M., Werner, E. and Teissié, J. (1990) Biochim. Biophys. Acta 1088, 135–138.
[8] Eynard, N., Sixou, S., Duran, N. and Teissié, J. (1992) Eur. J. Biochem. 209, 431–436.
[9] Xie, T.D., Sun, L. and Tsong, T.Y. (1990) Biophys. J. 58, 13–19.
[10] Xie, T.D. and Tsong, T.Y. (1992) Biophys. J. 63, 28–34.
[11] Xie, T.D., Sun, L., Zhao, H.G., Fuchs, J.A. and Tsong, T.Y. (1992) Biophys. J. 63, 1026–1031.
[12] Weaver, J.C., Harrison, G.I., Bliss, G. Mourant, R. and Powell, K.T. (1988) FEBS Lett. 229, 30–34.
[13] Bartoletti, D.C., Harrison, G.I. and Weaver, J.C. (1989) FEBS Lett. 256, 4–19.
[14] Brown, R.E., Bartoletti, D.C., Harrison, G.I., Gamble, T.R., Bliss, J.G., Powell, K.T. and Weaver, J.C. (1992) Bioelectrochem. Bioenerg. 28, 235–245.
[15] Delorme, E. (1989) Appl. Environ. Microbiol. 55, 2242–2246.
[16] Reddy, A. and Maley, F. (1993) Anal. Biochem. 208, 211–212.
[17] Meilhoc, E., Masson, J.-M. and Teissié, J. (1990) Bio/Technology 8, 223–227.
[18] Shapiro, H.M. (1988) Practical flow cytometry, 2nd Edn., Alan R. Liss, New York.
[19] De Nobel, J.G., Dijkers, C., Hooijberg, E. and Klis, F.M. (1989) J. Gen. Microbiol. 135, 2077–2084.
[20] Cabib, E., Roberts, R. and Bowers, B. (1982) Annu. Rev. Biochem. 51, 763–793.

[21] Kopecka, M., Phaff, H.J. and Fleet, G.H. (1974) J. Cell Biol. 62, 66–76.
[22] Zlotnik, H., Fernandez, M.P., Bowers, B. and Cabib, E. (1984) J. Bacteriol. 159, 1018–1026.
[23] De Nobel, J.G., Klis, F.M., Priem, J., Munnik, T. and Van Den Ende (1990) Yeast 6, 491–499.
[24] Valentin, E., Herrero, E., Pastor, F.I.J. and Sentandreu (1984) J. Gen. Microbiol. 130, 1419–1428.
[25] Tomov, T.C. and Tsoneva, I.C. (1989) Bioelectrochem. Bioenerg. 22, 127–133.
[26] Klis, F.M. (1994) Yeast 10, 851–869.
[27] Stratford, M. (1994) Yeast 10, 1741–1752.
[28] Dimitrov, D.S. and Sowers, A.E. (1990) Biochim. Biophys. Acta 1022, 381–392.
[29] Gift, E.A. and Weaver, J.C. (1995) Biochim. Biophys. Acta 1234, 52–62.