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Extensive electroporation abolishes experimentally induced shape transformations of erythrocytes: a consequence of phospholipid symmetrization?

Stephan Schwarz, Cees W.M. Haest, Bernhard Deuticke *

Institut für Physiologie, Medizinische Fakultät der RWTH, Universitätsklinikum, Pauwelsstr. 30, D-52057 Aachen, Germany

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Abstract

As shown in earlier work (M.M. Henszen et al., Mol. Membr. Biol. 14 (1997) 195–204), exposure of erythrocytes to single brief electric field pulses (5–7 kV cm^{-1}) enhances the transbilayer mobility of phospholipids and produces echinocytes which can subsequently be transformed into stomatocytes in an ATP-dependent process. These shape transformations arise from partly reversible changes of the transbilayer disposition of phospholipids, in agreement with the bilayer couple concept. Extensive membrane modification by repetitive ($\leq$20) field pulses followed by 20 h incubation at 37°C is now shown to produce discocytic cells which are resistant to many established shape-transforming treatments, including (A) single electric field pulses, Ca^{2+} incorporation and exposure to membrane active amphiphiles, but also (B) metabolic depletion, binding of band 3 ligands, alkaline pH and contact with glass surfaces. The suppression of type A effects can readily be interpreted by a complete symmetrization of the phospholipids in extensively field pulse-modified cells which prevents shape transformations related to the asymmetric disposition of the phospholipids. This symmetrization could be further substantiated by more direct determinations of the transbilayer distribution of phospholipids. Suppression of shape transformations of type B may indicate an involvement of phospholipid asymmetry in these processes on a yet unknown mechanistic basis. Alternatively we discuss field pulse-induced alterations of the disposition of peripheral proteins or of the conformation of integral membrane proteins as mechanisms interfering with shape transformations of type B.

Keywords: Erythrocyte membrane; Phospholipid asymmetry; Electroporation; Phospholipid scrambling; Erythrocyte shape

1. Introduction

The peculiar biconcave discocytic shape of the non-nucleated mammalian erythrocyte and its pathophysiological and experimentally induced alterations have attracted the interest of hematologists and

Abbreviations: CHES, 2-[*N*-cyclohexylamino]-ethanesulfonic acid; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; DNDS, 4,4′-dinitrostilbene-2,2′-disulfonic acid; DLPC, dilauroylphosphatidylcholine; GSH, glutathione; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]; HEPPS, *N*-[2-hydroxyethyl]-piperazine-*N*′-[3-propanesulfonic acid]; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; MES, 2-[*N*-morpholino]ethanesulfonic acid; MMI, mean morphological index; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, Sphingomyelin; TAPS, tris[hydroxymethyl]methylaminopropanesulfonic acid

* Corresponding author. Fax: +49-241-8888-434; E-mail: deuticke@physiology.rwth-aachen.de

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membranologists for many decades [1,2]. While defective proteins seem to constitute the major primary basis for shape changes observed in hereditary hematological diseases such as spherocytosis, elliptocytosis, pyropoikilocytosis and others [3], the characteristic experimentally induced shape transformations, echinocytosis and stomatocytosis, have been assigned in many cases to alterations of the lipid bilayer ([4,5], see [2,6] for further refs.).

As first proposed by Sheetz and Singer [5] on the basis of various experimental observations [1,2,6], native or induced differences in area between the two lipid leaflets which constitute the major element of the erythrocyte membrane as of any biological membrane, will impose on the bilayer a curvature or induce a change in curvature which in case of the erythrocyte affects cell shape. Due to its putative physical basis, this concept of cell shape changes has been termed 'bilayer couple concept'. Experimentally induced area differences may arise from preferential insertion of exogenous amphiphiles, including phospholipids, into one leaflet [7–9], or selective removal of native lipids from one layer [10]. Partial dissipation of the native asymmetric transbilayer orientation of membrane phospholipids (termed 'scrambling') is a further reason for shape changes [11–13]. This native asymmetry, now firmly established [14], is characterized by a preferential localization of the aminophospholipids (phosphatidylethanolamine, phosphatidylserine) in the inner leaflet of the bilayer, predominantly due to the action of an ATP-dependent translocase, i.e., a flippase [15,16], and a large excess of the choline phospholipids (phosphatidylcholine, sphingomyelin) in the outer layer, produced by mechanisms not yet fully understood. This pattern of an asymmetric transbilayer phospholipid disposition is responsible for an asymmetric arrangement of net negative surface charges in the lipid bilayer (density about 2.6×10^{13} charges/cm^2 at the inner surface, in contrast to essentially 0/cm^2 at the outer surface. A difference in surface charge densities provides an attractive explanation for the opposite shape effects of negatively (echinocytogenic) and positively (stomatocytogenic) charged permeant amphiphiles [9], based on their opposite patterns of distribution between the negatively charged inner and the neutral outer leaflet of the bilayer [5].

While the 'bilayer couple concept' of erythrocyte shape [5] can explain the effects of many exogenous shape-transforming amphiphilic ionic agents, it is still unclear whether or to what extent shape transformations occurring under other experimental conditions require the native asymmetric distribution of the membrane phospholipids. Such conditions comprise, e.g., echinocytosis resulting from metabolic depletion [17,18], Mg^{2+}-depletion [19,20], alkaline pH [21,22] or exposure to vanadate [23], echinocytosis in the presence of agents binding to band 3 protein with high selectivity (DNDS, DIDS, dipyridamole [24,25]), echinocytosis upon cell contact with glass surfaces [26,27], but also the puzzling stomatocytogenic effect of serum albumin [28] and stomatocytosis at acid pH [21,22].

To address this problem we have now taken advantage of a procedure, developed in our laboratory, to dissipate partially or even completely the transbilayer gradients of the erythrocyte membrane phospholipids. The method is based on the observation that strong electric field pulses (4–10 kV cm^{-1}) applied to erythrocyte suspensions not only induce membrane leaks permeable to ions and to hydrophilic nonelectrolytes [29–31], but also enhance the rates of transbilayer reorientation of endogenous phospholipids and related probes to a considerable and partly irreversible extent [32,33], which depends on the field strength as well as the duration and number of pulses applied. In recent work we could further demonstrate that this enhancement of flip rates goes along, at 0°C, with the formation of echinocytes, which not only return to the discocytic state upon incubation of the cells at 37°C, but even become stomatocytic in a process dependent on the supply of metabolic energy and inhibited by vanadate [34]. This indicates the involvement of an ATPase and we have presented evidence [34] that initial echinocyte and subsequent stomatocyte formation most likely originate from a primary incomplete scrambling of glycerophospholipids at 0°C, leading to an excess of phospholipids in the outer leaflet, and a subsequent preferential accumulation of phospholipids in the inner leaflet at 37°C, based on the uphill movement of aminophospholipids via the ATP-dependent translocase and the downhill movement of phosphatidylcholine via a passive leak.

In extending these studies we have now used extensive modification of erythrocytes by electric field

pulses to produce 'phospholipid-symmetrized cells' in which the phospholipids are most likely completely scrambled. The discocytic shape of these symmetrized cells proved to be insensitive to many modifications going along with shape transformation in native erythrocytes.

2. Materials and methods

2.1. Materials

Human erythrocyte concentrates prepared from the blood of healthy donors, anticoagulated with citrate and suspended in a conventional storage medium, were obtained from the local blood bank, kept at 4°C and used within 10 days. All standard reagents were of high or highest purity. Ionophore A23187, sodium orthovanadate, dilauroylphosphatidylcholine, merocyanine 540, *n*-dodecyl-α-D-maltoside, *N*-dodecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate (laurylsulfobetaine), tetracaine, dipyridamole, trypsin and DIDS were from Sigma–Aldridge. DNDS was from Pfaltz and Bauer, Waterbury, Canada, bovine serum albumin from Päsel and Lorei, Hanau, N-NBD-aminohexanoylphosphatidylcholine (NBD-PC) from Avanti Polar Lipids, Birmingham, AL, USA, *N*-NBD-aminohexanoylsphingomyelin (NBD-SM) from Molecular Probes, Eugene, OR, USA, and Dextran 4 (M_r 4–6 kDa) from Serva Electrophoresis (Heidelberg, Germany). NBD-PE and -PS were prepared from NBD-PC as described earlier [35]. Phospholipase A_2 (bee venom) and sphingomyelinase (*Staphylococcus aureus*) were obtained from Sigma.

2.2. General procedure

Erythrocytes were isolated by centrifugation and washed twice in isotonic saline. Cells were usually suspended in one of the following media at pH 7.4 (concentrations in mM): KCl (90), NaCl (45), NaH_2PO_4/Na_2HPO_4 (12.5), dextran 4 (8.8 g dl^{-1}) (= medium A) or sucrose (45) instead of dextran 4 (= medium B). In some experiments, phosphate buffer was replaced by HEPES (20) (medium C). If necessary, dextran 4 was present at 17.6 g dl^{-1} (medium D). The monomeric glucose content of the dextran 4 provided a glucose level (about 5 or 10 mM) sufficient to meet the energetic demands of the cells.

2.3. Additional treatments

ATP depletion was achieved by incubation of a cell suspension (Hct 10%) with iodoacetate (5 mM, 37°C, 30 min), followed by two washings with medium B and further incubation for 60 min in medium B containing 5 mM inosine.

Mg^{2+} was removed from the cells by a 30 min incubation at 37°C in medium B (Hct 10%) containing 5 mM EDTA and 10 μM ionophore A23187. This procedure was shown to lower total intracellular Mg^{2+} below 100 μM. Subsequently, the cells were washed twice with medium B.

Ca^{2+} was introduced into the cells by incubation of the cells in medium A in the presence of ionophore A23187 (5 μM) and Ca^{2+} (2 mM).

Intra- and extracellular pH were varied by incubation of the cells in medium A modified by exchanging phosphate against appropriate zwitterionic sulfonate buffers (MES pH 6, HEPES pH 7.4, HEPPS pH 8.0, TAPS pH 8.5, CHES pH 9.0) at 30 mM.

2.4. Exposure to electric field pulses

Cell suspensions (medium A or D, Hct 10%, 0–4°C) were filled into an acrylic glass discharge chamber with two stainless-steel electrodes arranged at a distance of 0.5 cm. The suspensions were subjected to a single or repetitive brief, exponentially decaying ($\tau = 40$ μs), field pulses (usually 7 kV cm^{-1}) by means of a discharge equipment consisting of a high voltage generator, a high-voltage storage capacitor ($C =$ 4 μF) and a mercury switch. Joule heating was essentially negligible in all cases.

Following the field pulse(s), the cell suspensions were immediately removed from the discharge chamber, put into a vessel thermostatted to 37°C and incubated for various periods of time in order to induce 'recovery' from the electroporation [34] produced by the field pulses. When incubation periods exceeded 120 min, gentamycin was added to prevent microbial growth.

Since repetitively electroporated erythrocytes lose a considerable fraction of their stores of adenine nucleotides and Mg^{2+} (S. Schwarz, unpublished data),

ATP (2 mM) and Mg^{2+} (5 mM) were added to the suspension media whenever a loss of these substances following the electroporation was expected to interfere with the further experiment.

Loss of GSH, which also occurred during and after electroporation, was compensated in special experiments by adding 10 mM GSH to the suspension media.

2.5. Investigation of shape changes

The capability of native or field pulse-modified cells to undergo shape changes upon exposure to established amphiphilic shape-transforming agents was studied by adding to a dilute cell suspension (Hct 10%) in medium A or D appropriate amounts of an aqueous or ethanolic stock solution of the agents. More hydrophobic shape transformers, such as the phospholipid probe DLPC, were spread as a dry film and dispersed in the incubation medium by sonication.

For the study of the transbilayer movement of shape-transforming phospholipids inserted into the membrane, the cells were washed with medium A or D after a loading procedure at 0–4°C (5 min for NBD-SM, 15–30 min for DLPC) and subsequently incubated in fresh phospholipid-free medium at 37°C for appropriate periods of time.

2.6. Microscopy

Cell morphology was assessed by interference contrast microscopy (Orthoplan Leitz, Wetzlar) before and after the various modification procedures. Aliquots of cell suspensions (Hct 10%) were diluted with 9 volumes of glutardialdehyde solution (0.5 g dl^{-1} in medium A) in order to stabilize the actual shape by fixation. Subsequently, 10 μl of the suspension were deposited between a glass slide and a glass coverslip. Cells were inspected at 400× or 1000× magnification. A morphological score MI as defined in [34] was assigned to each of 100 cells. In this classification positive values indicate echinocytes, negative ones stomatocytes. A 'mean morphological index' (MMI) was calculated as $\sum(n_i \bullet MI_i)/\sum n_i$, where i stands for the classes of shape and n for the number of cells in the ith class.

As an exception, the effects of glass and of serum albumin on erythrocyte shape were studied on unfixed cells.

2.7. Assessment of the transbilayer distribution of endogenous phospholipids and phospholipid probes in symmetrized cells

2.7.1. Endogenous phospholipids

In order to obtain information on changes of the transbilayer distribution of phospholipids in extensively field pulse-treated cells, the cells were treated with phospholipases as described earlier [32,36] to cleave selectively phospholipids in the outer leaflet. Since field pulse-treated cells lyse much more rapidly than native cells under this treatment, 'saturating' cleavage had to be avoided. Differences in the time-dependent fractional extent of cleavage between native and modified cells may, however, also serve as an indicator [36]. Thus, native and modified cells were exposed to sphingomyelinase (*S. aureus*, 0.25 U/ml medium) in presence of 0.25 mM Ca^{2+} for 5 min. Similarly, cells were exposed for 5 and 10 min to phospholipase A_2 (bee venom, 2 U/ml medium) in the presence of 0.25 mM Ca^{2+}. Lysis was less than 5% during this brief time interval, but increased rapidly thereafter.

After terminating the reaction by addition of 1 mM EDTA, the cells were extracted with isopropanol/chloroform [37]. Phospholipids and their cleavage products were separated by thin layer chromatography and quantified by phosphate determinations as described earlier [38].

2.7.2. Phospholipid probes

The transbilayer distribution of NBD-phospholipids inserted into the outer leaflet prior to field pulse exposure was routinely assayed by the albumin extraction procedure after field pulse exposure and subsequent incubation at 37°C for various time periods, as recently described in detail [33].

In special experiments, sidedness and accessibility of NBD-SM was studied by treatment of probe-loaded cells with sphingomyelinase (5 min) as described above. Cleavage of NBD-SM was determined by thin layer chromatographic isolation of NBD-ceramide and subsequent fluorimetric quantification.

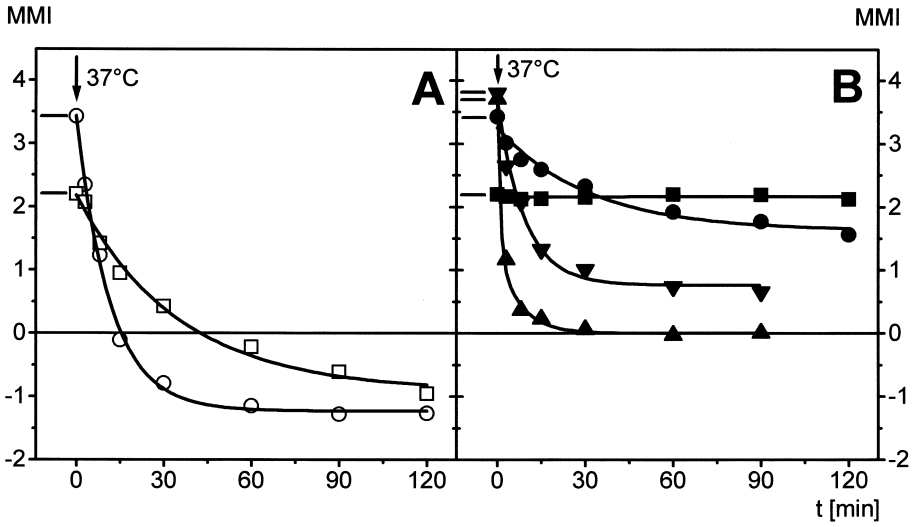

Fig. 1. Shape changes of erythrocytes following exposure to electric field pulses at 0°C and subsequent incubation at 37°C, beginning at 0 min. (A) No additions. □, 1×5 kV cm^{-1}; ○, 1×7 kV cm^{-1}. (B) Addition of 1 mM orthovanadate prior to resealing at 37°C. ■, 1×5 kV cm^{-1}; ●, 1×7 kV cm^{-1}; ▼, 10×7 kV cm^{-1}; ▲, 20×7 kV cm^{-1}. The cells were discocytic (MMI = 0) prior to the field pulse. After the pulse they assumed the shapes indicated by the horizontal lines. For further details see Section 2.

2.8. Further analytical techniques

Membrane protein patterns were characterized by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) as described in [38], GSH content of cells and media according to [39], and methemoglobin formation according to [40]. To detect the possible formation of lipid peroxidation products, dienes and fluorescent chromolipids were determined according to [41,42].

3. Results

3.1. Extensive electroporation symmetrizes the phospholipid bilayer of the erythrocyte membrane

Human erythrocytes exposed to a single electric field pulse of 5 kV cm^{-1} at 0°C become echinocytes (MMI = +2) within less than 1–2 s. After warming to 37°C (Fig. 1A), the cells return to the discocytic shape within about 40 min and then slowly become stomatocytic. In the presence of vanadate (Fig. 1B) or metabolic inhibitors [34], this reversal is completely suppressed. When a field pulse of 7 kV cm^{-1} is applied, echinocyte formation is more pronounced (MMI = +3.5), the reversal reaction is accelerated (Fig. 1A), and the cells exhibit some shape normalization even in the presence of vanadate (Fig. 1B). When field pulses of 7 kV cm^{-1} are applied repetitively, a rapid shape reversal at 37°C is observed in spite of the presence of vanadate (Fig. 1B). The reversal, however, does not exceed the dis-

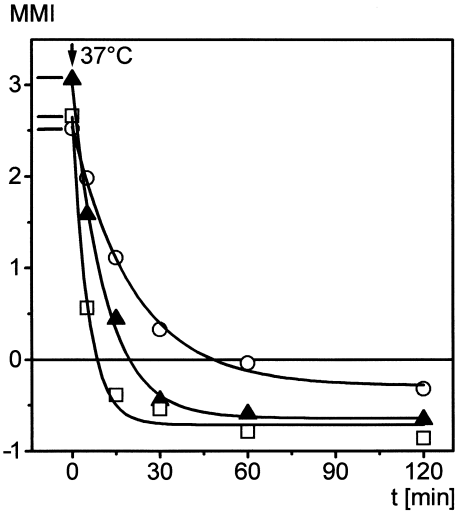

Fig. 2. Shape normalization (at 37°C) of echinocytes produced at $t = 0$ min by insertion, at 0–4°C, of NBD-SM into erythrocytes initially modified by repetitive electric field pulses (7 kV cm^{-1}) at 0°C and subsequent incubation ('resealing') at 37°C. The different stomatocytic shapes of the resealed cells prior to the insertion of NBD-SM are not shown in the diagram for reasons of clarity. ○, 1 pulse; ▲, 10 pulses; □, 15 pulses.

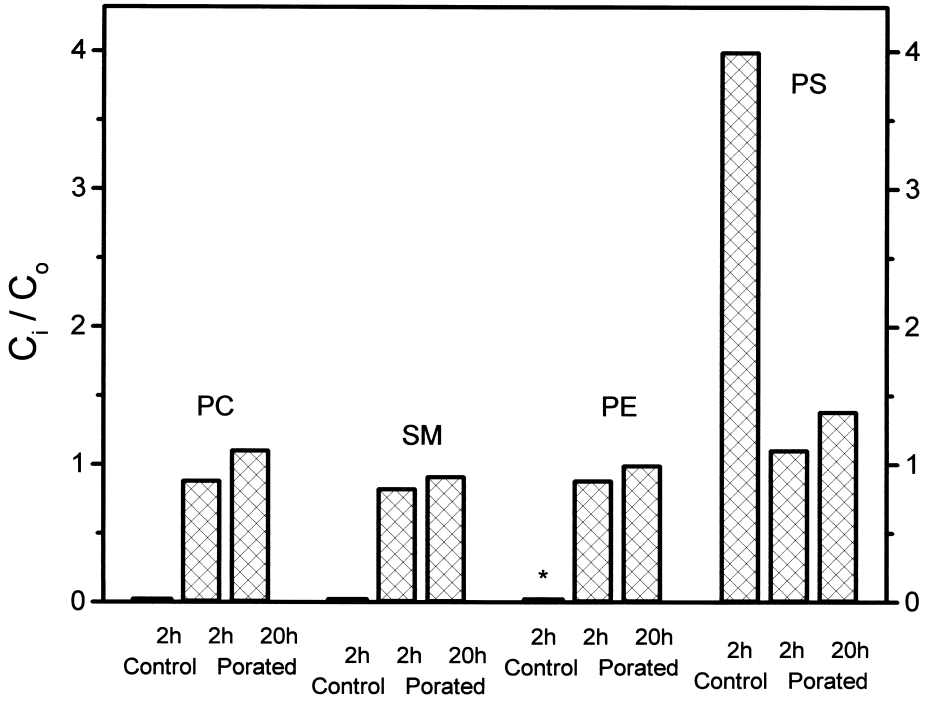

Fig. 3. Transbilayer disposition of exofacially inserted NBD-phospholipids in control and electroporated (20×7 kV cm^{-1}, 0°C) erythrocytes after 2 or 20 h incubation at 37°C. For experimental details see Section 2. *The NBD-PE probe is not a substrate of the aminophospholipid translocase and therefore not transported to the inner leaflet in control cells within 2 h.

cocytic shape. This does not change even when the cells are subsequently incubated at 37°C for 20 h (data not shown).

These observations are consistent with the concept, worked out in detail elsewhere (S. Schwarz, M.D. Thesis, Aachen, 1999), that a preferential passive outward movement of aminophospholipids, which occurs in moderately field pulse-treated cells at 0°C due to a high passive transbilayer mobility of these lipid species and accounts for echinocyte formation, is compensated in strongly field pulse-modified cells at 37°C by a passive inward movement of the exofacial choline phospholipids lecithin and sphingomyelin. This passive inward movement can fully replace the flippase-mediated active inward translocation of aminophospholipids, since shape normalization under these conditions is not affected by vanadate. As a result, both leaflets of the bilayer can finally reach equal composition and will have areas equal to the native area. The cells should therefore assume discocytic shape. This is indeed observed (cf. Fig. 1B). The major factors accounting for this 'symmetrization' are the partial irreversibility of the scrambling of phospholipids occurring during field pulse exposure and the permanently enhanced transbilayer mobility after the pulse(s). These factors have been investigated in detail in a previous study [33].

In order to substantiate the proposed phospholipid symmetrization in extensively field pulse-treated cells, we used non-invasive techniques mainly based on the transbilayer distribution of exogenous phospholipid probes, but also invasive strategies involving enzymatic cleavage of native phospholipids in the outer membrane leaflet.

Non-invasive methods for the determination of the sidedness of native phospholipids are available only for PS. They have been successfully applied to demonstrate the reversible appearance of PS in the outer leaflet of moderately electroporated cells [34]. To substantiate the symmetrization of the other phospholipids by non-invasive techniques, indirect evidence was adduced, taking advantage of the highly elevated transbilayer mobility of phospholipids in electroporated cells [33]. As shown by Daleke and Huestis [8,19], the transbilayer equilibration of an exogenously inserted phospholipid probe can be studied, at least semi-quantitatively, by following the time-dependent shape normalization of the echi-

nocytes induced by a primary incorporation of the probe into the outer leaflet of the erythrocyte membrane. Using this approach it was demonstrated [8,19] that phosphatidylcholine and in particular sphingomyelin probes are essentially not translocated in native cells, while phosphatidylserine and phosphatidylethanolamine probes are translocated inward at measurable rates by the aminophospholipid translocase.

Analogously, we could show (see Fig. 2) that cells exposed to repetitive strong field pulses and subsequently resealed at 37°C become echinocytes when the probe NBD-sphingomyelin is inserted into the outer leaflet, but return to their original stomatocytic shape within short time ($t/_2 < 10$ min after 15 pulses), indicating rapid transmembrane equilibration of the probe. Echinocytes induced by DLPC in such erythrocytes returned to the discocytic shape even faster (data not shown). These data are fully in line with the earlier direct demonstration of markedly enhanced flip rates of various fluorescent NBD-phospholipids in field pulse-treated erythrocytes [33] and indicate rapid symmetrization of NBD-SM and DLPC.

In further indirect support of a rapid phospholipid symmetrization we could demonstrate (Fig. 3) that the NBD labeled analogs of the four major phospholipids will rapidly attain, after insertion into the outer membrane leaflet, a symmetric transbilayer distribution in extensively field pulse-treated cells. In the non-modified controls, on the other hand, NBD-SM remains completely confined to the outer leaflet, NBD-PC slowly reorients inward to a minor extent, while NBD-PS is concentrated in the inner leaflet due to the action of the ATP-dependent aminophospholipid translocase. This pattern corresponds to the well-established transbilayer distribution of endogenous phospholipids. From the low half-times of redistribution of the NBD probes in the field pulse-treated cells, which are in the minute range, combined with estimates of the ratio between the passive mobilities of NBD probes [33] and of endogenous phospholipids of about 3:1 or less, depending on the acyl moieties [43], one would estimate approximate half times of symmetrization of endogenous phospholipids in the field pulse-treated cells in the range between 0.5–2 h. Twenty hours of incubation of such cells should therefore suffice for an essentially complete symmetrization.

Partial dissipation of the asymmetric distribution of aminophospholipids after single electric field pulses and subsequent brief resealing at 37°C has been demonstrated directly in previous studies [32] by the enzymatic cleavage of the outer membrane leaflet. This technique was now also applied to the repetitively pulse-treated cells, although only within the limits set by incipient lysis of these cells, when exposed to phospholipases. This lytic response was also observed when the cells were exposed to phospholipase C from *Bacillus cereus*, which does not attack the native asymmetric erythrocyte membrane from outside [44].

As evident from Table 1, brief (5–10 min) treatment of unmodified cells with phospholipase A_2 or sphingomyelinase cleaved almost all of the SM and about 50% of the total PC, equivalent to about 75% of the exofacial PC, in line with earlier data [32], while PS and PE, known to be completely or predominantly confined to the inner layer, remained intact.

In electroporated and subsequently incubated cells, PS and PE were rapidly cleaved to an extent indicat-

Table 1
Accessibility of the endogenous phospholipids to phospholipases in erythrocytes exposed to 20 repetitive field pulses (7 kV cm^{-1}, 0°C) and subsequently incubated for 20 h at 37°C

	Controls		Field pulse-treated/incubated	
	% cleaved after 5 min	% cleaved after 10 min	% cleaved after 5 min	% cleaved after 10 min
PE	0	9	69	83
PS	0	0	38	49
PC	46	52	64	77
SM	90	n.d.	65	n.d.

Exofacial glycerophospholipids were cleaved by phospholipase A_2, sphingomyelin by sphingomyelinase. Brief exposure times had to be used to avoid cell lysis. For details see Section 2. Mean values from two experiments. n.d., not determined.

ing extensive symmetrization. Cleavage of SM was less than in controls but not that expected in the case of complete symmetrization. PC, on the other hand, was cleaved more extensively than in controls. This finding seems to contradict symmetrization. It is, however, most likely an artifact resulting from the poration-induced breakdown of the barrier between the inner and the outer leaflet of the bilayer, which allows endofacial phospholipids to move outward following the gradient created by enzymatic cleavage of the outer leaflet. This interpretation is supported by the following two pieces of evidence.

1. It can readily be calculated from the data in Table 1 that the total cleavage of phospholipids by exofacially acting phospholipases considerably exceeds the expected maximum of 50% for a bilayer. It amounts to 61% after 5 min and 71% after 10 min of treatment.
2. In a direct experimental approach, the inner leaflet of the membrane of electroporated/incubated cells was loaded with NBD-SM during a flip period, followed by extraction of probe still residing in the outer leaflet by albumin. Subsequent treatment of these cells with sphingomyelinase went along with rapid cleavage of the endofacial NBD-SM (3% min^{-1}). Since SM is the slowest phospholipid to move across the erythrocyte membrane under all conditions studied so far [33], PC will probably be affected by this artifact to an even greater extent.

The phospholipase technique is thus not appropriate for a quantitative estimate of phospholipid sidedness in extensively field pulse-modified cells. It tends to overestimate the true amount of phospholipid in the outer leaflet. Nevertheless, it provides valuable semi-quantitative evidence for phospholipid symmetrization.

All these analytical data and considerations support the view that the asymmetric distribution of the major erythrocyte membrane phospholipids is essentially abolished during a prolonged incubation at 37°C after extensive field pulse treatment. Whether and to what extent this is also the case for the inositol phospholipids (predominantly located in the inner leaflet [45]), and the glycosphingolipids (assumed to be located in the outer leaflet) remains to be clarified. Since their headgroups are larger and more polar than those of the major phospholipids, their transverse mobility may not be sufficiently enhanced after field pulse treatment to provide for symmetrization. In any case, the discocytic shape of the phospholipid-symmetrized cells indicates, that these two lipid species do not impose a major spontaneous curvature on the erythrocyte membrane even if still distributed asymmetrically.

3.2. *Expected consequences of phospholipid symmetrization*

Lipid-'symmetrized' cells should lose the capacity to become echinocytic upon the application of a further field pulse at 0°C, since the net shift of phospholipids from the inner to the outer leaflet, which is required for this type of echinocyte formation, should not be possible in a symmetrized membrane. As shown in Fig. 4, this was indeed the case. While cells exposed to a single field pulse and then converted into stomatocytes by 2 h incubation at 37°C responded to a new single field pulse by echinocyte formation like native cells, cells exposed to five repetitive field pulses responded much less. Twenty pulses almost abolished the response. Cells incubated for 20 h at 37°C after 20 initial field pulses were disco-

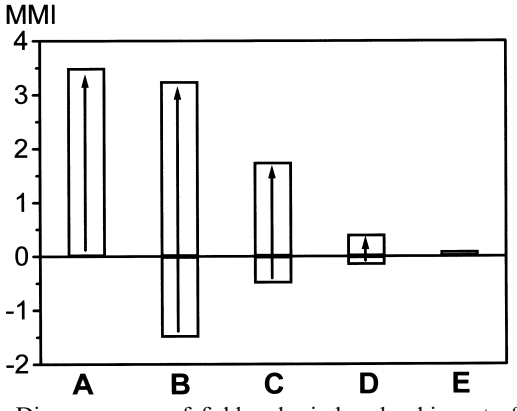

Fig. 4. Disappearance of field pulse-induced echinocyte formation as an indicator of field pulse-induced dissipation of transbilayer phospholipid asymmetry. Erythrocytes suspended in a medium protecting against colloid-osmotic lysis (see Section 2) were exposed to an increasing number of repetitive field pulses of 7 kV cm^{-1} at 0°C. (A) control; (B) 1 pulse; (C) 5 pulses; (D,E) 20 pulses. After a subsequent resealing at 37°C, for 2 h (B–D) or 20 h (E), the cells were again exposed to a single field pulse of 7 kV cm^{-1}. The base line of the columns indicates the shape after the initial field pulse (s) and resealing, the top marks the shape after the final single pulse.

cytic and fully resistant to a new field pulse, as would be expected in the case of a cell with a phospholipid-symmetric membrane. Since aminophospholipid translocase, which in native cells restores asymmetry by active transport, was not inhibited by vanadate in these experiments, it may be concluded that any active transport of the aminophospholipids was overrun by the high passive flip rates in the strongly modified membrane.

Further support for our claim that extensively field pulse-treated cells are indeed fully symmetrized came from experiments in which the echinocytogenic effect of intracellular Ca^{2+} was tested. This long-known effect [46] is thought to result from the activation, by cytoplasmic Ca^{2+}, of a 'scramblase' [47], which mediates a partial dissipation of the preexisting asymmetry of the phospholipids, in particular the endofacial aminophospholipids [12,13,48]. Consequently, cytoplasmic Ca^{2+} should lose its echinocytogenicity after field pulse treatments of increasing intensity. This was in fact observed (Fig. 5A).

These experiments also provided evidence (Fig. 5B) that the phospholipid symmetrization after field pulse treatment (20×7 kV cm^{-1}) requires about 4 h incubation at 37°C, since only after that time interval Ca^{2+}-induced echinocyte formation was completely abolished.

3.3. The bilayer couple mechanism is still operative in symmetrized membranes

It might be argued that extensively field pulse-treated cells are in general prevented from becoming echinocytic by some unknown mechanism not involving the proposed symmetrization. This objection can be refuted on the basis of the following results. From a number of reports it is known that long-chain amphiphiles with a large polar or anionic headgroup preventing their transbilayer reorientation, such as merocyanine 540, dodecylmaltoside or laurylsulfobetaine, will induce time-stable echinocytes, presumably by a permanent selective expansion of the outer leaflet of the bilayer [49,50]. Echinocytogenic agents of this type should in principle still be effective in phospholipid-symmetric cells according to the bilayer couple mechanism. As shown in Figs. 6 and 7, this proved to be true. Other than in native unmodified cells, however, the echinocytogenic effect only lasted for short time intervals after addition of the agents. The cells returned to their initial disco-

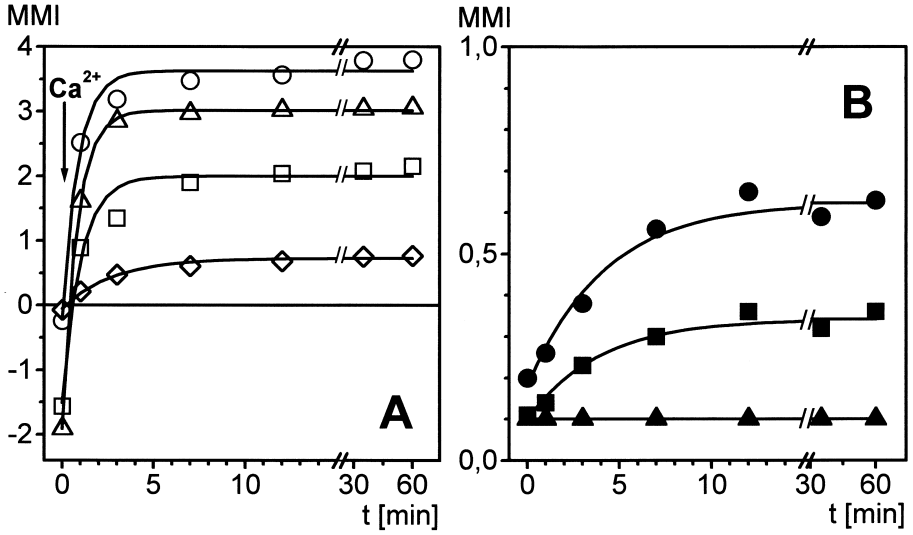

Fig. 5. Disappearance of calcium-induced echinocyte formation as an indicator of field pulse-induced dissipation of transbilayer phospholipid asymmetry. (A) Erythrocytes exposed, at 0–4°C, to 1 (△), 5 (□), or 15 (◇) repetitive field pulses of 7 kV cm^{-1} were resealed for 120 min at 37°C. ○, no field pulse (=control). Subsequently, extracellular Ca^{2+} (2 mM) was introduced at 0 min into the cell interior by 5 μM A23187 and cell shape changes observed for 60 min. (B) Erythrocytes exposed to 20 repetitive field pulses (7 kV cm^{-1}) and incubated at 37°C for 2 h (●), 4 h (■), or 20 h (▲) before addition, at 0 min, of Ca^{2+} (2 mM) and A23187 (5 μM). Note the different scales on the ordinates in A and B.

cytic shape within 10 to 60 min depending on the probe used (compare Figs. 6 and 7) and the incubation temperature (data not shown). This indicates that after extensive field pulse treatment the membranes had become leaky even to these normally im-

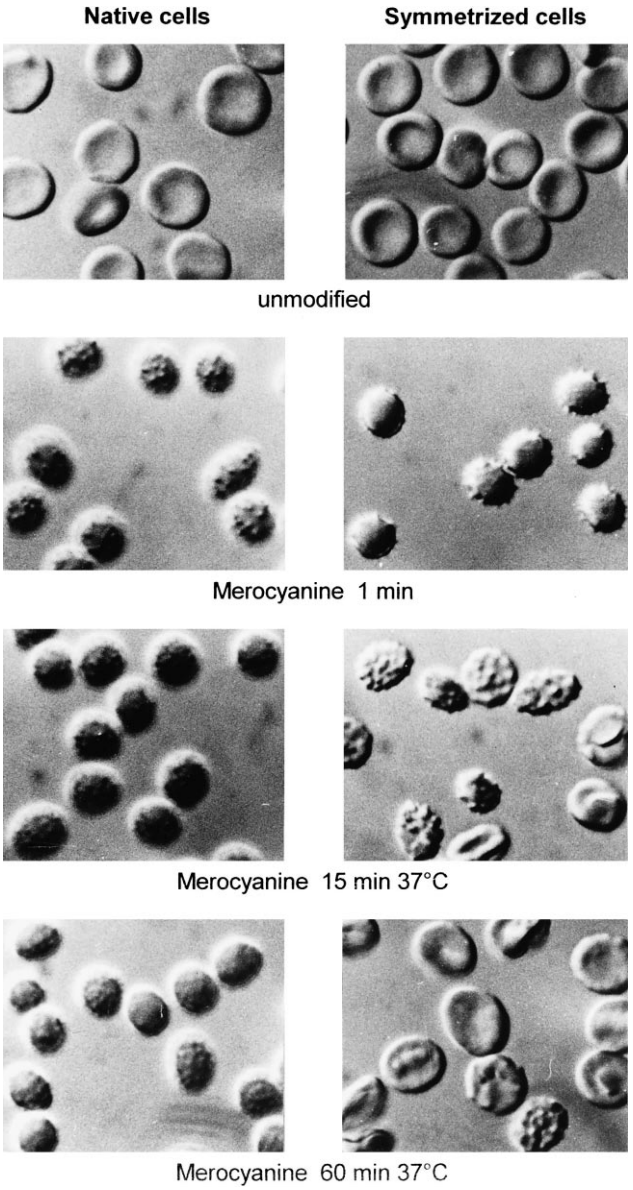

Fig. 6. Shape changes of erythrocytes following membrane insertion of merocyanine 540 into native cells (left panel) and into cells symmetrized by exposition to 20 repetitive field pulses (7 kV cm^{-1} at 0°C) followed by resealing at 37°C for 20 h (right panel). Merocyanine (20 μM) was inserted at room temperature (Hct 1%) and the cell suspension incubated at 37°C for the time period given below each panel.

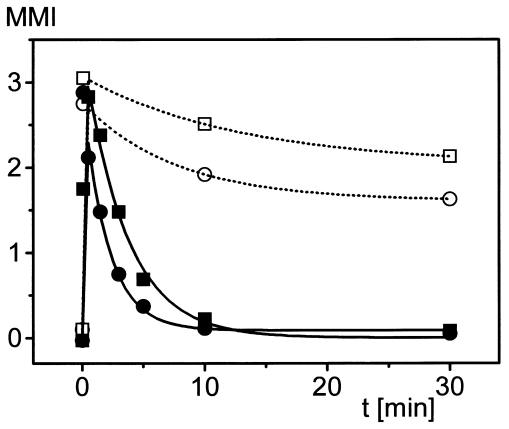

Fig. 7. Time course of the spontaneous shape reversal, at 37°C, of echinocytes produced by 4 μM docecylmaltoside (□,■) or 50 μM laurylsulfobetaine (○,●) in native (open symbols, hatched line) or field pulse-modified (closed symbols, continuous line) erythrocytes. Field pulse modification as described in Fig. 6.

permeant amphiphiles, allowing for a symmetrization of the probe.

3.4. *Influence of phospholipid symmetrization on shape changes explainable by the original bilayer couple concept*

If, as claimed above, all major membrane phospholipids, including the anionic species phosphatidylserine, are distributed symmetrically after extensive field pulse treatment, permeant anionic and cationic agents, which in the native asymmetric membrane act echino- or stomatocytogenic due to their surface charge-dependent asymmetric distribution between the inner and the outer leaflet [5,9,49], should lose their effect. This further test for a phospholipid symmetrization was performed using two anionic (salicylate, dinitrophenol), two cationic (tetracaine, octylammonium) and an uncharged (Triton X-100) shape-transforming agent.

As shown in Fig. 8, neither the anionic nor the cationic compound induced its typical effect in the field pulse-treated cells, presumably because the negative charges of the anionic phospholipids, which determine the transbilayer distribution ratio of the shape-transforming ionic agents, are no longer asymmetrically confined to the inner leaflet of the bilayer after the field pulse treatment, but are distributed randomly. In a reverse argumentation, this finding may be taken as evidence for Sheetz and Singer's

[5] original explanation of the shape-transforming effect of anionic and cationic membrane-active drugs.

According to Fig. 8, the yet unexplained stomatocytogenic effect of the nonionic detergent Triton X100 is also lost in field pulse-treated cells. This would seem to indicate that some asymmetrically distributed element(s) of the native bilayer promote an asymmetric bilayer distribution of this agent in native cells, too.

A further consequence of field pulse treatment readily explainable in terms of phospholipid symmetrization concerns erythrocyte ghosts. Lange and Steck [51] have collected evidence that the 'echinocytic' ghosts formed when 'discocytic' ghosts prepared by hypotonic lysis are reconstituted to normal ionic strength, are likely to arise from a constriction of the inner membrane leaflet due to a screening of the anionic phospholipid charges exclusively present at the inner surface. In line with this concept, symmetrized ghosts prepared from field pulse-treated/resealed cells (20×7 kV cm^{-1}, 20 h, 37°C) did not become echinocytic upon reconstitution of normal strength (S. Schwarz, M.D. Thesis, Aachen, 1999).

3.5. *Influence of phospholipid symmetrization on shape changes primarily not explainable by the bilayer couple concept*

All types of shape transformation and their disappearance after field pulse treatment described in the preceding paragraphs are consistent with the bilayer couple concept of erythrocyte shape. There are, however, other types of experimentally inducible erythrocyte shape changes which so far could not be reconciled with this concept. The echinocyte formation upon depletion of erythrocytes of ATP [17,18] or magnesium [19,20] and upon treatment with vanadate [23] belong to these types. In all of these modifications aminophospholipid translocase is inhibited [15,16]. According to the currently available data, however, this inhibition for itself does not induce the scrambling of aminophospholipids that would be required for echinocyte formation [52,53]. As an alternative explanation, changes in the state of phosphorylation of inositol phospholipids have been proposed [23] but this issue still lacks final experimental proof. Mechanisms not directly involving the membrane lipids might be responsible for these shape transformations. Phospholipid-symmetrized erythrocytes provide an instrument to gain new insights into possible mechanisms behind these shape changes.

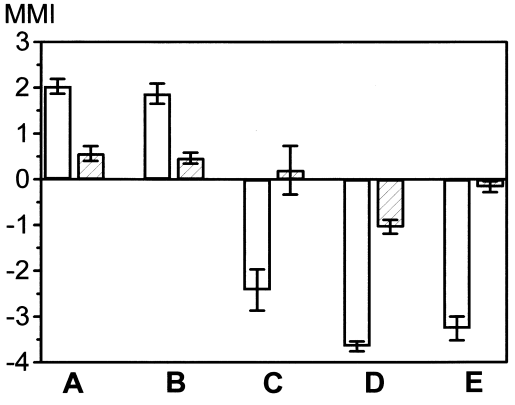

Fig. 8. Loss, in field pulse-modified erythrocytes, of the capacity for undergoing shape transformations in the presence of membrane-active amphiphilic agents. □, Native cells; ▨, field pulse-modified cells (20×7 kV cm^{-1} at 0°C, 20 h incubation at 37°C). (A) Salicylate 20 mM; (B) dinitrophenol 2 mM; (C) tetracaine 2 mM; (D) octylamine 2.5 mM; (E) Triton X-100 7.5 mg dl^{-1}. Mean values ± S.D., $n=4$.

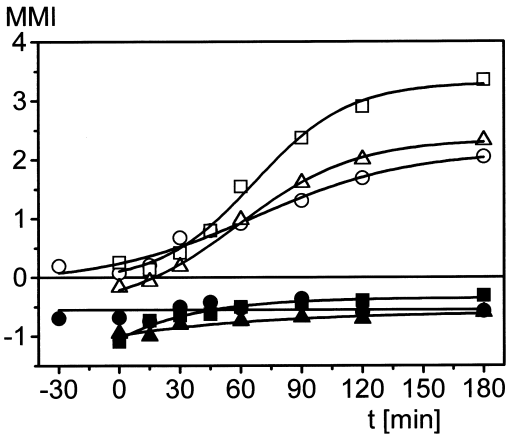

Fig. 9. Loss of the echinocytogenic effect, in field pulse-modified cells, of ATP depletion (○,●), Mg^{2+} depletion (□,■), and exposure to vanadate (△,▲). For experimental details see text. Open symbols, controls; closed symbols, field pulse-modified cells (20×7 kV cm^{-1} at 0°C, followed by 20 h incubation at 37°C).

Fig. 9 demonstrates the response of extensively field pulse-treated cells to depletion of ATP and Mg^{2+} and to vanadate treatment. For reasons of comparability of control and field pulse-treated cells, the latter ones were prepared under preservation of their normal levels of ATP and Mg^{2+} during poration and resealing, using the procedure outlined in Section 2. ATP and Mg^{2+} were only depleted after

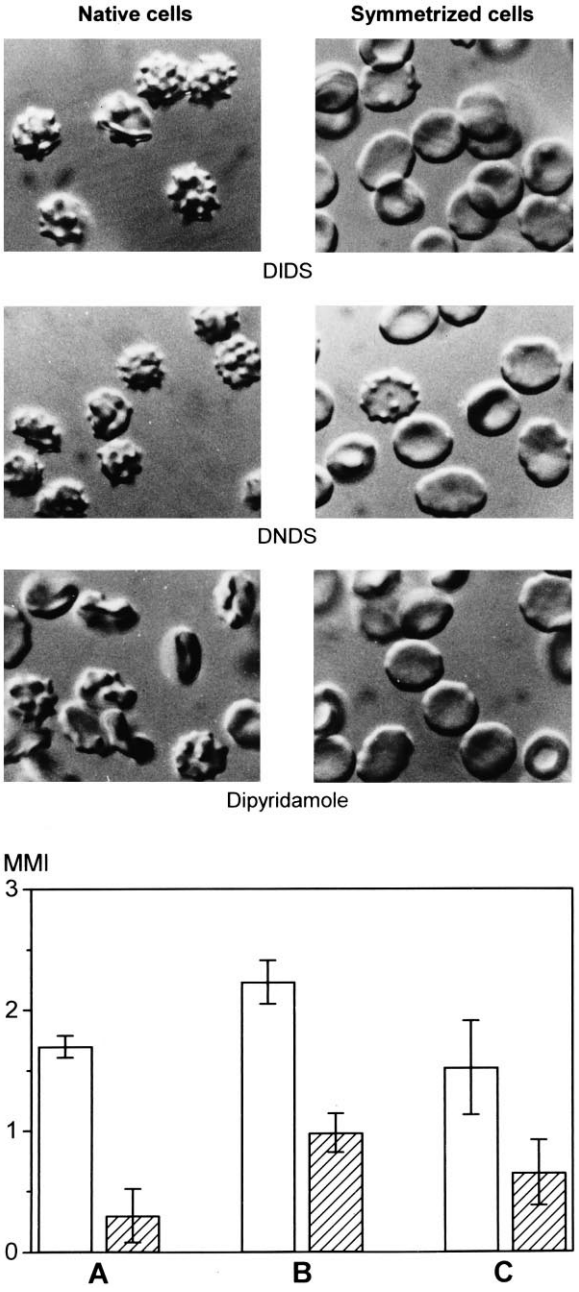

Fig. 10. Field pulse modification (20×7 kV cm^{-1} at 0°C, followed by 20 h incubation at 37°C) suppresses the formation of echinocytes induced in native erythrocytes by stilbene disulfonates (DIDS (10 μM, 37°C, 30 min), DNDS (2.5 mM, 22°C)) or cationic dipyridamole (20 μM, 22°C). Upper panel: direct microscopic evidence. □, Controls; ▨, field pulse-modified cells. (A) DIDS; (B) DNDS; (C) dipyridamole. Mean values ± S.D., $n=4$.

the 20-h resealing period as described in Section 2. As evident, these modifications as well as exposure to vanadate, all of which induce echinocyte formation in native cells, are no longer effective in symmetrized cells.

This loss of reactivity of the phospholipid-symmetrized cells might be taken as evidence that in the native membrane a Mg-ATP-dependent process involving a vanadate-sensitive enzyme provides for the energy-dependent asymmetric distribution of a specific membrane constituent, which becomes symmetrized when metabolic energy is no longer available. Considering that only about $1–2\times10^6$ phospholipid molecules out of a total of about 10^8 in the inner leaflet would have to be reoriented outward for the induction of echinocytes [7], the unaltered low passive transbilayer mobility of phospholipids in the energy-depleted cells might be sufficient – contrary to the currently prevailing opinion – for such a minor, analytically hardly detectable, passive net translocation after depletion of metabolic energy stores. Endofacial phosphatidylethanolamine might be a candidate for such a translocation, but other components of the bilayer cannot be excluded. In any case, a fully symmetric membrane would not show an echinocytic response, on the basis of this interpretation, to the three types of interference with energy supply discussed here. Alternatives to this speculative explanation can, however, not be excluded.

A second type of shape transformation that is difficult to explain in terms of a differential expansion of the lipid leaflets concerns the echinocyte formation by certain inhibitors of anion exchange via band 3 protein, specifically the stilbene disulfonates, DIDS and DNDS, and the weak cation dipyridamole [24,25]. Although these inhibitors have been shown to bind to (artificial) lipid–water interfaces [54,55], several pieces of evidence recently obtained in our laboratory indicate that they exert their echinocytogenic effect via their binding to band 3.

1. The echinocytogenic effect of dipyridamole, like its inhibitory effect on anion transport [56], disappears when chloride in the medium is replaced by sulfate (B. Deuticke, unpublished data).
2. Cells become echinocytic when treated with DIDS under conditions which only lead to its covalent

binding to band 3, while non-covalently bound agent, which might insert into the outer leaflet of the lipid bilayer, is again removed from the membrane (A. Sohnius, B. Deuticke, unpublished data).

3. DNDS binding saturates when the number of bound copies of DNDS equals the number copies of band 3 per cell [57]. In line with this observation the effect of the stilbenedisulfonates and dipyridamole saturates at the echinocyte stage 2–3 (B. Deuticke, unpublished data; see also [24]), while 'ordinary' echinocytogenic agents are increasingly more effective at increasing concentrations, i.e., finally induce crenated or smooth spheres [9]. In the same direction, the combined addition of both DIDS and dipyridamole does not produce a stronger effect than each of the agents for itself, while shape-transforming amphiphiles acting by their insertion into the lipid domain show additivity [9].

From Fig. 10 it becomes evident that the echinocytogenic effect of the ligands of band 3 is essentially lost in field pulse-treated/resealed erythrocytes. There is not even the brief phase of echinocyte formation expected for echinocytic agents slowly redistributing from the outer to the inner lipid bilayer (cf. Figs. 6 and 7). This unexpected finding cannot yet be incorporated easily into mechanistic interpretations for the shape effect of these ligands.

Serum albumin has been shown to convert discointo stomatocytes [28]. The question is unsolved, however, how this impermeant protein, which is claimed to adsorb to the erythrocyte surface [58], can affect membrane curvature. The stomatocytogenic effect, which is not always very pronounced in normal cells, can be demonstrated more sensitively after trypsination of the cells [28]. Removal, by trypsin, of exofacial glycopeptides from glycoproteins of the erythrocyte, in particular from glycophorins, as well as desialylation by neuraminidase have been shown [59] to shift erythrocyte shapes in the stomatocytic direction, supposedly by removing repulsive electrostatic forces in the outer membrane surface. In our experiments, native cells as well as erythrocytes preexposed to symmetrizing field pulses (20×7 kV cm^{-1}, 20 h, 37°C) were tested for their response to albumin without fixation either directly or after a treatment with trypsin.

These experiments provided the results compiled in Table 2.

1. The slight stomatocytogenic effect of albumin in field pulse-modified cells (−0.45 vs. −0.28 in the absence of albumin) was essentially similar to that in native cells (−0.27 vs. −0.14).
2. Trypsin treatment per se, which acted slightly stomatocytogenic in native lipid-asymmetric cells (−0.58 vs. −0.14), had a more pronounced stomatocytogenic effect in symmetrized cells (−1.83 vs. −0.28).
3. In the stomatocytic symmetrized and trypsinized cell, albumin had no additional stomatocytogenic effect. Albumin even shifted cell shape in the echinocytic direction compared to cells without albumin (−0.68 vs. −1.83).

From these findings it may be concluded that

1. the stomatocytogenic effect of albumin does not depend on a lipid-asymmetric membrane;
2. the membrane constituents removed by trypsin counteract a stomatocytogenic driving force present in the native membrane. Surprisingly, this stomatocytogenic force seems to be more pronounced in the symmetrized membrane, although the original, probably stomatocytogenic, influence of an endofacial excess of negative surface charges is no longer operative;
3. the direction of the shape effect of albumin seems to vary with the state of the membrane.

Although not yet conclusively interpretable, these

Table 2
Influence of bovine serum albumin (5 g dl^{-1}) and trypsination on the shape of erythrocytes before and after exposure to repetitive electric field pulses (20×7 kV cm^{-1}) and subsequent incubation at 37°C for 20 h

	MMI			
	No trypsin		After trypsin	
	control	albumin	control	albumin
'Native'	−0.14	−0.27	−0.58	−1.85
Field pulse-modified	−0.28	−0.45	−1.83	−0.68

Cells were treated with trypsin (1.5 mg/ml, Hct 5%, 37°C, 60 min) after the field pulse modification. Cell shapes quantified by the MMI. For further experimental details see Section 2 and text. Mean values from two experiments.

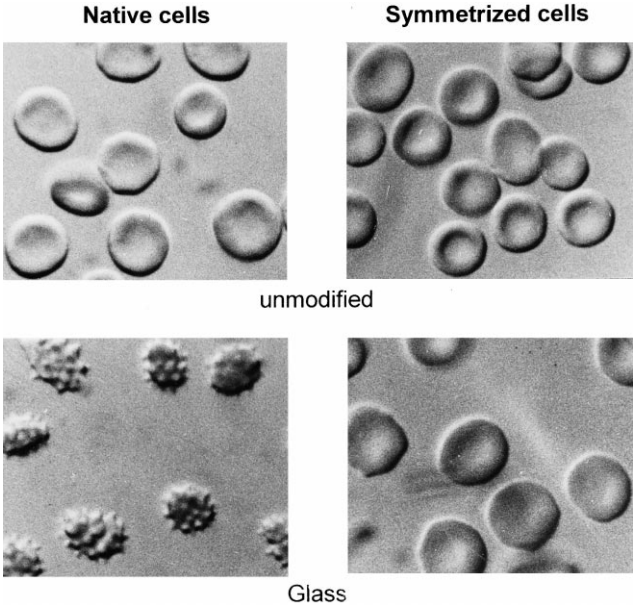

Fig. 11. Loss of the echinocytogenic effect of glass after field pulse modification (20×7 kV cm^{-1} at 0°C, followed by 20 h incubation at 37°C). Cells were not fixed in these experiments.

observations will have to be considered in future models of shape control in erythrocytes.

A further, long-known [1,26], but still puzzling type of echinocyte formation occurs upon close contact of red cells with glass (e.g., slides and coverslips) and other surfaces [26,27]. This so-called 'glass-effect' was completely suppressed in the field pulse-treated/resealed cells (Fig. 11).

It is presently not possible to provide a reasonable interpretation for this surprising consequence of field pulse treatment in terms of a phospholipid symmetrization. The situation becomes even more complex in the light of our further observation (data not shown) that pH effects on red cell shape are partly abolished in symmetrized cells. As established by earlier studies [21,22] cytoplasmic acidification acts stomatocytogenic, whereas alkalinization acts echinocytogenic. Surprisingly, the former effect is still present in electroporated/resealed cells, while echinocyte formation at alkaline pH (pH 8.5–9) is no longer observed. Swelling of the porated cells, which might counteract echinocytosis, was prevented in these experiments by the extracellular dextran. Gedde et al. [21] have recently compiled evidence against an involvement of titratable lipid charges in the shape response to cell pH. In the light of their arguments, the disappearance of high-pH-induced echinocytosis upon field pulse treatment is difficult to interpret in terms of a direct consequence of lipid symmetrization. Since the same is probably true for the suppression of the glass effect and of the echinocytogenic influence of ligands of band 3 (see below), additional or indirect effects of an extensive field pulse treatment may have to be considered to explain some of our findings. The following paragraph reports some data collected in attempts to demonstrate or exclude such additional effects.

3.6. *Other consequences of extensive field pulse treatment and their role in suppression of shape transformations*

Among the possible field pulse-induced alterations of the erythrocyte affecting its capability for shape changes one might consider

1. loss of essential cytoplasmic constituents;
2. peroxidative damage of membrane lipids;
3. oxidation and crosslinking of membrane proteins or perturbation of their supermolecular organization within the membrane skeleton and its interaction with integral membrane proteins.

3.6.1. *Loss of glutathione and its possible relevance*

Cells subjected to repetitive field pulses not only lose ATP and Mg^{2+} but also GSH (data not shown). This depletion is mainly due to release into the medium through the induced leaks immediately after poration. During the subsequent 20-h incubation at 37°C, some further decrease of the GSH level is due to its oxidation to GSSG. A comparable slight decrease also occurred in the control cells. Porated and resealed cells contained only 5–10% of the original GSH level of 2.2 μmol/ml cells. Glutathione (10 mM) added to the extracellular medium prior to poration maintained the cellular level at its original value. Glutathione depletion, however, is not related to the above reported loss of response of the field pulse-treated cells to shape changing agents and treatments, since GSH-supplemented cells exhibited the same lack of response as GSH-depleted ones (data not shown).

3.6.2. Possible role of lipid peroxidation

Peroxidation of membrane lipids upon electroporation has been observed in erythroleukemic (K 562) and Chinese hamster ovary (CHO) cells using chemoluminescence and determination of oxidative reaction products [60,61]. To our knowledge, erythrocytes have so far not been analyzed in this respect.

In order to exclude or demonstrate lipid peroxidation as a basis of the disappearance of shape responses we extracted lipids from erythrocytes after 20 pulses of 7 kV cm^{-1} and 20 h incubation at 37°C, and measured the formation of conjugated dienes as indicated by the absorption ratio A_{216}/A_{204} [41] and formation of fluorescent oxidation products as indicated by the fluorescence at 434 nm (excitation at 358 nm) [42]. Neither of these parameters was changed in the field pulse-treated cells relative to the controls. The absence of significant oxidative phospholipid degradation was further substantiated by measuring the relative content of phosphatidylethanolamine and phosphatidylserine, known to be the first ones to disappear upon oxidative damage [62]. The molar ratio PE/SM was 1.19 and that of PS/SM 0.54 in both native and pulse-treated cells. In the same line, formation of methemoglobin during the 20 h incubation at 37°C was the same in pulse-treated and control cells ($< 2\%\ h^{-1}$). Taken together, these results suggest that even repetitive electric field pulses do not constitute an effective oxidative stress for human erythrocytes.

3.6.3. Field pulse-induced protein modifications

Considering the observed depletion of GSH following field pulse treatment, the membrane proteins of such cells might no longer be protected against oxidative modification or crosslinking. Indeed, the analysis of membrane protein patterns after repetitive field pulses and subsequent 20 h incubation at 37°C revealed the appearance of small amounts of a high molecular mass band in SDS gels, apparently consisting of spectrin oligomers (data not shown). In the porated cells, this oligomerization could be prevented by addition of GSH (10 mM) to the incubation media used for the field pulse treatment and by reduction of the SDS-solubilized membranes with dithioerythritol. Non-pulsed cells incubated for 20 h exhibited some spectrin oligomerization, too.

Since, as reported above, field pulse-modified, GSH-depleted and GSH-supplemented cells do not differ in their altered response to shape-transforming conditions it may be concluded that the level or type of spectrin crosslinking induced by field pulse treatment cannot be responsible for the suppression of shape transformation.

4. Discussion

The results presented above provide new corroborative evidence for the bilayer couple mechanism underlying erythrocyte shape and some of its experimental transformations, but on the other hand, also raise the question to what extent the suppression, by field pulse treatment, of some shape transformations so far inexplainable by the simple bilayer couple mechanism can be assigned to the membrane phospholipid symmetrization on the basis of our findings.

The first of these two statements rests crucially on the validity of our claim that repetitive exposure to electric field pulses and 'resealing' at 37°C produce phospholipid symmetrization. Several pieces of direct and indirect evidence have been provided above, which support this issue.

1. Endogenous phospholipids, including sphingomyelin, are mobilized by repetitive field pulses and assume a more symmetric disposition, detectable by the phospholipase technique. Due to an enhanced sensitivity of field pulse-treated cells to the lytic action of phospholipases, and the high transbilayer mobility of phospholipids in these cells, however, complete phospholipid symmetrization cannot de demonstrated by the enzyme technique.
2. Phospholipid probes attain fully symmetric disposition within short time after extensive field pulse treatment (Fig. 3), readily detectable by the albumin extraction technique and shape analyses.
3. The capacity for spontaneous stomatocyte formation during resealing of electroporated cells at 37°C, primarily caused by reaccumulation of phosphatidylserine in the inner membrane leaflet [34], is gradually lost after repetitive electroporation and resealing, presumably due to the increasing transbilayer mobility of phospholipids [33] and

the progressively increasing loss of phospholipid asymmetry.
4. Exogenous phospholipid probes and amphiphiles, which in native cells produce permanent echinocytosis due to their virtual transverse immobility [8,49,50], only induce transient echinocytosis in field pulse-treated/resealed cells, followed by a return to the initial cell shape in parallel with transbilayer symmetrization of the probes due to their enhanced mobility.
5. Extensively porated and resealed cells lose the capacity to respond to shape-transforming maneuvers known [12,13,20,33,34] to arise from a partial loss of phospholipid asymmetry and to be reversible upon partial reconstitution of phospholipid asymmetry (single field pulse, Ca^{2+} loading).[1]

Further observations on field pulse-treated cells that also fit into the concept of phospholipid symmetrization comprise

1. the disappearance of the characteristic 'antagonistic' [9] shape transformations induced in native cells by permeant anionic and cationic drugs, and
2. the disappearance of the conversion of discocytic ghosts, prepared by hypotonic lysis, into echinocytic ghosts upon reconstitution of normal ionic strength.

Concerning other types of shape changes, their disappearance upon lipid symmetrization is less well understandable. This applies to some extent to the suppression of the slow echinocyte formation in erythrocytes after depletion of ATP or Mg^{2+} or treatment with vanadate, which inhibits phosphoryl transfer reactions including the operation of the aminophospholipid translocase.

This reversible effect has been interpreted, alternatively, either as a consequence of a slow dissipation of the steep outward transbilayer gradients of the aminophospholipids, leading to the minimal increase of the outer leaflet required for echinocyte formation [7], or as a consequence of a dephosphorylation of endofacial phosphoinositol phospholipids, decreasing the area of the inner leaflet with the same consequence [23].

The lack of shape changes in phospholipid-symmetrized cells after modifications impeding the operation of aminophospholipid translocase supports the former concept. Echinocyte formation due to dephosphorylation of endofacial phosphoinositol phospholipids should not be completely abolished following phospholipid symmetrization, even if this process would include the inositol phospholipids, which is presently unknown. At least 50% of the inositol phospholipids would still be oriented to the inner surface after symmetrization and be accessible to dephosphorylation.

While a lack of response to metabolic deprivation in symmetrized cells can thus be reconciled with the lipid bilayer couple concept, the lack of shape transformation at alkaline pH, by certain ligands of band 3 proteins, and by contact with glass seem hard to reconcile with this concept in its most simple form.

A first alternative explanation coming to mind, that the membrane of the field pulse-treated cell is either rigidified or instantaneously compensates all changes of membrane curvature leading to shape transformations by virtue of its high transbilayer mobility of lipids, can be discarded in view of our observation that such cells can still undergo shape changes (cf. Figs. 2, 4, 6 and 7).

It has therefore to be considered whether indirect consequences of lipid symmetrization or additional membrane alterations by extensive field pulse treatment account for, or contribute to the field pulse-induced resistance to shape-transforming membrane modifications. Since a causal involvement of simple side effects of field pulse treatment, such as loss of essential cytoplasmic constituents, lipid peroxidation, or SH-oxidation and spectrin polymerization, can be discarded (see above), one might consider altered interactions between the lipid bilayer and the sub-bi-

[1] Cytoplasmic Ca^{2+} has previously been proposed to exert its shape-transforming effect by promoting dephosphorylation and degradation of phosphoinositol phospholipids [63]. Since Ca^{2+}-induced echinocytosis is fully reversible [13] and can be repeated (S. Schwarz, M.D. Thesis, Aachen, 1999), while the Ca^{2+}-induced decomposition of phosphoinositol phospholipids is not reversible [13], the above interpretation seems no longer tenable. Since such transiently Ca_i^{2+} exposed phosphoinositol phospholipid-depleted cells also still respond to field pulses by reversible echinocyte formation like native cells, it seems very unlikely that changes of the inositol phospholipid pattern are involved in the disappearance of shape responses following field pulse treatment.

layer protein layer or even cytoplasmic proteins as causal factors. A significance of the membrane skeleton for red cell shape and its experimental alterations has been proposed repeatedly on the basis of theoretical arguments [64,65] and experimental work [22,66,67] but is still less safely proven for the intact erythrocyte than the role of the lipid bilayer. Stomatocyte formation in intact cells at low pH_i – as opposed to echinocytic shapes in ghost membranes in this range of pH – has recently been assigned by Gedde et al. [68] to an expansion of the inner membrane leaflet by increased binding of a cytoplasmic protein. In terms of this concept our observation that low pH stomatocyte formation is not abolished by field pulse treatment would indicate that this protein–lipid interaction does not require the particular phospholipid composition of the asymmetric bilayer. Echinocyte formation at high pH_i, contrasting to the occurrence of stomatocytic shapes in ghost membranes and explainable, according to Gedde et al. [68], by a decreased interaction of the cytoplasmic protein with the bilayer, is suppressed in field pulse-treated cells. This finding would seem to indicate that the alkaline branch of the pH dependence of the red cell shape is based on a mechanisms different from that operative at acid pH. There is evidence that alkaline pH_i lowers the interactions between the bilayer and the sub-bilayer protein domain [69]. If these interactions are opposed by the density of negative charges in the inner lipid bilayer, phospholipid symmetrization, going along with a decrease of charge density, might stabilize these interactions and thus counteract echinocyte formation.

We have thus some indications, at least, how phospholipid symmetrization could affect shape changes at high pH_i. In contrast, it is presently not clear, how the binding of stilbenedisulfonates and of dipyridamole leads to shape changes suppressed by symmetrization. The large number of copies of small ligands such as dilauroyl phospholipids required for a certain degree of echinocytosis via insertion into the lipid domain [7], as compared to the small number of maximally available binding sites for ligands of band 3 [57] makes it very unlikely that a simple, 'stoichiometric' expansion of the outer leaflet can account for the echinocytosis produced by the ligands of band 3. Since binding of stilbenedisulfonates and dipyridamole induces allosteric conformation changes in the membrane domain of band 3 [70–72], the expansive effect of the binding of a single ligand molecule to band 3 might be amplified. It is not clear, however, how phospholipid symmetrization could interfere with this mechanism. An involvement of the interactions of band 3 with membrane skeletal proteins, influencing consecutively the interaction of the skeleton with the inner leaflet of the bilayer, provides a working hypothesis that might be worth to be followed. The demonstration of such indirect mechanisms of shape changes originating from the cell surface but affecting the bilayer via its interaction with the skeleton may finally also lead to an explanation of the suppression, by field pulse treatment, of the glass effect that has puzzled investigators for more than a century [1].

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