Biochimica et Biophysica Acta, 406 (1975) 424–432

BBA 77097

DIELECTRIC BREAKDOWN MEASUREMENTS OF HUMAN AND BOVINE ERYTHROCYTE MEMBRANES USING BENZYL ALCOHOL AS A PROBE MOLECULE

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(Received April 4th, 1975)

SUMMARY

Dielectric breakdown of intact erythrocytes and subsequent haemolysis in the presence of increasing concentrations of benzyl alcohol were investigated by means of an electrolytical discharge chamber and a hydrodynamic focusing Coulter Counter.

Low concentrations of the drug stabilized human and bovine erythrocytes against haemolysis induced by dielectric breakdown of the cell membrane in isotonic solutions, while high concentrations caused lysis similar to hypotonic and mechanical haemolysis. The stabilizing effect of the drug on electrically induced haemolysis depends on the pulse length of the applied electric field. The critical dielectric breakdown voltage of the membranes of intact cells decreases progressively with increasing benzyl alcohol concentrations, at which the membrane is also more stabilized against electrical and osmotic haemolysis. Occasionally, an increase in the dielectric breakdown voltage is observed at drug concentrations at which lysis occurs. A similar dependence of the breakdown voltage on drug concentration was found for human erythrocyte ghost cells prepared by dielectric breakdown.

The results are consistent with the electromechanical model suggested for the dielectric breakdown mechanism and with the assumption of Metcalfe, using NMR and ESR techniques, that the fluidity of the membrane increases with increasing benzyl alcohol concentration.

INTRODUCTION

Dielectric breakdown of cell membranes occurs when a critical membrane voltage is reached by applying short voltage or current pulses of increasing strength directly (using micro-electrodes) or indirectly (by external electric fields) to the cell [1–3]. Dielectric breakdown is manifested by a marked, reversible permeability change of the membrane during which cellular content and the external medium are exchanged bi-directionally. Thus, as shown previously, haemoglobin release from red blood cells can be induced by dielectric breakdown of the cell membrane [2, 4], while external molecules (proteins, etc.) are incorporated simultaneously into the resealing

ghosts [5]. After resealing, dielectric breakdown can be repeated many times on the same cell with a very reproducible breakdown voltage [6].

The large temperature dependence of the critical breakdown voltage [7] as well as the differences between the breakdown voltages for bacteria harvested from the logarithmic and stationary growth phase [2] suggest that the dielectric breakdown of cell membranes may be a very sensitive tool for detecting changes in membrane structure and composition. In this communication we investigated the effect of varying concentrations of the local anaesthetic benzyl alcohol on the red blood cell membrane in relation to the dielectric breakdown voltage. At lower concentration local anaesthetics such as benzyl alcohol, as well as tranquilizer and anti-inflammatory compounds, are well known to have a stabilizing effect on the red blood cell membrane [8–11]. This effect is documented by a biphasic dose response curve for osmotic haemolysis.

Using NMR and ESR techniques, Metcalfe [12–14] has studied structural perturbations caused by the insertion of benzyl alcohol into the isolated erythrocyte membrane, both in the prelytic concentration range of the drug, where a stabilizing effect against haemolysis is observed, and in the lytic range. Both magnetic resonance techniques indicated a progressive fluidization of membrane components when benzyl alcohol was applied in prelytic concentrations. Measurements of dielectric breakdown of red blood cell membranes treated with benzyl alcohol, therefore, may provide information about the sensitivity of this electrical method to detect structural perturbations of the membrane.

METHODS

Since the experimental procedure for the determination of the critical membrane breakdown voltage of red blood cells was described in detail previously [2, 15, 16], only a brief description is required here. In contrast to the experiments of Metcalfe [12–14], measurements of the critical dielectric breakdown voltage were performed with fresh intact erythrocytes or ghosts obtained by electrical haemolyis. It should be further noted that the experiments were carried out in isotonic solutions (NaCl, 138.6 mM; Na_2HPO_4, 12.3 mM; NaH_2PO_4, 2.7 mM; pH 7.4) to which increasing concentrations of benzyl alcohol were added.

The volume ratio of red blood cells to medium was about 1 : 100 in the discharge cell and about 1 : 500 in the Coulter Counter experiments, corresponding to about 10^8 and $2 \cdot 10^7$ human erythrocytes per ml, respectively. As indicated in the figures, the incubation times were 5 and 10 min, respectively.

The dielectric breakdown voltage of the membrane was calculated from measurements of the size distributions of the red blood cell suspension as a function of increasing electric field strengths in the orifice of an improved Coulter Counter instrument. At the critical field strength in the orifice a shift in the size distribution to smaller sizes occurs, caused by dielectric breakdown of the cell membranes. The critical external electric field strength in the orifice is volume dependent. From the critical electric field strength the critical membrane breakdown voltage can be calculated by solving the Laplace equation [2] in spherical coordinates. The membrane breakdown voltage, however, is volume-independent.

It is a reasonable approximation that the shape factor, for red blood cells (1.08), is unaltered in the presence of the drug.

The haemoglobin release from the red blood cells induced by dielectric breakdown at different concentrations of benzyl alcohol was measured in an electrolytic discharge cell in which two flat platinum electrodes were placed. The chamber was part of a high-voltage discharge circuit. Short voltage pulses were applied to the electrodes and to the suspension in the chamber by discharging the high voltage storage capacitor via a spark gap. The pulse length (the time constant) was varied by changing the capacitance of the capacitor. The released haemoglobin was measured photometrically at 415 nm in the supernatant after centrifugation of the samples collected.

Erythrocyte ghosts were prepared by dielectric breakdown of the membrane as described elsewhere [5]. The erythrocytes were suspended in a solution containing Na_2HPO_4, 7.6 mM; NaH_2PO_4, 2.4 mM; $MgCl_2$, 4.0 mM; NaCl, 20 mM; KCl, 105 mM and glucose 10 mM, pH 7.2. An electric field strength of 12 kV $\cdot$ cm^{-1} was then applied to the suspension (pulse length 40 μs). This field is sufficiently strong to induce dielectric breakdown also in the membranes of small cells [5]. The haemoglobin content of the ghost cells was about 10 %, compared to intact erythrocytes, as measured with a Zeiss photometer at 415 nm. After resealing, the dielectric breakdown measurements were carried out in the isotonic buffer described above.

RESULTS AND DISCUSSION

Fig. 1 shows the effect of increasing benzyl alcohol concentrations on the haemolysis of human red blood cells induced by dielectric breakdown. The measurements were carried out in the electrolytical discharge chamber at a critical electric field strength of 14 kV $\cdot$ cm^{-1} and a pulse length of 10 μs. The degree of haemolysis is expressed in percent of total haemolysis determined independently by osmotic lysis in water. At concentrations below approx. 130 mM, benzyl alcohol stabilizes the erythrocyte membrane against haemolysis induced electrically, particularly in the concentration range above 100 mM. Above approx. 130 mM benzyl alcohol, increased haemolysis is observed. The values are corrected for the slight haemolysis of the cells caused by the drug in the absence of the field. At about 150 mM benzyl alcohol, spontaneous and complete lysis of the cells occurred also in the absence of an electric field. The modal (mean) volume was constant within the whole concentration range. For human red cells the results were quite reproducible. A similar biphasic dose-response curve was obtained for bovine erythrocytes [15]. With this species the stabilizing effect of the drug on the electrical fragility of the membrane is observed below a concentration of 80 mM. That the effects were not as reproducible as with human erythrocytes may be due to some artifacts, since the blood was obtained from the slaughter house.

The extent of the stabilizing effect of benzyl alcohol on the electrically induced haemolysis depends on the pulse length of the capacitor discharging process of the high voltage circuit. In Fig. 2, the maximal stabilizing effect of benzyl alcohol occurring at about 130 mM is plotted against increasing pulse lengths for human erythrocytes. The values for maximal protection (Fig. 2) were obtained by measuring haemoglobin release as function of increasing drug concentrations at different pulse lengths. The

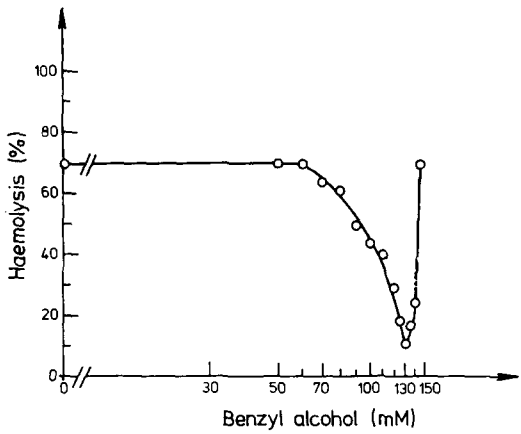

Fig. 1. Effect of increasing benzyl alcohol concentration on the haemolysis of human erythrocytes induced by dielectric breakdown in an electrolytical discharge chamber. The electric field strength was 14 $kV \cdot cm^{-1}$; the pulse length of the applied field was 10 μs. Note that electrically induced haemolysis was performed in isotonic buffer solution (NaCl, 138.6 mM; Na_2HPO_4, 12.3 mM; NaH_2PO_4, 2.7 mM; pH 7.4) to which the various concentrations of the drug were added. The volume ratio of red blood cells to medium was about 1 : 100, corresponding to about 10^8 cells per ml. The ordinate represents the percentage amount of haemolysis relative to osmotic lysis in distilled water. The values for haemoglobin release at concentrations of benzyl alcohol from 130 mM to 150 mM are corrected for the slight lysis caused by the drug in the absence of the electric field. The data points are average values from two different sets of measurements.

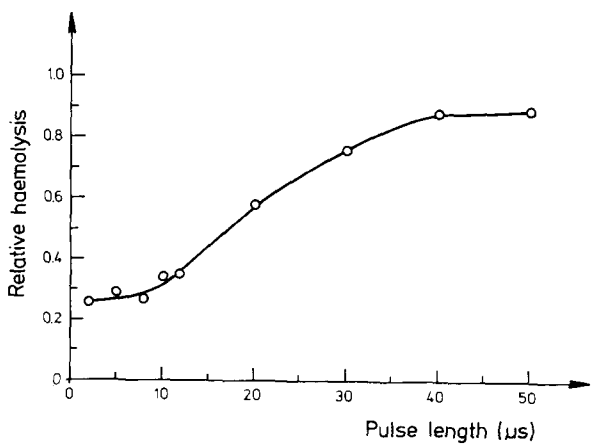

Fig. 2. Influence of the pulse length of the applied electrical field in the discharge chamber on the electrically induced haemolysis of human erythrocytes in the presence of 130 mM benzyl alcohol. At this concentration the maximal protection effect of the drug against haemolysis is observed according to Fig. 1. A relative haemolysis of 1.0 indicates an absolute degree of haemolysis of about 70 % in the absence of benzyl alcohol. Therefore, the electric field strength was varied for each pulse length in order to produce 70 % haemolysis in the absence of the drug. As indicated in the figure, the stabilization effect of benzyl alcohol on the erythrocyte membrane decreases with increasing pulse length and disappears at a pulse length of about 50 μs. The data points are average values from six different sets of measurements.

incubation time was 10 min. As shown previously [16], the haemoglobin release depends on the pulse length due to the capacity properties of the erythrocyte membranes, which become the rate-limiting step for the temporal build-up of the critical membrane breakdown voltage below 10 μs pulse length. Therefore, the experiments were arranged in such a manner that the electric field strength for each pulse length was varied to produce approx. 70 % haemoglobin release in the absence of benzyl alcohol to eliminate this effect.

As shown in Fig. 2, the stabilizing effect of the drug on the erythrocyte membranes is observed only for a small pulse length range. For technical reasons measurements below 5 μs pulse length were not possible. The protection effect against electrically induced haemolysis decreases continuously from 10 to 50 μs as indicated by the increase of haemolysis. Above a pulse length of 50 μs, the protection effect disappears and the degree of haemolysis reaches a value which is comparable to that in the absence of benzyl alcohol. This surprising result is not yet completely understood. However, we feel that this finding points to different mechanisms involved in the electrical and osmotic haemolysis, although both processes are phenomenologically equal if one considers the membrane stabilizing effect of the drug at low pulse length.

In Figs 3 and 4 the dielectric breakdown voltages of the membranes of human and bovine erythrocytes are plotted versus increasing benzyl alcohol concentrations. Using the Laplace equation the critical breakdown voltage (V_c) was calculated from the size distribution measured in the hydrodynamic focusing Coulter Counter at

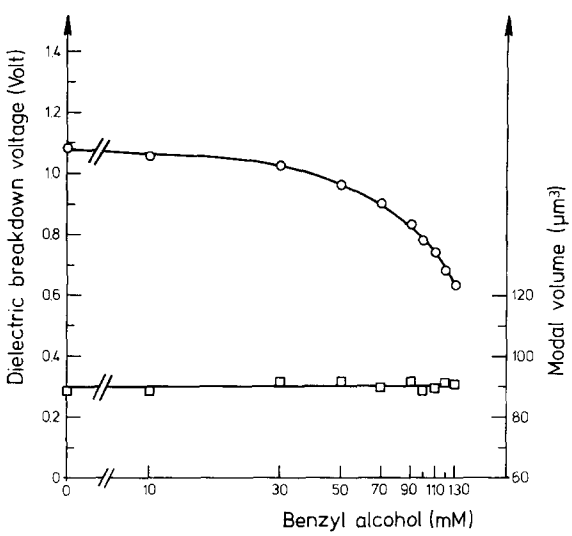

Fig. 3. Dielectric breakdown voltage of the membrane of human erythrocytes as a function of increasing benzyl alcohol concentration measured with a hydrodynamic focusing Coulter Counter with an orifice of 60 μm in diameter and length. The erythrocytes were preincubated for 5 min in the isotonic solution described in Fig. 1 to which various concentrations of the drug were added. The volume ratio of red blood cells to medium was about 1 : 500, corresponding to about $2 \cdot 10^7$ erythrocytes per ml. As indicated in the figure, the dielectric breakdown voltage (○-○) decreases with increasing benzyl alcohol concentration. The modal volume of the size distribution of the erythrocytes (□-□) is concentration independent. The data points are average values from three different sets of measurements.

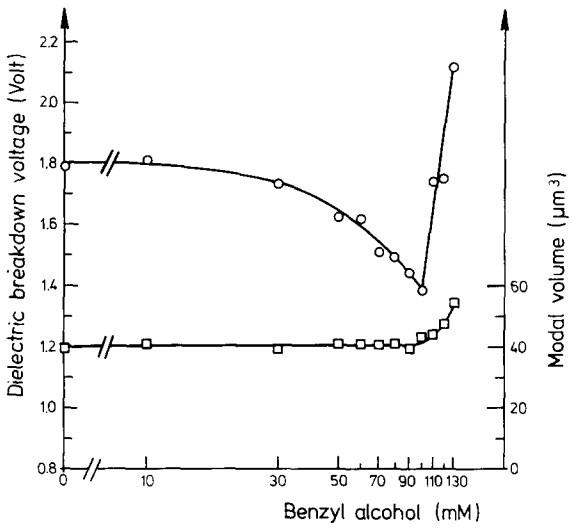

Fig. 4. Dielectric breakdown voltage of the membrane of bovine erythrocytes as a function of increasing benzyl alcohol concentrations measured with a hydrodynamic focusing Coulter Counter with an orifice of 40 μm in diameter and length. The erythrocytes were preincubated for 5 min in the isotonic solution described in Fig. 1, to which various concentrations of the drug were added. As indicated in the figure, the dielectric breakdown voltage (○-○) decreases with increasing benzyl alcohol concentration. The modal volume of the size distribution of the erythrocytes (□-□) is constant over a large concentration range, but increases slightly at high benzyl alcohol concentrations of about 100 mM. The data points are average values from four different sets of measurements.

different electrical field strengths in the orifice (1–5 kV · cm^{-1}) [2]. In this experiment cells passed through the orifice (length = 40 μm) in about 30 μs, i.e. the pulse length of the applied electric field corresponds to 30 μs.

As indicated in both figures, the concentration range of benzyl alcohol in which the dielectric breakdown voltage of the membrane is progressively decreasing coincides well with that at which the membrane is more stabilized against electrical (and osmotic) haemolysis. The same inverse correlation between the dielectric breakdown voltage and the drug concentration in the prelytic range was also obtained for human erythrocyte ghosts prepared by dielectric breakdown (Fig. 5), emphasizing that the effects described arise from perturbations within the membranes.

In bovine erythrocytes a rise in the dielectric breakdown voltage was sometimes observed, together with a corresponding volume increase which coincided with the onset of lysis. It is possible that the pronounced upswing of the relaxation rate observed by Metcalfe in NMR experiments at lytic concentrations is related to our observation using the dielectric breakdown voltage technique. Metcalfe [12–14] interpreted his effect by assuming that the membranes become so perturbed at these lytic concentrations that new protein-binding sites are exposed, resulting in irreversible perturbations of the membranes. Since measurements of the dielectric breakdown voltage can be made only with intact cells, it is not surprising that the rise of the dielectric breakdown voltage could not be observed for human erythrocytes; human erythrocytes lyse immediately at concentrations of approx. 150 mM whereas, in the absence of the electric field, lysis of bovine erythrocytes induced by lytic benzyl alcohol concentrations takes several minutes.

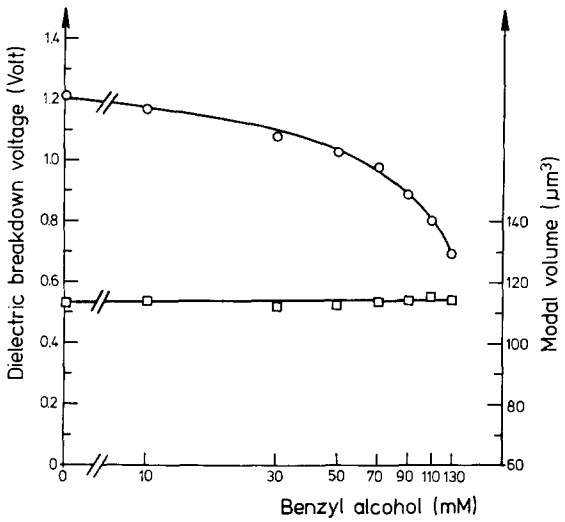

Fig. 5. Dielectric breakdown voltage of human erythrocyte ghosts as a function of increasing benzyl alcohol concentrations measured with a hydrodynamic focusing Coulter Counter with an orifice of 60 μm in diameter and length. The ghosts were prepared by dielectric breakdown in the electrolytical discharge chamber, using an electric field strength of 12 kV · cm^{-1} and a pulse length of 40 μs. After a resealing time of 30 min the ghosts were preincubated for 5 min in the isotonic solution described in Fig. 1 to which increasing drug concentrations were added. As indicated in the figure, the dielectric breakdown voltage (○-○) decreases with increasing benzyl alcohol concentration. The modal volume of the size distribution of the ghost cells (□-□) is concentration independent. The data points are average values from two different sets of measurements.

That prelytic benzyl alcohol concentrations caused reversible changes in the structure of the membrane was also reported by Metcalfe [12]: intact human erythrocytes pretreated with 100 mM benzyl alcohol for 5 min, washed in isotonic solution several times followed by incubation in a solution to which 50 mM of the drug was added, exhibited the same dielectric breakdown voltage as measured in non-pretreated cells.

By measuring the rotational rate of the benzyl alcohol molecules with NMR and ESR in the prelytic range, Metcalfe found that the motion of the molecules within the isolated erythrocyte membrane becomes progressively freer, interpreting this effect in terms of an increased membrane fluidity. This conclusion is not in conflict with the model developed for the dielectric breakdown mechanism [2, 7]. At present this model explains the dielectric breakdown data by assuming a dimensional equilibrium between the electric compressive forces in a membrane and the elastic forces created by the compression of the membrane.

On the basis of this model, the dielectric breakdown voltage* V_c depends on the ratio of the elastic, compressive modulus Y (normally orientated to the membrane plane) to the dielectric constant ε and the membrane thickness δ_0 at zero membrane

* The equation of the dielectric breakdown voltage [7, 16] on the basis of the electromechanical model is given by:

$$V_c^2 = \frac{0.3679 \cdot Y \cdot \delta_0^2}{\varepsilon \cdot \varepsilon_0}$$

potential. Assuming that the thickness and the dielectric constant are unaltered by benzyl alcohol, the decrease of the dielectric breakdown voltage with increasing drug concentration in the prelytic range could be caused by a decrease in the elastic compressive modulus. On this assumption, the value of the elastic modulus would shift from $7 \cdot 10^6$ Nm^{-2} in the absence to $2.4 \cdot 10^6$ Nm^{-2} in the presence of benzyl alcohol where maximal protection is observed (approx. 130 mM, human erythrocytes); i.e. as a result of increasing perturbations, the membrane becomes more compressible. This model is not in contradiction to the possible mechanisms of erythrocyte stabilization by drugs discussed by others [17]. Investigations by Seeman indicated that drugs increase the critical haemolytic volume of the erythrocyte. This may be due to an actual increase in the area/volume ratio of the cell by insertion of benzyl alcohol into the membrane or due to a change in membrane visco-elasticity [18].

The data presented here suggest that perturbations in the membrane introduced by a probe molecule such as benzyl alcohol can be sensitively measured by dielectric breakdown of the cell membrane. It cannot be excluded that pertubations in the membrane lead to a decrease in membrane resistance. As outlined in detail elsewhere [7, 16] a change in membrane resistance can also result in a decrease of the dielectric breakdown voltage under certain experimental conditions. At present, a final conclusion cannot be made concerning the correlation betweenthe changes in the dielectric breakdown voltage and those on the membrane ultrastructural level, since it is not entirely clear from the results of Metcalfe and Seeman [12, 13] in which membrane region the probe is localized. From analysis of the temperature dependence of the partition coefficient data and from the NMR line width data, Metcalfe [12] supposed that the membrane protein is the major binding component for benzyl alcohol in the prelytic range of benzyl alcohol concentration. If this is true, we can conclude that proteins are also involved in the dielectric breakdown mechanism. However, this question may be answered more precisely by extending this study to a wider range of substances. In any case, we feel that dielectric breakdown measurements on cell membranes are a most promising technique for studying changes in cell membranes induced by chemical agents and physical parameters (temperature, osmotic stress, pH, etc.).

ACKNOWLEDGEMENTS

We should like to thank Dr P. K. Lauf (Duke University, Durham, North Carolina) for carefully reading and discussing the manuscript, Mr Buers for expert technical assistance and Mrs Alexowsky for typewriting. The work was supported by a grant from the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 160.

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