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Electrofusion of Chinese hamster ovary cells after ethanol incubation

G. Orgambide, C. Blangero and J. Teissié *

Centre de Biochimie et de Génétique cellulaires du CNRS, 118 route de Narbonne, 31062 Toulouse Cedex (France)

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Electrofusion of Chinese hamster ovary cells is obtained with cells growing in monolayers on a culture dish. The cells were incubated with increasing amounts of ethanol before the field pulsation. The electroperforation was apparently not affected by the incubation but the threshold field intensity required to induce the fusion was shifted to higher values. This effect is tentatively explained by a decrease in size of the field-induced pores as a consequence of an increase in the lipid matrix fluidity.

Introduction

Fusion of cells is now easily obtained by the applications of short-duration high-intensity electric field pulses. Different approaches have been proposed to induce the requisite contact between cells: natural contact [1], dielectric mediated interactions [2], chemically induced aggregations [3,4], antigen-antibody targeting [5] and contact inhibition for cells growing in monolayers [6,7]. But investigations of the molecular processes involved in this kind of fusion are rather speculative. In our laboratory, using Chinese hamster ovary cells (CHO) plated on culture dishes as a model system, we are developing such an approach. We first demonstrated that the interaction between cells and the electric pulse was vectorial, providing direct evidence that the trigger of the fusion was the external electric field [8]. Thus the speculation that the first step in the fusion was the electroperforation was strengthened. In fact we always observed in our experiments that dyes were penetrating inside the cells under fusing pulsing conditions [7], proving the induction of transient permeable structures. But a recent study [9] demonstrated that under the same pulsing conditions the fusion could be abolished by the ionic content of the external medium. It appears that different steps are present in the fusion mechanism, where the perforation is in fact required but not sufficient. Furthermore, the closing process of the pores must be dependent on the dynamic of the membrane. A short abstract [10] suggested using data on the temperature dependence of the electrofusion of lymphoma that the fluidity of the membrane was important for the occurrence of the process. Such an observation was in agreement with experiments on the Ca^{2+}-induced fusion of model systems [11]. In fact, the authors were speculating that the effect of the temperature was only to alter the 'fluidity' of the membrane. They neglected the well-known fact that many biological activities are strongly dependent on the temperature. In the present study we investigated the consequences on the electrofusion of the incubation of CHO with a fluidizing agent (ethanol) in order to obtain informations free of such an enthalpy-induced contribution.

Methods

The 'electrofusion' method we are using was as previously described [7]. It was shown with other

* To whom correspondence should be addressed.

cell systems that this method gives a high-yield cell hybridization [12]. In this approach, the electric pulse is applied on cells growing in monolayers on culture dishes (Nunc, Denmark). The field is homogeneous, being generated by a high voltage (Cober power supply, U.S.A.) applied on two parallel flat stainless-steel electrodes (distance 6 mm, length 30 mm) which are in contact with the bottom of the dish (Fig. 1). Five repetitive pulses (100 μs duration) are applied with a constant selected intensity (square wave pulse). The pulsing medium (2 ml) is 10 mM Tris buffer, 250 mM sucrose and 1 mM $MgCl_2$. The cells are in contact with the pulsing medium only during the pulsation, i.e., about 2 min. After pulsation cells are incubated in the culture medium at 37°C for 2 h. They are then fixed and observed under an inverted microscope. In the present study the cells at a density of 780 cells/mm^2 are incubated for 30 min (before the electric treatment) at 37°C in a culture medium where the indicated ethanol concentrations are present. No ethanol was present in the pulsing medium and its effect is induced during the pre-pulse incubation. Incubations are always operated in a 95% air/5% CO_2 atmosphere. In all experiments control samples are present with no ethanol treatment during the pre-pulse incubation period.

The extent of fusion is monitored either by computing the fusion index R, which is the percentage of nuclei belonging to polynucleated cells against the total number of nuclei, or by plotting the polynucleation histogram as a function of the number of nuclei per cell. One of the advantages of our method is that control cells which are not pulsed are present on the same dish as the pulsed ones. This was clearly illustrated in Ref. 7. About 500 cells are observed for each experimental condition.

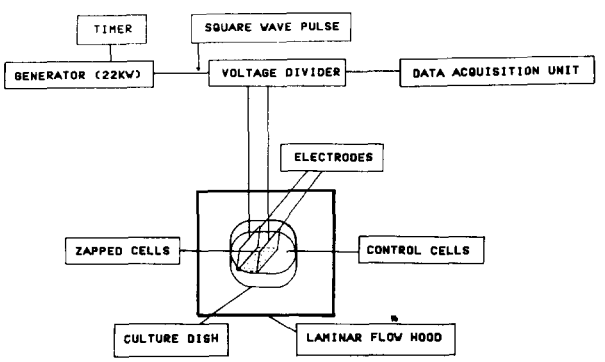

Fig. 1. A schematic drawing of the electrofusion device used in the present study. Square-wave voltage pulses generated by the Cober pulser are applied on the electrodes. A small fraction of the applied voltage is fed on a Datalab transient recorder and visualized on an Enertec oscilloscope. The cells are kept under sterile conditions in an ESI laminar flow hood.

Results

Electroperforation

The direct control of ethanol-treated cells under the microscope does not reveal any morphological change as a consequence of the incubation. Electric field-induced transient permeable structures are always induced. This is shown by pulsing the cells in a medium where a low concentration of a non-permeant dye was present. As shown in Fig. 2, this 'pore'-opening process is dependent on the intensity of the external field and a threshold value is present. 'Pores' are opened for the same field value whether the cells are incubated or not. The profile of the curve in Fig. 2 is not affected by the ethanol pretreatment.

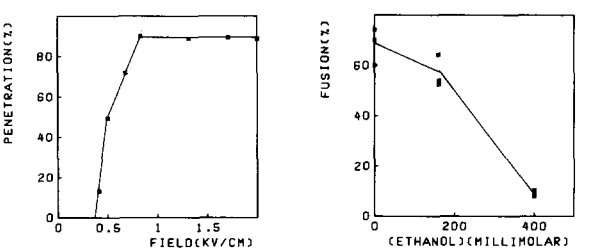

Fig. 2. (left) Dye penetration in plated CHO cells as a consequence of 100-μs electric pulses. Five pulses with the indicated intensities were applied to the cell culture. Eosin was present in the pulsing medium at a concentration of 2.3 mM. Permeated cells were observed under the microscope and displayed a red staining of the cellular components. The ordinate is the percentage of stained cells. The curve is similar whether the cells are treated with ethanol or not.

Fig. 3. Decrease in the polynucleation index of pulsed cells as a consequence of their preincubation in ethanol. Cells were pulsed five times (1.4 kV/cm, 100 μs) after a 30 min incubation in the presence of the indicated ethanol concentration. The yield is the polynucleation index expressed in percent.

Electrofusion

When the intensity of the electric field is kept at a value of 1.4 kV/cm, the ethanol incubation induced a decrease in the yield of fusion, as shown in Fig. 3; but this observation is not linked to an inhibition of the ability of cells to fuse. If the yield of fusion is plotted as a function of the magnitude of the applied field, its value for 2.5 kV/cm remains constant, with an index of polynucleation of about 30% (Fig. 4) except when we are using 400 mM ethanol, where the maximum extent of polynucleation is not reached. This 2.5 kV/cm intensity is the highest we are able to generate due to the power limitation of our generator.

In a previous study [7] we showed that the fusion was observed only when the cells were pulsed with fields stronger than those required to induce the perforation. The major change is that the threshold value needed to induce the fusion is shifted towards higher intensities. This threshold value seems to increase linearly with the ethanol concentration with a possible saturation effect for the highest concentration we were able to use in our experimental conditions (400 mM) (Fig. 5).

Under field saturating conditions (more than 2 kV/cm) the histogram of polynucleation was not affected by the prepulse ethanol incubation (Fig. 6). The fusion process occurs in the same manner as soon as field conditions powerful enough to trigger it are applied.

Discussion

Electrofusion can be triggered on CHO cultured in monolayers after an ethanol incubation when one uses strong electric fields with an intensity of the order of 2 kV/cm. The major conclusion of the present study is that the extent of the electrofusion under these strong pulsing conditions is not affected by the pretreatment because (i) the index of polynucleation R is the same under high field conditions (Fig. 4), and (ii) the extent of polynucleation is not altered (Fig. 6). But the mechanism which is the basis of this fusion is clearly altered, as higher field intensities are required for its induction (Fig. 5). This explains the apparent discrepancy with this conclusion that we observe when incubating the cells with 400 mM ethanol. In this case the power of the generator is not large enough to provide the required field.

The nature of the contacts between cells does not appear to be affected by the ethanol treatment. This conclusion is obtained from the direct observation of the cells under the microscope and from the polynucleation histogram. If the contacts were altered, the number of polyfusion events

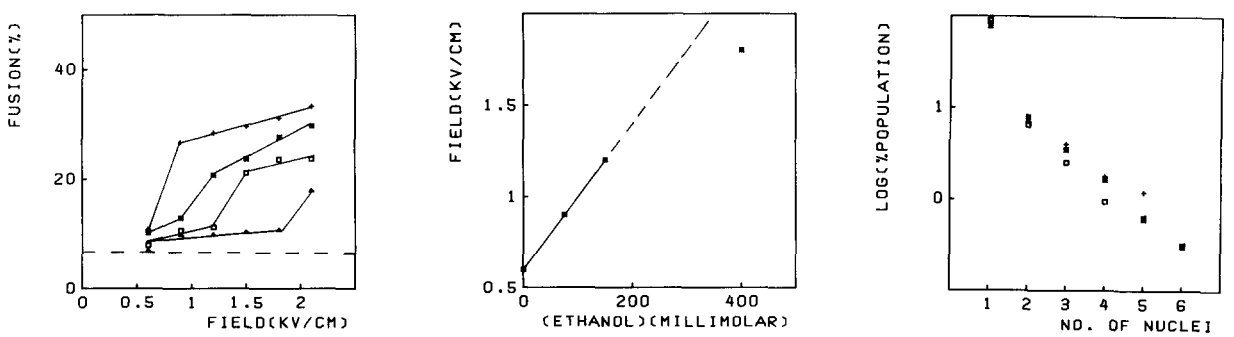

Fig. 4. (left) Changes in the polynucleation index of pulsed cells as a function of the amplitude of the applied field. Cells were pulsed five times (pulse duration 100 μs) after a 30 min incubation in the presence of different ethanol concentrations: control (+), 81 mM (*), 162 mM (□), 403 mM (△). The yield is the polynucleation index expressed in percent.

Fig. 5. Evolution of the fusing threshold intensity as a function of the prepulse incubation ethanol concentration. Cells were pulsed five times (pulse duration 100 μs) after a 30 min incubation at the indicated ethanol concentration. The field intensity should be greater than the threshold in order to elicit the fusion. These data are obtained directly from Fig. 4.

Fig. 6. Histogram of polynucleation of cells pulsed after incubation with different ethanol concentrations. Cells were pulsed five times (2.1 kV/cm, 100 μS) after a 30 min incubation with different ethanol concentrations: control (+), 81 mM (*), 162 mM (□).

would decrease, leading to a shift of the histogram to the low number of nuclei region (left side of the diagram). This is not the case.

The number of induced pores does not seem to be altered by the ethanol treatment. This conclusion is obtained from the results in Fig. 2, where the penetration of eosin is reported not to be affected. If the number of pores were decreased then we would expect a decrease in penetration linked to a limitation of the incoming flow of exogenous molecules. Furthermore, in a recent publication [26] it was proposed that the defects involved in the electric field-induced pore-opening were located at the sites of the discrete surface charges. The duration of the ethanol incubation is too short to induce any change in the electrical properties of the membrane, and the density of surface charges remains unaffected. As a consequence the density of defects is not altered and, as we show that the threshold field for perforation, i.e. the energy barrier required to trigger the opening of pores, remains unaffected, then the probability of pore-opening, which is directly related to this energy, is the same. As their density and their probability are unchanged, the number of evoked pores must remain the same.

The basic scheme for electrofusion is proposed to be: (i) electroperforation; (ii) closing of the pores either by an intercellular process (fusion) or by an intracellular one (reversibility). We showed that step (i) is not affected by the ethanol incubation (Fig. 2), at least in its initiation step. It was previously demonstrated that the perforation is in fact a two-step processes [13,14]. The first one is a growth of structural defects up to a critical size where a pore is generated [15,16] and the second is an increase in the diameter of the pores. This second step is under the control of the magnitude of the applied field and of the pulse duration [13,14]; furthermore it appears to be under the control of the ionic content of the external medium [17]. It could easily be imagined that this last step is strongly altered by the ethanol incubation. Experiments on model systems showed that ethanol was interacting with lipids at the level of the polar head groups, creating a hydrogen bond between the OH of the alcohol and the carbonyl of the lipid [18]. The order parameter of the three first segments of the hydrocarbon chain was decreased and the chains were distorted leading to an increase of the molecular area. This expansion of the film may explain the observed interdigitation [19,20]. Thus ethanol affects mainly the polar head region of lipids, which was shown by NMR studies to be affected by external electric fields [21]. In membrane vesicles a similar effect was observed [22] but it was decreased by the presence of cholesterol [23]. Nevertheless it must be emphasized that a microcompartmentation of lipids is postulated to exist around intrinsic proteins [23] and that this annulus of lipids would be strongly affected by ethanol even when the average properties of the membranes are not altered as a consequence of their high content of cholesterol, a well-known fact in eukaryotic cells. This localized effect of ethanol would explain its specific effect on transport systems as described in Ref. 24. In the case of electroperforation, ethanol would alter the increase in size of the pores after they have been created, or by increasing the 'fluidity' of the lipid matrix it would decrease their lifetime. Both effects would alter the fusion process by altering the nature of the electric field-induced pores which are a prerequisite for the mixing of the membranes. Such an effect would explain the shift to higher intensities of the threshold field required to induce

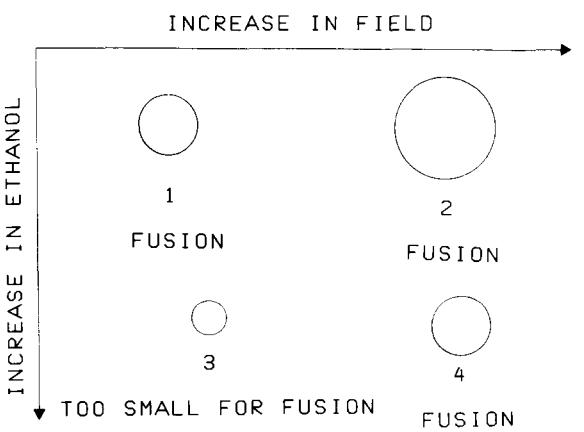

Fig. 7. Schematic drawing explaining our proposal of the effect of ethanol incubation on the size of an electric field-induced pore. Field pulses induce pores of a given size (state 1). Increasing the intensity of the applied field increases the size of the pore (state 2). After incubating the cells in ethanol before the pulsation, the field pulse induces smaller pores (state 3). Thus a higher field induces pores of the initial size if the cells are preincubated with ethanol (state 4).

the fusion (Fig. 5), as for a given pulse duration the final size of the pore is under the control of the intensity of the field [13]. Thus increasing the intensity of the field would either compensate for the decrease in size due to ethanol or return the pore lifetime to its initial value. It is difficult to decide between the two explanations (decrease in diameter or in lifetime), but it is known that the resealing of the pore is very slow (several minutes at 37°C) [2,25], too slow to be due to a simple lipid diffusion process. Thus the pore lifetime is rather long and its above postulated ethanol-induced change does not appear as a likely candidate to explain the decrease in fusion yield. In our experiments we compensated for this decrease in size by increasing the intensity of the field which keeps a constant value during the pulse (square wave pulsation) (Fig. 7). An implicit conclusion from this model is that the pores should be larger than a critical size in order to induce the fusion even though these pores are always large enough to allow the penetration of low molecular weight dyes (Fig. 2).

Fluidization of the membrane by the ethanol treatment apparently inhibits the cell electrofusion. This effect can be linked to a decrease in size of the induced pores. The temperature-induced increase in fusion yield reported elsewhere appears not to be linked to the fluidity of the membrane but rather to a more general cell reorganization [25].

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References

1 Neumann, E., Gerisch, G. and Opatz, K. (1980) Naturwissenschaften 67, 414–415
2 Zimmermann, U. (1982) Biochim. Biophys. Acta 694, 227–277
3 Berg, H., Augsten, K., Bauer, E., Forster, W., Jacob, H.-E., Muhlig, P. and Weber, H. (1984) Bioelectrochem. Bioenerg. 12, 119–133
4 Chapel, M., Teissié, J. and Alibert, G. (1984) FEBS Lett. 173, 331–336
5 Lo, M.M.S., Tsong, T.Y., Conrad, M.K., Strittmatter, S.M., Hester, L.D. and Snyder, H.S. (1984) Nature 310, 792–794
6 Teissié, J., Knutson, V.P., Tsong, T.Y. and Lane, M.D. (1982) Science 216, 537–538
7 Blangero, C. and Teissié, J. (1983) Biochem. Biophys. Res. Commun. 114, 663–669
8 Teissié, J. and Blangero, C. (1984) Biochim. Biophys. Acta 775, 446–448
9 Blangero, C. and Teissié, J. (1985) J. Membrane Biol., in the press
10 Ohno-Shosaku, T. and Okada, Y. (1984) Abstr. Int. Congr. Cell Biol. Kyoto, p. 459
11 Wilschut, J., Duzgunes, N., Hoekstra, D. and Papahadjopoulos, D. (1985) Biochemistry 24, 8–14
12 Finaz, C., Lefevre, A. and Teissié, J. (1984) Exp. Cell Res. 150, 477–482
13 Kinosita, K. and Tsong, T.Y. (1979) Biochim. Biophys. Acta 554, 479–497
14 Chernomordik, L.V., Sukharev, S.I., Abidor, I.G. and Chizmadzhev, Yu.A. (1983) Biochim. Biophys. Acta 736, 203–213
15 Abidor, I.G., Arakelyan, V.B., Chernomordik, L.V., Chizmadzhev, Yu.A., Pastushenko, V.F. and Tarasevitch, M.R. (1979) Bioelectrochem. Bioenerg. 6, 37–52
16 Sugar, I.P. and Neumann, E. (1984) Biophys. Chem. 19, 211–225
17 Kinosita, K. and Tsong, T.Y. (1977) Biochim. Biophys. Acta 471, 227–242
18 Pope, J.M., Walker, L.W. and Dubro D. (1984) Chem. Phys. Lipids 35, 259–277
19 Simon, S.A. and McIntosh, T.J. (1984) Biochim. Biophys. Acta 773, 169–172
20 Rowe, E.S. (1985) Biochim. Biophys. Acta 813, 321–330
21 Stulen, G. (1981) Biochim. Biophys. Acta 640, 621–627
22 Chin, J.H. and Goldstein, D.B. (1984) Lipids 19, 929–935
23 Silvius, J.R., McMillen, D.A., Saley, N.D., Jost, P.C. and Griffith, O.H. (1984) Biochemistry 23, 538–547
24 Dorio, R.J., Hoek, J.B. and Rubin, E. (1984) J. Biol. Chem. 259, 11430–11435
25 Blangero, C. (1984) Thèse de 3° cycle, Université de Toulouse 3
26 Miller, I.R. (1983) Bioelectrochem. Bioenerg. 11, 231–243