ELSEVIER

Biochimica et Biophysica Acta 1238 (1995) 81–85

BBA Biochimica et Biophysica Acta

Dimensional distribution of human erythrocytes obtained from electropermeabilization experiments

I. Turcu *, Silvia Neamtu

Institute of Isotopic and Molecular Technology, P.O. Box 700, 3400 Cluj-Napoca, Romania

Received 27 February 1995; accepted 28 March 1995

Abstract

Electropermeabilization of erythrocyte membrane was performed using square electric field pulses of 50 μs and 1 ms pulse length. The electrohemolysis efficiency has a sigmoid dependence on the electric field strength given mainly by the statistical distribution of erythrocytes radius. The normalized dimensional distribution function for a human erythrocyte population was determined from electropermeabilization data.

Keywords: Electropermeabilization; Erythrocyte membrane; Shape; Size distribution; Electrohemolysis; Hemolysis; (Human)

1. Introduction

In the last two decades the electropermeabilization and the electrofusion, techniques based on high voltage pulsed electric fields, became a very useful and efficient tool for genetic engineering, medicine, biology, agriculture and biotechnology [1–4]. A lot of experiments have been done on human erythrocytes in order to obtain information about the values of their specific electrical parameters [1], to prepare ghosts [5] or to use them as drug carriers [3,5–7].

The erythrocyte membrane electropermeabilization can be induced by exponentially decaying or square pulses with high electric field strengths (kV/cm) and short lengths (μs). The electropermeabilization phenomenon reveals two specific mechanisms responsible for the uptake and release of molecular material that penetrate permeabilized regions of the cellular membrane as a consequence of the applied electric field pulse. Ions and small molecular species penetrates the membrane as soon as the threshold value of the electric field strength, needed to induce the dielectric breakdown, is swept past.

The macromolecules having large dimensions penetrate with difficulty the cell membrane, the permeabilization being essentially a complex electro-osmotic phenomenon, the final stage corresponding to the colloid osmotic swelling of the electroporated cells [8–10].

A specific example for this second type of process is the electrohemolysis. Using rectangular pulses of different lengths, the range of electric field strength values, needed to induce the complete hemolysis of a normal erythrocyte population was determined. The electrohemolysis efficiency depends in a sigmoid manner on the applied electric field strength. Our goal was to use this dependence in order to obtain the dimensional distribution function of the investigated cellular population.

2. Electropermeabilization –a cell size dependent phenomenon

Usually the electropermeabilization techniques are based on pulsed electric fields. The maximum transmembrane potential difference induced on a cell of radius R by a square electric pulse having a strength E and a length T is given [4,11–13] by:

$$U = fgER(1 - e^{-T/\tau}) \quad (1)$$

where f is a shape dependent factor (3/2 for spheres), g a dimensionless parameter of order of unity, controlled mainly by the membrane electric conductivity and τ the polarization relaxation time.

Considering the cell membrane a pure dielectric the product fg in Eq. (1) is taken simply 3/2. Lower values

* Corresponding author.

SSDI 0005-2736(95)00090-9

of this adjustment parameter were reported recently [13] and it was shown also that they are specie dependent. The dimensionless parameter g is also radius dependent but the dependence is very weak and it will be neglected in the following. A stronger dependence has the characteristic time constant τ that to a good approximation is proportional to R. Nevertheless we can obtain a negligible effect of this dependence on the transmembrane potential by using longer electric field pulses. In our experiments we have used pulses of 50 μs and 1 ms pulse length. As erythrocytes have the characteristic time constant of order of microseconds the contribution of the exponential factor in Eq. (1) become negligible.

Taking into account all that we conclude that to a good approximation the transmembrane potential has a linear dependence on cell radius.

Above a threshold value of the transmembrane electric potential U_c the membrane permeability increase dramatically. The change in the membrane permeability is accompanied by the release of the molecular material of the cell and by the uptake of some molecular species from the external medium.

Along the years different values have been proposed from about 1 V by Zimmermann [8] down to 200 mV by Teissié [13]. A possible explanation is that the adjustment parameter g in Eq. (1) was overestimated.

In order to avoid all that we will define an effective critical transmembrane potential:

$$U_c^{\text{eff}} = \tfrac{3}{2}\left(fg\left(1 - e^{T/\tau}\right)\right)^{-1} U_c \tag{2}$$

which incorporate all the particularities of the cell membrane, internal medium and suspending medium excepting the linear dependence of the cell radius.

That means that U_c^{eff} have the same value for any cell, in a given cellular population, so that the electropermeabilization phenomenon becomes only dimensional dependent. Increasing the electric field strength, the critical condition:

$$U_c^{\text{eff}} = \tfrac{3}{2}ER \tag{3}$$

is first accomplished for cells with great radius their membrane being electropermeabilized. Increasing again the field strength for more and more cells the critical condition (Eq. (3)) is accomplished so that in a certain range of the field strength values all the cells are permeabilized. For a normally distributed cellular population the electropermeabilization curve have a sigmoid dependence.

We will further describe the way of obtaining the dimensional distribution of a cellular population from electropermeabilization data.

Let suppose that we have experimentally determined the electropermeabilization efficiency $\eta(E)$, at a given value of the electric field strength, as the ratio between the number of permeabilized cells and the total number of cells. As the critical value of the effective transmembrane potential is supposed to be the same for all the cells, the membrane of any cell having a radius greater than the value $R(E)$ (given by Eq. (3)) is permeabilized.

Following this reasoning, the connection between the normalized dimensional distribution function and the electropermeabilization efficiency is given by the equation:

$$\eta(y) = 1 - \int_0^x f(x')\,\mathrm{d}x' \tag{4}$$

where $f(x)$ is the normalized dimensional distribution function, x and y being the following dimensionless variables:

$$x = R/R_{1/2};\ y = E/E_{1/2} \tag{5}$$

The quantities $E_{1/2}$ and $R_{1/2}$ are the values of the electric field strength at 1/2 efficiency and the middle cell radius:

$$\int_0^1 f(x)\,\mathrm{d}x = \tfrac{1}{2} \tag{6}$$

respectively. It must be emphasized that the middle radius defined in Eq. (6) and the mean radius of a given erythrocyte population have in general different values. The critical condition given by Eq. (3) becomes in the new dimensionless variables:

$$x = 1/y \tag{7}$$

By differentiating the equality (4) in respect with x the dimensional distribution function is given by:

$$f(x) = \frac{1}{x^2}\frac{\mathrm{d}\eta}{\mathrm{d}y} \tag{8}$$

This formula is the main result of this work and it will be used to numerically compute the normalized dimensional distribution function.

It is a remarkable fact that using normalized variable Eq. (8) has a universal validity, being independent on the specific values of the membrane conductivity, cell size and also on the electric properties of the internal and external media.

3. Materials and methods

Human blood was collected from apparently healthy donors preserved with sodium citrate and used on the same day. The red blood cells were centrifuged and washed three times with an isosmotic solution (NaCl/mannitol, 2%/98%). The experiments were performed with a 1% erythrocytes suspension in the same medium.

The cell suspension was subjected to a rectangular electric field pulse with fixed length and variable amplitude. At each pulse length the amplitude was increased until a threshold value of the electric field strength was attained and the lysis of the first cell was observed. The erythrocytes lysis was detected by the drastic change in the refractive index. Increasing further the strength of the field

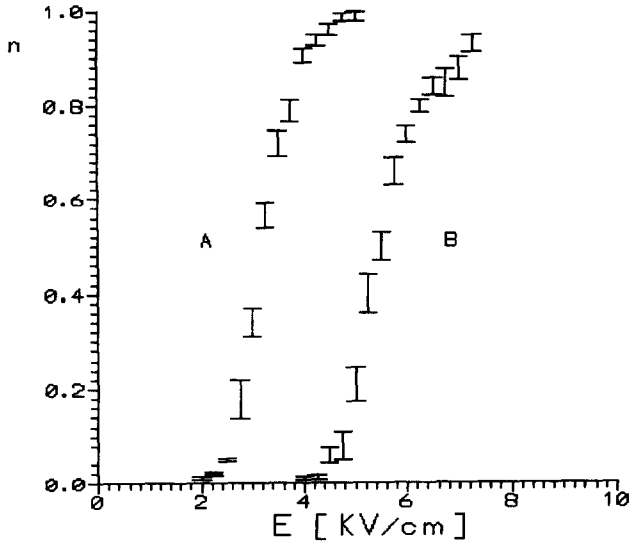

Fig. 1. The sigmoid dependence of the electrohemolysis efficiency on the pulsed electric field strength. The length of the used square pulses was: (A) 1 ms; (B) 50 μs.

the number of lysed cells increases until all of them are lysed.

The electrohemolysis efficiency was determined by two methods:

(a) Using a very dilute suspension (0.01%) we were able to bring between electrodes only one cell. For each singular cell the threshold value of the electric field strength that induces hemolysis was determined.

(b) Using an aliquot of 1% cellular suspension we had about 60–80 cells between electrodes. The efficiency of the electrohemolysis phenomenon, was calculated for each value of the field by dividing the number of lysed cells to the number of all cells.

The electric field was obtained between two metallic electrodes, made from gold, deposited on a microscope glass slide by a microphotolithographic technique. The metallic film was about 5 μm thick and the gap between electrodes has 100 μm. The electric field strength was estimated as the ratio of the voltage pulse amplitude versus the gap distance. The pulses were generated by a complex apparatus (SPEC-1) build in our laboratory and monitored on line by a Hameg 205 oscilloscope. The decay for the longer pulses was no more than 5%.

The electrohemolysis was observed directly at a research microscope MC-5A.

4. Results

The dependence of the electrohemolysis efficiency on the electric field strength is shown in Fig. 1, for two values of the pulse length: 50 μs and 1 ms, respectively.

The curves A and B obtained using the multicellular and unicellular method respectively, have both a sigmoid shape. It can be seen that the electric field strength needed to induce hemolysis is almost double for the shorter pulse. The range of the values of the electric field strength, starting at the first lysed cell and ending at the complete lysis is roughly speaking 2 kV/cm–5 kV/cm for the longer pulse and 4 kV/cm–7.5 kV/cm for the shorter one.

The experimentally obtained values for the $E_{1/2}$ electric field strength are 3.2 kV/cm for 1 ms pulse length and 5.5 kV/cm for 50 μs pulse length. By direct observation at the microscope we have obtained, for a population of about 200 cells, the middle radius $R_{1/2}$ equal to 3.2 μm. The effective critical values of the transmembrane potential obtained from the given $E_{1/2}$ and $R_{1/2}$ are 1.5 V and 2.6, V respectively.

The error bars in Fig. 1 reveal a rather large dispersion of experimental data. Having in mind that the corresponding curves must be differentiated to obtain the dimensional distribution function we will present the electrohemolysis data by histograms. Each value was obtained by averaging on at least three experimental measurements.

Fig. 2 presents the efficiency of electrohemolytic process versus the dimensionless parameter y as histograms

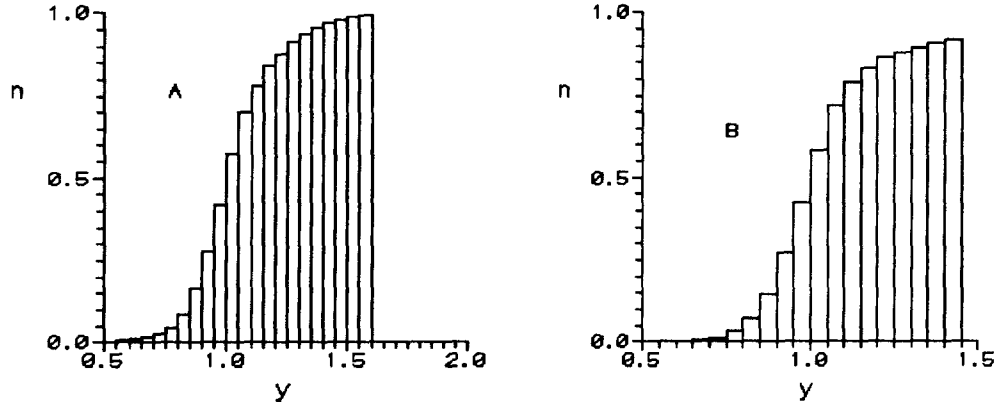

Fig. 2. The histograms describing the dependence of the electrohemolysis efficiency on the electric field strength.

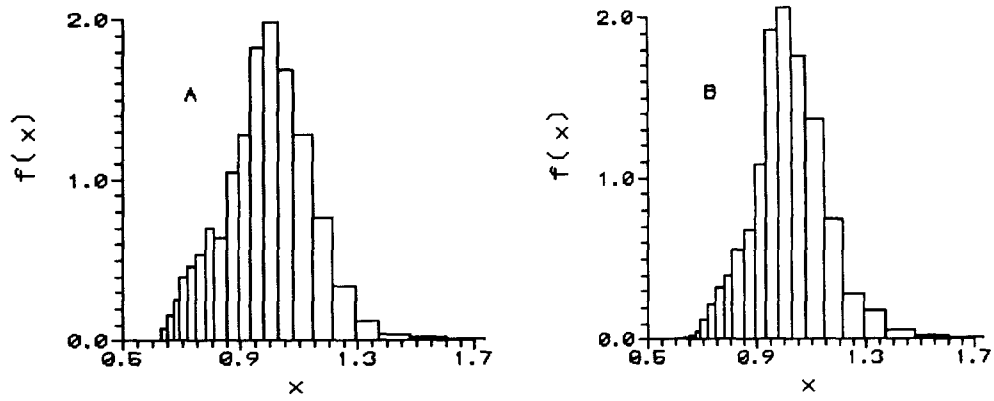

Fig. 3. The normalized dimensional distribution function obtained by numerical differentiation of the electric field dependent electrohemolysis efficiency histograms.

for the two pulse lengths. Using Eq. (8), by numerical differentiation of the two histograms, we obtained the normalized dimensional distribution function corresponding to the two sets of experimental data (Fig. 3).

Although the width of the y increment in Fig. 2 is constant, the width of the x increment in Fig. 3 increase, the parameters x and y being related by Eq. (7).

5. Discussion and conclusions

The electrohemolysis experiments reveal some unexpected facts, apparently in contradiction with the known data on the electric parameters of human erythrocytes membrane. The typical accepted value for the electric field potential that produces the biomembrane dielectric breakdown are generally of 0.2–1 V [1,13]. Our results give values that are several times higher. On the other side the pulses that we have used are much more longer than the erythrocyte membrane electric polarization time therefore we expect a very small difference between the curves A and B corresponding to the two pulses. The experimental values (1.5 V and 2.6 V) obtained from the 50% haemoglobin release electric field strengths $E_{1/2}$ are in disagreement with the previous hypothesis.

All these disagreements can be solved if the electrohemolysis is understood as an colloid-osmotic phenomenon [2,7,9,10], the dielectric breakdown being only the promoter of the process. The values obtained in our experiments are smaller than those obtained by Zimmermann and al. [8] in a set of alike experiments on bovine and human erythrocytes. A possible source of this discrepancy can be found in the two major differences between our works: a saline external medium and an exponentially decaying electric pulse on one side, a nonelectrolytic medium and a square electric pulse on the other side.

Let return now to our main goal in this paper and make some comments on the normalized dimensional distribution functions represented as histograms in Fig. 3. By comparing the two distribution one remarks that they have both a single relatively narrow peak, centered on the middle radius $R_{1/2}$ and a similar shape. The second histogram, obtained for a 50 μs pulse length by the unicellular method, is a little higher and tighter. The width of both distribution is about $0.3R_{1/2}$ that means 1 μm. A normally distributed human erythrocyte population width is in general smaller that our value. This means that besides the dimension there are other parameters that enlarge the width by their statistically distributed values. A possible candidate that can be suspected is the nonuniformity of the electric field. What we know precisely is the potential applied on electrodes but the position of each cell is dictated by chance, the local electric field being influenced by the proximity of an other cell or of the electrodes. However, the importance of this aspect is ruled out by our results that show almost identical widths for both unicellular and multicellular types of experiments.

The single parameter that can be suspected to enlarge the width of the dimensional distribution is the effective critical transmembrane potential which values seems to vary a little bit from cell to cell. A possible explanation can be find in the dependence of the g value (Eq. (1)) on the physiology of each cell of the population. It is a well known fact that the erythrocyte membrane composition and functions changes during maturation and aging [14]. In general it is accepted that with increasing age there are decreases in the total lipid, cholesterol and phospholipid of the red cell. During the aging process there is also a significant shift in the fatty acid patterns related to the increase in some short-chain acids compensated by a decrease in other long-chain acids. It was demonstrated also that the membrane fluidity decreases during in vivo aging. Consequently, small differences in the critical transmembrane potential during erythrocytes maturation and aging are to be expected.

Acknowledgements

The authors are indebted to Dr. V.V. Morariu for drawing their attention to the subject.

References

[1] Zimmermann, U. (1982) Biochim. Biophys. Acta 694, 227–277.
[2] Tsong, T.Y. (1983) Biosci. Rep. 3, 487–505.
[3] Zimmermann, U., Arnold, W.M. and Mehrle, W. (1988) J. Electrostat. 21, 309–345.
[4] Neumann, E., Sowers, A.E. and Jordan, C.A. (1989) Electroporation and Electrofusion in Cell Biology, Plenum Press, New York.
[5] Zimmermann, U., Riemann, F. and Pilwat, G. (1976) Biochim. Biophys. Acta 436, 460–474.
[6] Zimmermann, U., Scheurich, P., Pilwat, G. and Benz, R. (1981) Angew. Chem. Int. Edn. Engl. 20, 325–345.
[7] Zimmermann, U., Pilwat, G. and Wienken, J. (1980) in Recent Results in Cancer Research, Vol. 75 (Mathe, G. and Muggia, F.M., eds.), pp. 252–259, Springer-Verlag, Heidelberg.
[8] Riemann, F., Zimmermann, U. and Pilwat, G. (1975) Biochim. Biophys. Acta 394, 449–462.
[9] Kinosita, K., Jr. and Tsong, T.Y. (1977) Biochim. Biophys. Acta 471, 227–242.
[10] Kinosita, K., Jr. and Tsong, T.Y. (1979) Biochim. Biophys. Acta 554, 479–497.
[11] Schwan, H.P. (1957) Adv. Biol. Med. Phys. 5, 147–209.
[12] Turcu, I. and Lucaciu, C.M. (1989) J. Phys. A 22, 985–1003.
[13] Teissié, J. and Rols, M.P. (1993) Biophys. J. 65, 409–413.
[14] Shiga, T., Maeda, N., Suda, T., Kon, K. and Sekya, M. (1979) Biochim. Biophys. Acta 553, 84–95.