Electric-Field-Induced Fusion of Enzyme-Treated Human Red Cells: Kinetics of Intermembrane Protein Exchange

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Abstract. Fusion of neuraminidase or trypsin pretreated human erythrocytes and untreated cells was performed. The obtained doublets had a permanent dipole moment which disappeared as a function of time. Since the dipole moment disappearance is due to protein interdiffusion the lateral diffusion constant may be determined. This was performed by fixing fused cells in different time intervals following the electric pulse and by measuring the reorientation of fixed cell doublets in a static electric field. A surprisingly high diffusion constant was obtained, about 3×10^{-9} cm^2/s. A delay in interdiffusion kinetics was observed in neuraminidase treated cells; this may be due to a restricted growth of the fusion site perimeter. Our data demonstrate that protease treatement promotes electric-field-induced cell-to-cell fusion.

Key words: Membrane protein — Lateral diffusion — Breakdown — Electric--field-induced fusion

Introduction

In recent years, methods of cell fusion have gained increasing interest in basic research and biotechnology (Zimmermann and Vienken 1982; Zimmermann et al. 1980; Vienken et al. 1981; Yamaizumi et al. 1978; Zimmermann 1982; Zimmermann et al. 1981). One of these techniques is electric-field — induced cell fusion. This particular technique avoids the use of chemical or viral agents, and it guarantees a synchronous onset of the fusion process. In addition due to interfacial orientation, relatively pure doublet hybrids can be obtained (Vienken and Zimmermann 1982).

With human red cells, all the known techniques of cell fusion result in hemolysis (Loyter and Lalazar 1980). The nature of this accompanying hemolysis is probably osmotic. Electric field short-pulse-treatment also generates slow osmotic hemolysis. This process itself is of interest (Kinosita and Tsong 1977a; Kinosita and Tsong 1977b; Zimmermann et al. 1976). It is however possible to create fusion conditions under which minimum hemolysis and extensive fusion occur (Scheurich and Zimmermann 1981; Zimmermann 1982).

To improve the largely empirical technique of the electric-field-induced cell

fusion and to understand the mechanism of the fusion process the post-electric pulse time period should be studied with respect to the behaviour of the resulting fused membranes. It is well established now that pulses of sufficiently high field strength induce immediately pores in the opposing membranes; these pores may be transient, and they also may result in direct hemolysis of cell fusion (Zimmermann 1982; Zimmermann et al. 1974; Zimmermann et al. 1976). To form completely fused membranes, the diameter of the pores has to increase simultaneously with the fusion. This step in the electric-field-induced fusion has so far not been thouroughly studied. This promoted us to study intermembrane protein exchange as a function of time after the application of an electric pulse. A special experimental technique was developed to study this membrane protein mixing process. Twin cells having different surface charge densities were used and the orientation of these cell doublets in a static electric field was investigated. The orientation time is a measure of the membrane protein asymmetry between two cells. This allows to investigate the kinetics of the membrane fusion process by recording the extent of lateral interdiffusion of charged membrane proteins between the two cells.

Materials and Methods

Red cell treatment: Human blood from healthy donors was supplied by the blood bank. Plasma and buffy coat were removed after centrifugation $500 \times g$, 5 min followed by two subsequent washings in physiological NaCl-solution, pH 7,4. The surface charge was removed by treating the cells with neuraminidase 5.5 E/mg, from Atrobacter, Staatliches Inst. Immunpräp. Nährmed., Berlin-Weissen--see; or 5 E/mg from *Vibrio cholerae*, SERVA or with trypsin Fritz Leidholdt, Kleinmachnow, 80 E/mg. Enzyme treatments were performed at 37 °C in 147 mmol/l NaCl, 5.7 mmol/l, 0.3 mmol/l phosphate buffer, pH 7.4 under continuous stirring for 1 hr. The volume concentration of the cells was 45%. To adjust the actual enzyme activity, electrophoretic mobility measurements were made. For neuraminidase treatment the mobility decrease was 55% using trypsin, 10 mg/20 ml cell suspension a maximum mobility reduction of only 32% was obtained. The enzyme treatment was stopped by four washings in ice-cold physiological NaCl-phosphate buffer solution $500 \times g$; $3 \times 2000 \times g$.

Dielectrophoresis and cell fusion: The chamber was made of plexiglass with two platinum wire electrodes ∅ 1.5 mm inserted. The gap between the two cylindrical electrodes was 1 cm in length and 0.1 mm in distance. The dielectrophoretic frequency was 1 MHz and the dielectrophoretic field strength in the gap was in the range of 200—750 V/cm. Dielectrophoretic collection of the cells was performed in isotonic 290 mOsm sucrose solution supplemented with 1 mmol/l Tris-buffer. The pH was adjusted with HCl to 7.5. The temperature was about 21 ± 1 °C. At pH values below 7 the pearl chain formation was disturbed by red blood cell aggregation. Dielectrophoresis lasted no longer than 10 min. Fusion pulses were generated by a device constructed in our laboratory. The shape of the pulses was highly rectangular. Both the length and the field strength of the pulses were varied to find conditions of low hemolysis and maximum fusion efficiency. Acceptable condition found were 3 KV/cm 20 μs pulse duration. The dielectrophoretic field strength was decreased to 100 V/cm 5 s before the fusion pulse to avoid rotation of the cells after the pulse. This low field strength was still required to stabilize the pearl chains at the platinum electrodes.

Glutardialdehyde fixation: At different time intervals after the pulse, a 25% glutardialdehyde solution was added directly to the chamber in the immediate neighbourhood of the pearl chains. This was done using a very thin capillary ∅ about 300 μm inserted through a hollow in the chamber wall. The final concentration of glutardialdehyde was 5%. The fixation time was kept below 10 s. This was estimated using the effect of dielectrophoretic stretching of the cells under the action of increasing dielectrophoretic field strength. There was no cell deformation under varying field strength other 10 s. Due to hydrodynamic disturbances during the glutardialdehyde application the pearl chains at the electrodes were largely destroyed. Electrophoretic rotation measurements: Fixed fused cell aggregates and single cells were transferred to the electrophoretic chamber using another thin capillary ∅ 100 μm. The number of doublets per transfer was about 50. An electrophoretic chamber of a very small volume had therefore to be used. The chamber was made of glass plates sticked together by Phthalat G (Chem. Werke Buna). The electrodes were made of copper and they were separated by a gelatine plug from the bulk of the chamber. The total volume of the chamber was 0.28 ml. A new chamber had to be built for every new experiment, since the chamber could not cleaned. The total volume of the cell suspension transferred (diluted in 260 μm of 40% sucrose solution with 14 mmol/l NaCl) was 50 μm. The final density of the solution was 1.14 g/cm^3, pH was 7.3 and the ionic strength was about 10 mmol/l. This test solution has the advantage of preventing almost entirely cell sedimentation. Control measurements with a Zytopherometer OPTON have proved that this rather viscous solution satisfies the linear dependence of the electrophoretic velocity on field strength, viscosity and electrokinetic potential, respectively. Doublets near the upper stationary layer were found by systematically moving the microscope stage and for each doublet, the reorientation time after changing the direction of the electric field by 180° was measured 6 times. About 30 different doublets were investigated in every experiment. As a rule, only few doublets were suitable for these measurements and the necessary number of transfers was therefore made. The electric field strength was 10 V/cm. Brownian motion and small convection reduced the upper limit of the reorientation time which still could be reproducibly measured (about 60 s).

Results

Pilwat et al. (1981) and Zimmermann and Pilwat (1981) have established that surface coat protein degradation promotes cell fusion. Also fused cells with protease-treated membrane seem to have a higher stability against osmotic hemolysis induced by dielectric membrane breakdown. We therefore tried to compare the fusion kinetics of different enzyme-treated red cells. Our preliminary experiments have indeed confirmed the results of Zimmermann and his group (Zimmermann et al. 1976; Zimmermann et al. 1981, Scheurich and Zimmermann 1981; Zimmermann and Vienken 1982). A high degree of hemolysis was observed in trypsin-treated red cells after the application of electric pulses of 4.5 KV/cm and a duration exceeding than 50 μs only. Contrary to this, untreated and neuraminidase-treated cells, respectively already exhibited visible hemolysis at field strengths exceeding 2.5 KV/cm and pulse duration times exceeding 20 μs. All the above observations were made directly in the chamber by counting ghosts occurring within the pearl chains of the dielectrophoretically collected cells. The pearl chains remained stable regardless of the presence of ghosts within the chain sequence. Cell swelling always was observed, whenever hemolysis occurred. This strongly suggest the osmotic nature of the hemolysis observed.

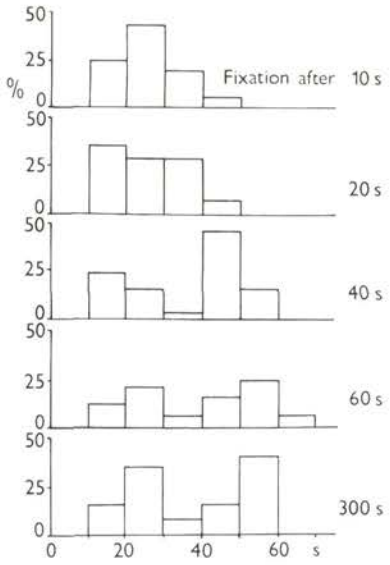

Fig. 1. Distribution frequency of orientation time over fixation interval after the electric pulse application. Fused cell pairs: trypsin-treated and untreated cells. Increasing time intervals after the pulse application. The class width: 10 s. The number of cell pairs has been chosen to meet the condition that the square root of the total sample number be not less than the number of classes.

As it is extremely difficult and uncertain to establish by direct microscopic observation whether fusion or aggregation occurred, particularly shortly after the pulse, membrane protein exchange between two adjacent cells was investigated. If the cell pair is only aggregated, no protein transfer should be possible. Consequently, if there is real membrane fusion lateral diffusion should result in an intermixing of the membrane components. Generally, the method of fluorescence recovery after photobleaching is used to study lateral diffusion. This method requires labelling of the proteins or lipide. A more serious disadvantage of the method is that it is difficult to work with intact red cells due to the photochemical reaction of hemoglobin (Peters 1981). This method can therefore be succesfully applied only to red cell ghosts. We therefore developed another method based on surface charge differences between two adjacent or fused cell membranes of intact cells. We used a mixture of untreated and enzyme-treated red cells. Enzyme-treated red cells have a lower surface charge density and, consequently, a lower electrophoretic mobility. If now a doublet is formed consisting of two different cells it has a permanent dipole moment with respect to the surface charge densities of the component cell. Consequently, it would orientate in a static electric field. However, when membrane constituents can diffuse across some established fused membrane bridges this dipole moment and, consequently, the orientation, should disappear continuously

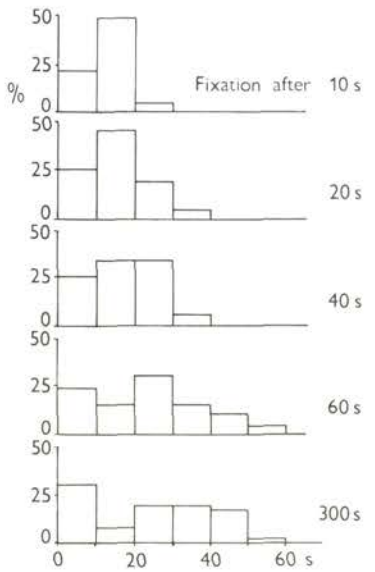

Fig. 2. Distribution frequency of orientation time over fixation interval after the electric pulse application. Fused cell pairs: neuraminidase-treated and untreated cells. Also see Fig. 1.

with the time after the application of a pulse. As for doublets collected in the electrophoretic chamber, only about one half of them will orientate. As measurements of orientation time and the necessary manipulations take quite a lot of time, and since the fused pairs are very sensitive to hemolysis, even when collecting them by gentle sucking into a capillary, it is necessary to fix the cells in the fusion chamber as described above. Marikovsky and Weinstein (1981) showed that the method described is suitable to study a current membrane lateral protein distribution in red cells. The action of this fixative seems to be quite rapid. Sutera and Mehrjardi (1975) used this method to freeze deformation in turbulent flow. We therefore concluded that this method can be well used for our purpuse of long-term investigations of lateral protein distribution.

Fig. 1 shows our results obtained with the combination of trypsin-treated and untreated cells. Initially, 10 s after the electric pulse, the distribution frequency of orientation time was homogeneous. The distribution became bimodal with the time. Some of the doublets had still the same orientation time as initially, even after 300 s. We interpret these doublets as aggregated cells. Other doublets showed a continuous increase in their orientation time consequently this portion of the doublets represented fused cells. Unfortunately, orientation times exceeding 60 s are difficult to measured with the time, it is getting more and more difficult to find orientating doublets. From this figure it can generally be concluded that the intermembrane diffusion was largely completed after 300 s. Fig. 2 shows results of

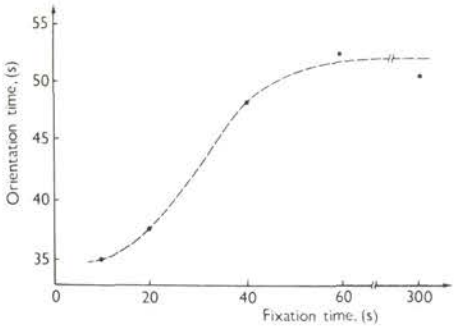

Fig. 3. Orientation time of fused pairs (trypsin-treated and untreated erythrocytes) as a function of fixation interval after the pulse application.

a combination of neuraminidase-treated and untreated cells; a comparison of Fig. 1 and Fig. 2 reveales that this latter combination showed a bimodal distribution after 60 s only. This process seemed not to be complete even after 300 s. Initially, the orientation time was considerably shorter than in the trypsin-treated cells. This can be explained by a larger dipole moment because of a lower surface charge density after neuraminidase-treatment as compared to trypsin-treatment. Thus, an orientation time of neuraminidase-treated/untreated cells of about 60 s is not equivalent to an orientation time of 60 s in trypsin treated-untreated cells. Neuraminidase-treated cell/untreated cell pairs having the some orientation time as trypsin-treated-untreated pairs are closer to an equilibrium intermembrane protein distribution.

Considering the position of the minimum in the distribution over the orientation time of the combination of trypsin-treated/untreated cells as a criterion for the decision whether fusion has taken place a minimum orientation time of 35 s is obtained for fused cells. A minimum fusion orientation time of 20 s is obtained for the combination of neuraminidase-treated/untreated cells (Fig. 2). A plot of the mean orientation time of doublets, orientating more slowly than the critical time, against the fixation interval after the pulse application, gives the kinetics of membrane protein exchange of fused unequal cell pairs. This procedure is illustrated in Fig. 3 and 4 for trypsin and neuraminidase-treatment, respectively. With the increasing time interval between the fixation and pulse application the orientation time should theoretically increase to infinity. In practice, measurements are limited to orientation times around 60 s. If the fixation is carried out in the order of minutes after the pulse less and less doublets are observed due to polyerythrocyte fusion. Nevertheless, we consider an orientation time of 60 s to be sufficiently close to a complete intermixing of membrane proteins because, under these condition, more and more twins do not orientate at all.

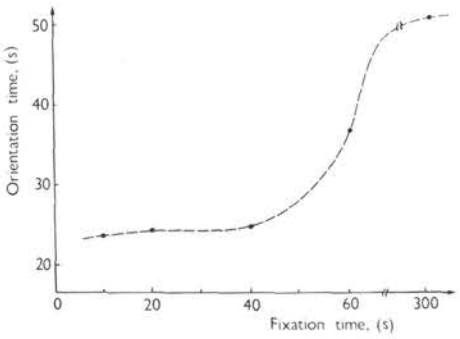

Fig. 4. Orientation time of fused pairs (neuraminidase-treated and untreated erythrocytes) as a function of fixation interval after the pulse application.

There is one basic difference between neuraminidase and trypsin-treatment with respect to the kinetics of intermembrane protein exchange. With trypsin, the equilibration process of the intermembrane protein distribution starts immediately after the pulse application, indicating a relatively fast and unhindered course of the fusion process. With neuraminidase-treated cells, the dipole moment is significantly decreased after about 40 s only. This indicates a latency period of 40 s with a subsequent increase in intermembrane protein mixing. The equilibration half time of the protein distribution, however, is similar in both cases (about 30 s). Assuming a simple two-dimensional diffusion process, the lateral diffusion coefficient of membrane proteins may roughly be estimated using the Einstein equation

$$D = \frac{x^2}{4t} \tag{1}$$

where x is the assumed characteristic length of the diffusion path; t represents the characteristic time; and D is the diffusion coefficient. Setting x equal to 1/4 of the erythrocyte perimeter ($x = 6\ \mu m$) and t to the half time, a value of about $3 \times 10^{-9}\ cm^2/s$ is obtained for D.

Discussion

The mechanism of cell fusion induced by electric field pulses is of considerable interest with respect to the improvement in the technique and in the understanding of membrane events underlying in vivo membrane fusion. We therefore developed a new method to study membrane protein diffusion between two cells. This intermixing kinetics is both a measure of the lateral mobility of membrane proteins (Fowler and Branton 1977; Edidin 1981) and of the contact surface perimeter growth. If only lateral diffusion determines the equilibration kinetics of membrane

protein distribution were determined by lateral diffusion only, a simple saturation kinetics similar to a diffusion process on a sphere should be obtained (Koppel et al. 1980; Soumpasis 1983). In our experiments however, sigmoid curve shapes were obtained. This indicates that there may be an additional process cooperating with the diffusion. At the beginning of the intermembrane diffusion process, immediately after the pulse application, the contact surface perimeter should be very small, limiting the diffusion transport of membrane proteins. With the proceeding cell fusion, the contact surface perimeter increases, promoting the intermembrane diffusion. The system presented here is thus more complex than systems studied by the FRP-technique. Especially, we have not yet been able to describe theoretically the curve shapes obtained. The main uncertainty is how to introduce a time-dependent contact surface perimeter taking into account the changing geometry of the system and; consequently, the possible convective membrane flow. Because of these uncertainties our estimation of the lateral diffusion constant is probably very rough. Nevertheless, we believe that the diffusion constant has not very much been overestimated because the contact surface perimeter may actually be small and we assume free diffusion in a plane, applying the Einstein equation. On the other hand, a possible convective membrane flow would result in an overestimation of the diffusion constant value. Taking into account these difficulties, let us now compare our value with literary data obtained by other methods on similar membranes.

First, we certainly measured the distribution of glycophorin as this membrane glycoprotein is largely responsible for the electrophoretic mobility. The majority of works done with other methods have however, been carried out with labelled band-3 protein. At physiological salt concentration, Koppel et al. (1980), Koppel et al. (1981) and Sheetz et al. (1980) have obtained values ranging between 2.2×10^{-11} and 9.2×10^{-11} cm^2/s, i.e. by two orders of magnitude smaller than our value. However, the lateral mobility of the band-3 splits into two fractions, one of them being high. Cherra (1979) e.g., has found, by fluorescence polarization measurements, a value of 3×10^{-9} cm^2/s. As contrary to the FRP-technique, fluorescence polarization measurements provide information about the local mobility and they do not necessarily coincide with data on the diffusion over large distances. Koppel et al. (1981) showed in mice mutants lacking erythrocyte spectrin a lateral diffusion coefficient of about 2.5×10^{-9} cm^2/s. Extremely interesting are the data of Golan and Veatch (1980) who studied the ionic strength and temperature dependence of the lateral mobility of the band-3 protein. They observed a sharp increase in the diffusion constant with the decreasing ionic strength in erythrocyte ghosts. Using 13 mmol/l phosphate at 37 °C they obtained a value of about 2×10^{-9} cm^2/s. At room temperature this increase was less pronounced. At high temperatures and at very low ionic strengh comparable to our fusion conditions the ghosts were not stable. Several authors dealing with protein

mobility of reconstituded model membranes reported values of the diffusion coefficient in the order of 10^{-9} cm^2/s (Chang et al. 1981).

Summarizing these data it may be suggested that it is the cytoskeleton that is responsible for a low lateral diffusion mobility, at least over larger distances. Whenever the cytoskeleton is absent, or when the interaction with the membrane is diminished by temperature, low ionic strength or other factors, a high lateral mobility is observed. Smith and Palek (1982) and Schindler et al. (1980) discuss a diffusion hindrance in a polymer network. In additon other authors assume chemical binding to the cytoskeleton (see Elson and Reidler 1979).

Based on the above, we may also conclude that our high value of the diffusion constant is due to a diminished interaction of the cytoskeleton with the cell membrane. This may have resulted from the very low ionic strength (about 1 mmol/l) in our dielectrophoresis and fusion experiment. Moreover, the fusion itself may significantly increase the lateral mobility as discussed by Edidin (1981). Maybe both these phenomena the fusion and the dimished cytoskeleton membrane interaction, are closely related to one another.

It is well established that pores induced in lipid model membranes by dielectric membrane breakdown are reversible if the electric field does not exceed a critical value (Chizmadzhev et al. 1982; Shchipunov and Drachev 1982; Benz and Zimmermann 1981). With intact cell membranes with a high protein content the breakdown process is more difficult (Coster and Zimmermann 1975; Zimmermann et al. 1974). In cell membranes, the breakdown usually occurs at the lipid-protein junctions or in the proteins themselves. The recovery of these field effects takes much longer (Zimmermann 1982).

Kinosita and Tsong (1977b) demonstrated with human erythrocytes that hemolysis of erythrocytes treated by electric field pulses still proceeds even after several hours. If membrane proteins exhibit a high lateral mobility and there is a small amount of material due to proteolytic degradation the possibility of lipid domain formation increases. Zimmermann and Vienken (1982) have postulated a mechanism of dielectrophoretic lateral protein displacement which results in lipid domain formation at the opposing membranes. As our results showed cell fusion of protease-treated cells to proceed more rapidly as compared to neuraminidase-treated cells, this mechanism of lipid domain formation is at least not contradictory to Zimmermann's postulate. If there is less protein material at the membrane fusion site, less hindrance will occur. On the other hand, the fact of the pronounced higher stability of protease-treated cells and their higher fusion efficiency demonstrated by Zimmermann and Vienken (1982) and Scheurich and Zimmermann (1981) and confirmed by us also supports the hypothesis of an increased reversibility of the breakdown, probably at lipid areas at the poles of the opposing cells. One can also speculate about the latency period observed (Fig. 4) with neuraminidase-treated cells: it may be due to a diffusion hindrance at the initially small fusion site or the

fusion itself may be slower because of the necessary displacement of the probably absorbed uncharged protein material (Donath and Lerche 1980). There is no final resolution to this question at the moment and further experiments are required.

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