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FUSION OF MAMMALIAN CELLS IN CULTURE IS OBTAINED BY CREATING THE CONTACT BETWEEN CELLS AFTER THEIR ELECTROPERMEABILIZATION

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Electropermeabilization of chinese hamster ovary cells (CHO) is a long lived process but is nevertheless fully reversible.
Electrofusion can be obtained by centrifuging the cells several minutes after the pulsation and then by incubating the pellet.
These results show that fusion is not directly created by the field but is due to the existence of a peculiar state of the plasma membrane. The permeabilized patches are local on the cell surface and do not spread or diffuse on it.

Electric field induced permeabilization (electropermeabilization) and fusion (electrofusion) are now routinely used in cell biology either for transformation (1,2) or hybridization (3). Both processes appear correlated but in fact very few is known about their molecular basis. In our laboratory, we called them transient permeant structures (TPS). Some thermodynamic descriptions have been given but which are difficult to link to the complexity of a cell (4-6). As a consequence of this indigence of basic understanding, experimental procedures were most all of the time rather empirical. It was then postulated that one requirement for fusion was the existence of a close contact between cells before the electric pulsation (3). Different procedures for creating this contact were used: spontaneous (7), chemical agglutination (8), dielectrophoresis of pronase treated cells (3), contact inhibition for cells growing on culture dishes (9), high density (10).

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These experimental conditions were often tedious and lead to low yields of viable hybrids.

In a recent paper, evidences were provided that with red blood cell ghosts contact can be created after the pulse and that nevertheless fusion was induced (11). As a consequence, the models proposed to explain the fusion where a contact before the pulse was needed were no more valid (3). In this communication, this conclusion is extended to mammalian cells in culture and similar results are obtained with CHO cells in suspension with the use of an ingenious procedure. Consequences on the organization of permeabilized cell membranes are discussed.

MATERIALS AND METHODS

CHO cells were grown in suspension up to a cell density of 10^6 cells per mL (exponential growth phase). The culture medium was Eagle's mimimum medium (MEM0111, Eurobio, France) supplemented with 6% new born calf serum (Boehringer, Germany), antibiotics and glutamine.

The cell suspension was then washed by centrifugation (100xg) (600 to 800 rpm, Jouan C500 centrifuge, France) at room temperature. The cell pellet was then resuspended in a pulsation medium (10 mM phosphate, pH 7.4, 1 mM MgCl2, 250 mM sucrose)(9). This low ionic content isoosmotic mineral medium was shown to be optimal for the electrofusion of CHO grown in monolayers on a culture dish(12). The viability of the cells was not affected by being kept in that medium during more than 2 hours. Viability was checked by plating efficiency because the strain we were using was able to grow in suspension or plated in monolayers.

Electropulsation was operated by use of an ATEIM-CNRS cell pulsator. In that system, the electrodes are brought into contact with a culture dish thus building an open discharge chamber. 200 µL of cell suspension are put between the electrodes and bridge then by a conducting link. Square wave electric fields with a 100 µS duration are then applied several times in a row with a delay which can be as short as 0.1 S. Advantage can be taken of the technology in the following way. If the electrodes are lifted upwards, the cell suspension remains as a drop on the dish due to hydrophobic repulsions; if now the dish is turned of 90° and the electrodes carefully dipped against the dish again, the field can be applied again in a direction perpendicular to the previous direction as referred to the dish and as a consequence to the sample if it is supposed that the rotation of the cells is a slow process (Fig.1). This technology was already used to show the vectorial character of the interaction between cells and electric pulse in the induction of cell fusion (13).The pulsed sample was then collected in a sterile tube by pipetting. 3 successive samples were pooled together in order to obtain a final volume of 0.6 mL. The tube was then centrifuged very gently in order to pellet the cells (25xg)(5 min., 400 rpm, Jouan C500, room temperature) and then incubated (30 min., 37°). The sample was then dispersed in 2 mL of culture medium and the obtained cell suspension was poured in a culture dish (35 mm diameter, Nunc) . It was then incubated for several hours at 37° in an air/CO2 95/5 atmosphere (Jouan incubator, France).

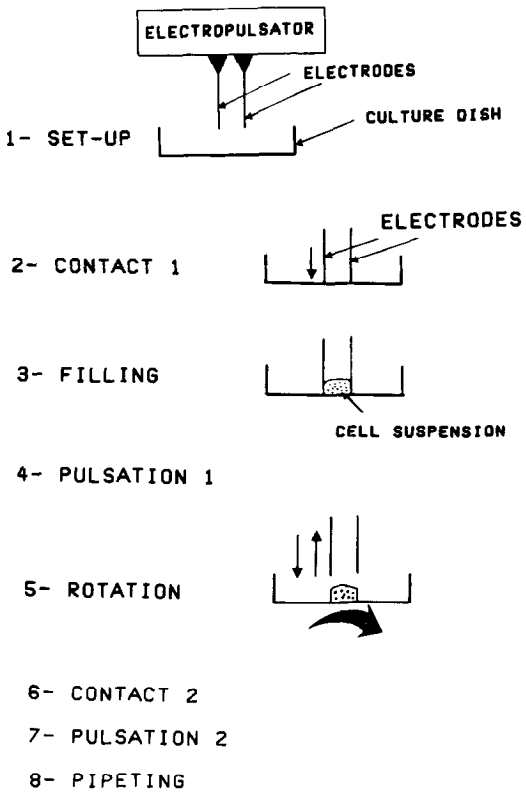


FIG.1. Description of the procedure used to pulse the cell suspension under crossed directions.
The electodes are part of the ATEIM CNRS cell pulsator. The culture dish is either for bacterial culture (Greiner, France) or for mammalian cells (Nunk, Denmark). Cell manipulation is operated under sterile conditions (Gilson pipetman, France or equivalent) in a laminar flow hood (ESI, France).

Cell fusion was quantitatified by the polynucleation index I (9):

$$I\ (\text{in } \%) = ((\Sigma_2^{\infty} nC(n))/(\Sigma_1^{\infty} nC(n)))*100$$

with n being the number of nuclei per cell and C(n) being the number of cells where n nuclei were present. Another representation was the polynucleation histogram. More than 300 cells were counted per experimental condition in order to obtain representative data. The results were reproductible within +/- 5% from our batch of cells to the other.

Viability of the cells was checked by controlling their growth on a culture dish during more than 24 h.

Electric field induced permeability was checked by the penetration of Trypan blue. The dye (4 mg/mL in the pulsing medium) was added to the cell suspension after a given delay following the electric pulsation. Labelling lasted 5 min. at room temperature. The percentage of permeabilization was taken as equal to the percentage of Trypan Blue labelled cells. It should be emphasized that several hundreds of cells were observed under each experimental condition, such a control being facilitated by use of an inverted microscope coupled to a video monitoring (Leitz Diavert, Germany; JVC, Japan). The level of labelling was zero in the control sample (unpulsed cells).

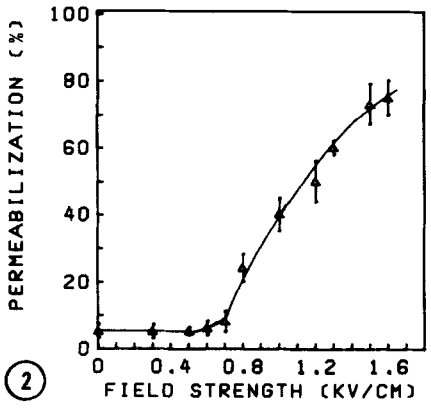
RESULTS

1- CHO electropermeabilization is fully reversible

As shown in Fig.2, permeabilization of CHO cells was observed only if the field strength was larger than 0.7 kV/cm (for a duration of 100 μS, 5 successive pulses, 1 S. delay) but all treated cells were permeabilized if the field intensity was above 1.5 kV/cm (other conditions being kept unchanged). Cell viability was not altered as long as the field magnitude remained smaller than 2 kV/cm.

This electric field induced permeability disappeared spontaneously when the cells were incubated at room temperature. As shown in Fig.3, all cells were still permeable 5 min. after the pulsations but 50 % had recovered their plasma membrane impermeability after a 20 min. delay. In other words, the TPS remained present for rather long periods and it can be taken advantage of that property to load the cells with exogeneous material as in transformation (2).

2- Fusion is obtained by creating contact between already electropermeabilized cells.



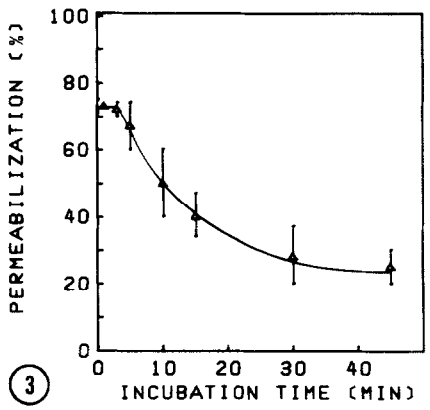


FIG.2. Induction of CHO permeabilization by electric field pulses. Permeabilization was observed by Trypan Blue penetation and counting the percentage of stained cells. The pulse duration was 100 μS and 5 successive pulses were applied with a 1 S. delay.

FIG.3. Annihilation of the permeabilization with time. Cells were pulsed (1.5 kV/cm, 100 μS, 1S. delay) and kept at room temperature . Treatment by Trypan blue was operated at the indicated time. Level of permeabilization was taken as the percentage of stained cells.

TABLE I

Polynucleation Index of centrifuged electropermeabilized CHO

Field (kV/cm)	Duration (μS)	Number	Centrifugation (a)	Polynucleation (%)
0			No	20
0			Yes	20
1	100	5	Yes	25
1	100	3+3(b)	Yes	37
1.6	100	5	No	22
1.6	100	1	Yes	50
1.6	100	5	Yes	52

a) The cells were centrifuged after the electric pulsations
b) The pulsations were applied in crossed directions
All conditions are those described in "Materials and Methods".
Note : a basic level of polynucleation is always present in CHO.

We took advantage of this long life-time of the TPS to induce the fusion of cells in suspension. As shown in Table I and Fig.4, centrifuging cells which were pulsed (1.6 kV/cm, 100 μS, 5 times, 1S. delay) in order to create a pellet where a large number of cell-cell contacts existed, triggered their fusion. If the centrifugation was not operated then no fusion was detected.

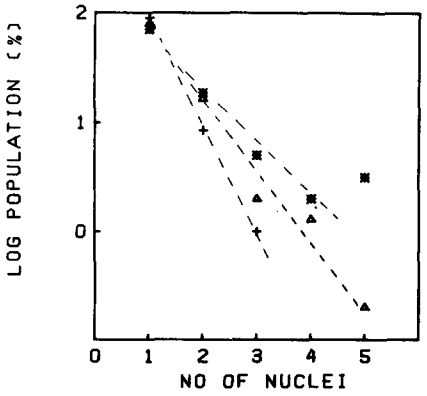


FIG.4. Polynucleation histogram.
(+) control sample; (*) 1.6 kV/cm, 100 μS, 5 times; (Δ) 1 kV/cm, 100 μS, 3 times and then 3 times in a crossed direction.

The delay between the pulsation and the centrifugation was about 5 min.. This means that the cells remained in their permeabilized state (STPS present) during that delay before being brought into contact.

Cell viability was not affected in any condition.

3- Orientation of the field drastically modulated the fusion

As described in "Materials and Methods", the cells in suspension could be submitted to successive pulses with different orientations. This physical parameter was shown to be very important in electric field effect due to the vectorial character of the electric field and the consequences on the symmetry of its effects.

As shown in Table I, pulsing the cells under two crossed orientations induced a drastic increase in the polynucleation index without affecting the cell viability. It should be noticed that a low field intensity was used as compared by the results in part 2 (1 kV/cm versus 1.6 kV/cm) but that nevertheless comparable polynucleation index increases were obtained.

4- Centrifugation speed control is critical

In routine experiments, washing of CHO suspension is always operated with the use of high centrifugation speed (700xg)(above 2000 rpm in the same centifuge). This was controlled not to affect the cell viability.

This is not at all the case with pulsed cells. If cells which were pulsed by fields with intensity of at least 1 kV/cm (100 μS duration, 5 times, 1S. delay) were spun down at a 800 rpm velocity, no viable cell was observed thereafter. Cells were unable to be plated as described in "Materials and Methods", even if the incubation in the culture medium was operated during several hours. Irreversible damages have been induced by the centrifugation. If the optical density of the supernatant was checked, a peak centered at 260 nm was observed. It is associated to the leak of the cytoplasmic content which is permitted by the permeabilization. We then associated the cytotoxic effect of high

speed centrifugation to a draining of the cytoplasm linked to the pressure induced by spinning. As a consequence the cells lost their ability of annihiling their TPS.

DISCUSSION

The present study provides a direct evidence that electrofusion is a consequence of electropermeabilization and that the two events occur in that order (permeabilization and then fusion) and not simultaneously. Electric field induced TPS confer to the cells the ability to fuse if good contact conditions exist or are created thereafter. Such an observation means that long-lived TPS are associated to a new metastable state of the plasma membrane where the repulsive hydration forces are reduced or annihiled (14). This means that the mixing of the membrane components, which occurs during the fusion, is not a direct effect of the electric field but is mediated by the TPS. The longest delay between pulsation and contact appears then to be as long as the TPS life-time.

A similar explanation can be provided for the effect of the field intensity and of the field direction (Table I). We already gave experimental proofs that the physical basis for permeabilization and fusion was the electric field mediated membrane potential change (13, 15). The previous results and the present ones suggest that TPS are created only where the membrane potential is brought to a value greater than a threshold. Our results with large unilamellar vesicles suggest a value close to 200 mV (16). Once created these TPS are going to keep the same position on the cell surface and not to diffuse away. In that case, due to the cosine relationship between the induced membrane potential increase and the external field (15), the percentage of the cell surface, where the potential is larger than the permeabilization threshold and where TPS are induced, is going to increase with the field

intensity. For similar reasons, if the field is applied under crossed directions, the regions, where TPS are present on the cell surface, are going to be not exactly the same as when the field is applied in only one direction. As TPS must face each other on each cell in order to induce fusion (9), fusion yield increases with the probablity of contact of TPS bearing membranes. Of course, this probability is directly linked to the percentage of the cell surface where TPS are present. As we observed, any experimental procedure, which increases this percentage (high field, crossed fields), will improve the fusion yield. The absence of diffusion and/or spreading of the TPS suggest a compartimentation of those structures and their connection to the cytoskeleton (17).

A more practical consequence of the present study is the description of a very simple technique to obtain fusion of cells in suspension. The advantages are: 1- the use of a pH buffered mineral pulsation medium as with plated cells (9, 12, 13), 2- the absence of chemical additives, 3- a large cell volume, 4- no need of a high cell density during the pulsation, 5- a very easy to use procedure where no control of the conditions under the microscope is required, 6- work under the laminar flow hood when sterile conditions are needed, 7- a high yield of fusion. A possibility for the hybridation of cells of very different sizes is offered by the present approach. It is known that the intensity of the field which induces the permeabilization is strongly dependent on the size of the cell (3, 15). But a high intensity field can be toxic (see the present results). A strong limitation was then present. From the present study, the following approach is possible. The two partners are pulsed separatly under their own optimal conditions (field intensities can then be very different), the pulsed samples are then mixed together and the mixture is centrifuged in order to create the contacts. This procedure appears very promising in the case of hybridoma where the size of the partners is very different.

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