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# DIELECTRIC BREAKDOWN OF THE ERYTHROCYTE MEMBRANE ENHANCES TRANSBILAYER MOBILITY OF PHOSPHOLIPIDS

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**Dielectric breakdown of erythrocytes is shown to result in a loss of asymmetry of phosphatidylethanolamine and in a markedly enhanced transbilayer mobility of exogenous lysophosphatidylcholine. The effect is much more pronounced in non-resealed cells than in cells resealed after the breakdown. A causal relationship between the structural defects in the lipid phase, indicated by these results, and fusion by dielectric breakdown is discussed.**

Phospholipids are distributed asymmetrically across the membrane of mammalian erythrocytes and probably other biomembranes [1]. The mechanisms responsible for the maintenance of this asymmetry are not yet understood. An important role, however, is certainly played by the low transbilayer mobility of the phospholipids, measurable as a flip-flop [1,2]. A simple technique to assess transbilayer mobility of phospholipids in erythrocytes consists of measuring the time-dependent decrease of the extractability, by albumin, of labelled lysophospholipids inserted at very low concentrations into the erythrocyte membrane. Lysophospholipids become inaccessible to albumin upon reorientation from the outer to the inner layer of the membrane [3,4]. Half-times of flip determined by this technique range from 4 to 16 h at 37°C, depending on the nature of the acyl chains and the polar headgroups. The flip rates are sensitive to modulation of the lipid phase of the erythrocytes [5], but particularly to reversible perturbations of the membrane skeleton, e.g., via oxidation and cross-linking of SH-groups by diamide [6]. These perturbations also produce a partial loss of phospholipid asymmetry [7].

In parallel to its effects on transbilayer mobility, diamide produces a marked but also reversible enhancement of the passive permeability of erythrocytes to small ions and non-electrolytes [8]. A common basis for both phenomena, which is indicated by numerous parallels in the molecular properties of pores and flip sites [8], may be the formation of structural defects acting as pores and flip sites in the lipid domain or at the interface between membrane lipids and intrinsic proteins. A large reversible leak is also induced in erythrocytes by exposure of the cells to intense electric fields (over 2 kV/cm) [9]. The structural equivalent of this electric breakdown may again be the formation of defects acting as pores [10]. A further consequence of electric breakdown of membranes is cell fusion, provided that cells are first brought into close contact [11]. Other fusion processes, for example, those induced by chemical agents, have long been claimed to involve perturbation and reorganization of the membrane lipid domain [12], and experimental evidence has been advanced in favour of this hypothesis [13–16]. Indications that the pores induced by electric breakdown can also serve as flip sites would provide an important

piece of evidence that reorganization of the membrane lipid phase is also essential for electric fusion. Such indications are presented here.

The almost exclusive orientation of phosphatidylethanolamine to the inner face of the erythrocyte membrane is completely lost in ghosts prepared by colloid-osmotic lysis after electric breakdown and subsequent resealing, but also in cells resealed after electric breakdown without lysis (Table I). Phosphatidylserine, the other endofacial aminophospholipid, is affected much less. The extent of the loss of phosphatidylethanolamine asymmetry is exclusively dependent on the voltage applied, but independent of time elapsed (at 0°C) after breakdown. Ghosts prepared by hypotonic lysis largely maintain the lipid asymmetry observed in intact cells.

The loss of asymmetry can be accounted for by a conspicuous enhancement of the flip-flop of phospholipids. The half-time of the transbilayer

TABLE I

LOSS OF THE ASYMMETRIC ARRANGEMENT OF PHOSPHOLIPIDS AFTER DIELECTRIC BREAKDOWN

Resealed ghosts were prepared by hypotonic lysis and reconstitution, or in isotonic KCl by application of high voltage (5–5.5 kV/cm) [25,26]. Cells resealed after electric breakdown were obtained as described in the legend of Fig. 1. Phospholipids in the outer layer of the membrane were detected by phospholipase $A_2$ from bee venom, or sphingomyelinase [7]. Mean values of 2–5 experiments. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; S, sphingomyelin.

| | % of phospholipid cleaved ( = present in the outer layer) | | | |
|---|---|---|---|---|
| | PC | PE | PS | S |
| Erythrocytes | 55 | 5 | 0 | 85 |
| Resealed ghosts (dielectric) | 57 | 42 | 10 | |
| Resealed ghosts (hypotonic) | 54 | 15 | 0 | |
| Erythrocytes resealed: | | | | |
| (a) immediately after | | | | |
| breakdown at: 3.5 kV/cm | 66 | 20 | 0 | |
| 4.0 kV/cm | 65 | 36 | 0 | |
| 5.2 kV/cm | 68 | 38 | < 10 | 85 |
| (b) after a 3.5 h incubation (0°C) following breakdown at 3.5 kV/cm | 64 | 23 | 0 | |

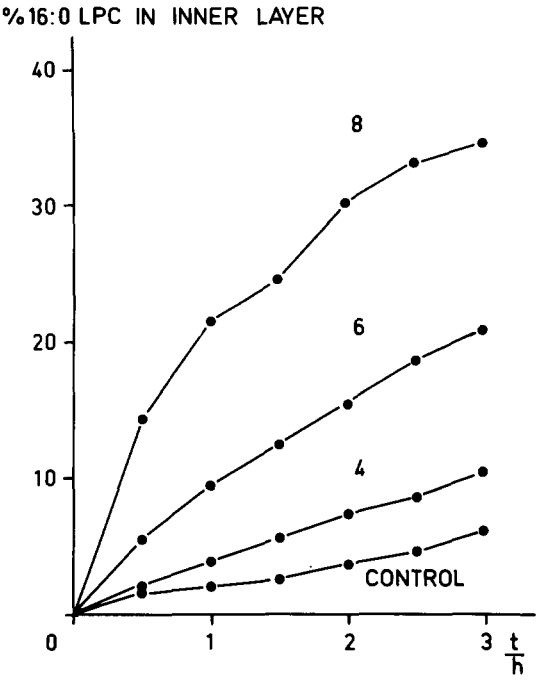


Fig. 1. Enhancement of the transbilayer reorientation of labelled lysophosphatidylcholine in erythrocytes resealed after electric breakdown. Human erythrocytes were suspended (hemocrit 30%) in media containing 115 mM KCl, 25 mM NaCl, 14.8 g/dl (approx. 30 mM) Dextran 4000 (Serva, Heidelberg) adjusted to pH 7.4 with imidazole. Breakdown was carried out at 0°C in a discharge chamber [26] (pulse length $\tau = 40$ μs (4 $\mu F \times 10 \Omega$)). Leaks induced by electric breakdown were characterized by the rate of leakage of $K^+$ at 0°C as determined by ion-selective electrodes (Schwister, K., unpublished data). Resealing (by 60 min incubation at 37°C) was ascertained by normalization of $K^+$ leakage. Resealed cells (40 μl) were washed and suspended in 40 μl of a medium containing 100 mM KCl, 50 mM NaCl, 44 mM sucrose, 12.5 mM $Na_2HPO_4/NaH_2PO_4$ (pH 7.4). A trace amount (0.7 nmol) of [$^{14}$C]palmitoyl-lysophosphatidylcholine (16:0 LPC) was added at 0°C. After 1 min exposure, the cells were spun down (13000 × $g$), the supernatant was removed and the cells were resuspended in 400 μl of the medium and incubated at 37°C. 50-μl samples were removed at different times and diluted into 400 μl ice-cold medium. Half of the sample was centrifuged, the cells were washed once in 1 ml medium at 0°C, lysed by 200 μl $H_2O$ and pipetted into scintillation fluid to determine the total counts taken up. Cells from the second half of the sample were washed, resuspended twice for 1 min in 200 μl of 1.5% solution of fatty-acid-free bovine serum albumin at 0°C (diluted with 1 ml medium prior to centrifugation) and then treated as the controls. The ratio of the counts in albumin-extracted cells normalized to total counts served as a measure of the fraction of lysophosphatidylcholine reoriented to the inner layer of the membrane. Figures on the curves are applied voltages in kV·cm$^{-1}$.

reorientation of palmitoyllysolecithin decreases from 16 h to 1.5 h in cells resealed after breakdown at 8 kV/cm (Fig. 1). A comparable enhancement of flip is obtained after breakdown using a shorter voltage pulse (1 μs, instead of 40 μs used in Fig. 1), at higher voltages (10–15 kV/cm). Rate coefficients of flip after breakdown and resealing are related exponentially to the field strength during breakdown (Fig. 2). The slope is very similar to the slope for the field dependency of the leak permeabilities induced by breakdown (Schwister and Deuticke, unpublished data). The activation energy of the flip of palmitoyllysolecithin after electric breakdown and resealing (18.9 kcal/mol) is lower than the activation energy of the normal flip (29.2 kcal/mol), indicating a change in the rate-limiting step of transbilayer movements after electric breakdown.

Flip rates in cells resealed after electric breakdown are readily and precisely measurable but do not reflect the situation prevailing in the leaky cells immediately after breakdown. Flip processes in leaky cells have to be measured at 0°C, in order to prevent resealing, and under protection against lysis. The enhancement of flip rates observed in such leaky non-resealed cells was much more pronounced than that in resealed cells (Fig. 3). Flip rates of oleoyllysophosphatidylcholine, as characterized by a loss of extractability of the phospholipid by albumin, were increased up to 2000-fold as compared to controls and reached a half-time of less than 15 min. Resealing reduced these values by at least 95% (only 1.3% flipped to the inner layer within 15 min, as compared to 43% in the non-resealed state).

The data presented here clearly suggest the formation of reversible structural defects in the lipid domain of cell membranes after electric breakdown. The concept that such structural defects in membranes may represent flip sites as well as pores has previously been forwarded for artificial lipid and lipid/protein systems [17–19]. It cannot yet be decided whether in the red cell

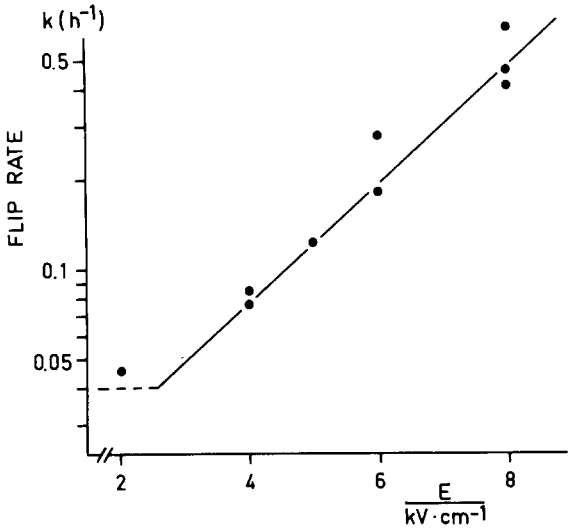


Fig. 2. Exponential relationship between flip rates and breakdown voltage. Rate coefficients for the transbilayer reorientation of palmitoyllysophosphatidylcholine were calculated from the data in Fig. 1 on the basis of a two-compartment model and a 1:1 distribution of lysophosphatidylcholine between the two halves of the bilayer at equilibrium.

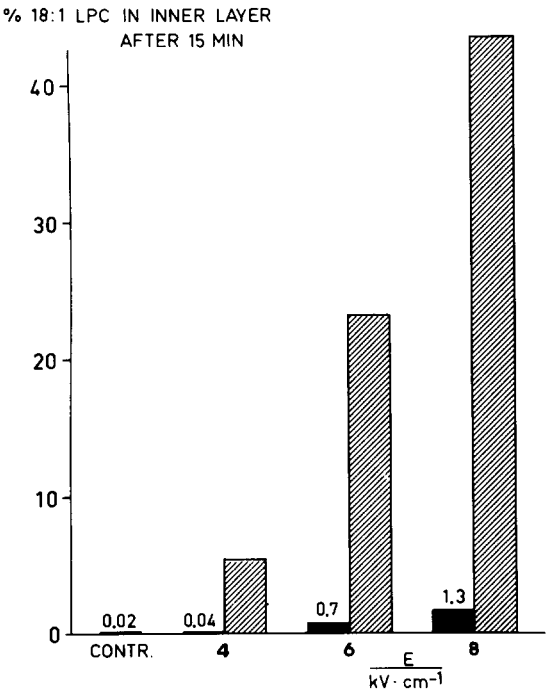


Fig. 3. Acceleration of transmembrane reorientation of oleoyllysophosphatidylcholine (18:1 LPC) in non-resealed erythrocytes (hatched bars) after electric breakdown without resealing. Cells were loaded with [$^{14}$C]oleoyllysophosphatidylcholine as described in Fig. 1, except that cells were suspended in media containing 30 mM Dextran 4000. After an exposure time, at 0°C, of 15 min the amount of oleoyllysophosphatidylcholine in the inner layer was determined as described in the text and Fig. 1. Data for the flip of the oleoyllysophosphatidylcholine (at 0°C) in resealed cells (filled – black – bars) are given for reasons of comparison. Mean values of 3–4 experiments.

membrane these defects should be envisaged as hydrophilic or hydrophobic pores in the lipid bilayer [20,21] or as some sort of induced mismatch between lipid and intrinsic proteins in their contact area [22]. The observation that electrically induced defects in pure lipid membranes reseal within the $\mu$s range [23], whereas the defects in erythrocyte membranes need seconds to minutes (Schwister and Deuticke, unpublished results) suggest essential differences between the defects in pure lipid systems and in the erythrocyte membrane.

Three major questions will have to be clarified in future work. Firstly, the real enhancement of transbilayer mobility induced by electric breakdown is still unknown. From our experiments, transbilayer flip rates are obtained for the whole cell membrane. The flip, however, is presumably restricted to sites of an unknown but certainly limited number and size. Secondly, a possible mechanistic link between enhancement of flip-flop and cell fusion will have to be elaborated. An enhanced mobility of phosphatidylethanolamine may facilitate the merging of two closely apposed bilayers, a process certainly required for fusion. Interestingly, an enhanced phospholipid mobility does not seem to be a general phenomenon after electric breakdown. Neither the strongly asymmetric distribution of sphingomyelin nor the exclusive endofacial orientation of phosphatidylserine is affected (Table I). In these two cases, either the forces maintaining the asymmetry are too strong, or the two phospholipids do not have access to the flip sites. Third, it remains to be worked out whether a local aggregation of proteins is somehow involved in the enhancement of flip rates and the occurrence of cell fusion. On the one hand, transbilayer mobility of phospholipids is increased by the presence of membrane-spanning proteins in many lipid bilayer systems [19], though probably not in membranes prepared from erythrocyte lipid [19]. On the other hand, fusion has been claimed to require protein-free lipid domains of membranes in close contact (Ref. 13, but see Ref. 24). Physical and micromorphological techniques will be needed to clarify this problem.

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## References

1 Op den Kamp, J.A.F. (1979) Annu. Rev. Biochem. 48, 47–71
2 Bergelson, L.D. and Barsukov, L.I. (1977) Science 197, 224–230
3 Mohandas, N., Wyatt, J., Mel, St.F. Rossi, M.E. and St.B. Shohet (1982) J. Biol. Chem. 257, 6537–6543
4 Haest, C.W.M., Bergmann, W., Plasa, G. and Deuticke, B. (1980) Biophys. Struct. Mechan. 6, 98 (Abstr.)
5 Dressler, V., Bergmann, W., Haest, C.W.M. and Deuticke, B. (1982) Hoppe-Seyler's Ztschr. Physiol. Chem. 363, 896 (Abstr.)
6 Haest, C.W.M., Bergmann, W. and Deuticke, B. (1981) Biochemical Society Transactions 9, 178P (Abstract)
7 Haest, C.W.M., Plasa, G., Kamp, D. and Deuticke, B. (1978) Biochim. Biophys. Acta 509, 21–32
8 Deuticke, B., Poser, B., Lütkemeier, P. and Haest, C.W.M. (1983) Biochim. Biophys. Acta 731, 196–210
9 Riemann, F., Zimmermann, U. and Pilwat, G. (1975) Biochim. Biophys. Acta 394, 449–462
10 Kinosita, K., Jr., and Tsong, T.Y. (1977) Nature 268, 438–441
11 Zimmermann, U. (1982) Biochim. Biophys. Acta 694, 227–277
12 Lucy, J.A. (1970) Nature 227, 814–817
13 Papahadjopoulos, Post, G. and Vail, W.J. (1979) in: Methods in Membrane Biology (Korn, E.D., ed), Vol. 10, pp. 1–121, Plenum Press, New York
14 Hope, M.J. and Cullis, P.R. (1981) Biochim. Biophys. Acta 640, 82–90
15 Bearer, E.L., Düzgünes, N., Friend, D.S. and Papahadjopoulos, D. (1982) Biochim. Biophys. Acta 693, 93–98
16 Cullis, P.R. and De Kruijff, B. (1979) Biochim. Biophys. Acta 559, 399–420
17 Taupin, C., Dvolaitzky, M. and Sauterey, C. (1975) Biochemistry 14, 4771–4775
18 Blok, M.C., Van der Neut-Kok, E.C.M., Van Deenen, L.L.M. and De Gier, J. (1975) Biochim. Biophys. Acta 406, 187–196
19 Van der Steen, A.T.M., De Jong, W.A.C., De Kruijff, B. and Van Deenen, L.L.M. (1981) Biochim. Biophys. Acta 647, 63–72
20 Petrov, A.G., Mitov, M.D. and Derzhanski, A.I. (1980) Adv. Liquid Crystal Res. Applic. 695–737
21 Sugár, I.P. (1981) J. Physiol. Paris 77, 1045–1042
22 Israelachvili, J.N. (1977) Biochim. Biophys. Acta 469, 221–225
23 Benz, R. and Zimmermann, U. (1981) Biochim. Biophys. Acta 640, 169–178
24 Chandler, D.E. and Heuser, J. (1979) J. Cell Biology 83, 91–108
25 Haest, C.W.M., Plasa, G., Kamp, D. and Deuticke, B. (1980) in Membrane Transport in Erythrocytes, Alfred Benzon Symposium 14, (Lassen, U.V., Ussing, H.H. and Wieth J.O., eds.), pp. 108–123, Munksgaard, Copenhagen
26 Jausel-Hüsken, S. and Deuticke, B. (1981) J. Membrane Biol. 63, 61–70