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Transbilayer reorientation of phospholipid probes in the human erythrocyte membrane. Lessons from studies on electroporated and resealed cells

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Abstract

In order to characterize in more detail the previously observed (Dressler et al. (1983) Biochim. Biophys. Acta 732, 304–307) increases in transbilayer mobility of phospholipids in the erythrocyte membrane following electroporation at 0°C and subsequent resealing at 37°C of the cells, we have studied rates of flip and flop as well as steady state distributions of the fluorescent *N*-(NBD)-aminohexanoyl-analogues of the four major membrane phospholipids. Measurements comprised the passive non-mediated components as well as those mediated by specific translocases (flippase and floppase). The major new findings and insights can be summarized as follows. (1) The enhancement of passive transbilayer mobility which increases with the strength, duration, and number of field pulses at 0°C, cannot be fully reversed by subsequent resealing at 37°C. Flip-flop remains considerably elevated relative to the original values.(2) Enhanced mobilities induced by electroporation differ for the probes studied in the sequence SM $\lll$ PS $\ll$ PC $<$ PE. Other membrane perturbations going along with enhanced flip-flop share only in part this pattern. (3) Mediated, ATP-dependent components of flip and flop of the probes are suppressed in electroporated/resealed cells, partly due to loss of cellular Mg^{2+}, partly – in case of flippase – due to competition by externalized endogenous PS. (4) Electroporated/resealed cells provide an elegant means to demonstrate the contribution of various components of flip and flop to the steady state transbilayer distribution of phospholipids, in particular the rôle of passive mobility. The new, detailed information on the displacements of phospholipid between the two leaflets of the membrane bilayer in porated/resealed cells will help to understand erythrocyte shape changes following poration and during resealing (Henszen et al. (1993) Biol. Chem. Hoppe-Seyler 374, 114).

Keywords: Erythrocyte membrane; Phospholipid; Flip-flop; Flippase; Floppase; Electroporation

Abbreviations: BSA, bovine serum albumin; NBD-PC, 1-oleoyl-2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino hexanoyl]-*sn*-glycero-3-phosphocholine; NBD-PE, 1-oleoyl-2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino hexanoyl]-*sn*-glycero-3-phosphoethanolamine; NBD-PS, 1-oleoyl-2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino hexanoyl]-*sn*-glycero-3-phosphoserine; NBD-SM, 6-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino hexanoyl]-sphingosyl-phosphocholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin; NEM, *N*-ethylmaleimide; DLPS, dilauroylphosphatidylserine; BHT, butylated hydroxytoluene

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1. Introduction

Exposure of erythrocytes to brief electric field pulses of high intensity has been known for more than two decades to induce formation of membrane leaks with the properties of aqueous 'pores', which at low temperature remain open and induce colloid-osmotic lysis of the cells, but reseal at least to a considerable extent at higher temperatures (see Refs. [1–4] for recent reviews). This phenomenon of 'electroporation' is probably based primarily on a perturbation of the membrane lipid domain that also promotes cell fusion under appropriate conditions of cell contact [1,3,5,6]. This perturbation could be substantiated by a variety of spectroscopic and other biophysical approaches [7–9]. Since, however, in pure lipid membranes field-pulse-induced leaks reseal rapidly [10,11], a contribution of proteins to the phenomenon of leak formation in biological membranes seems likely [2,3].

We and others have established earlier [12,13] in erythrocytes subjected to electroporation a partial loss of the asymmetric distribution of the aminophospholipids, phosphatidylserine and phosphatidylethanolamine, between the inner and the outer leaflet of the lipid bilayer. We assigned this loss of asymmetry to a marked increase of the passive transbilayer mobility (flip-flop) of phospholipids [12] which, at that time, however, could only be demonstrated using lyso-PC as a probe. Moreover, at that time much less was known than today about the kinetics, energetics and mechanisms of the flip-flop of phospholipids. In particular, it was yet unknown that a 'scrambling', i.e. a partial randomization, of phospholipid asymmetry and an energy-dependent phospholipid transport by flippases may contribute to certain types of experimentally induced alterations of erythrocyte shape and their 'spontaneous' normalization [14–18].

In the course of studies dealing with shape changes of erythrocytes subjected to electroporation and subsequent resealing at 37°C [19], we have now studied in greater detail the dynamics and equilibria of phospholipids in native and electroporated erythrocytes using fluorescent diacyl phospholipid probes which are sensitive qualitative indicators of the behaviour of their natural analogues under many conditions. It turned out in these studies that electroporation is also an instructive tool to investigate principles of the relationship between kinetics and steady-state distribution of phospholipids in plasma membranes.

2. Materials and methods

2.1. Materials

Human blood from healthy donors was obtained from the local blood bank and anticoagulated with citrate. Packed erythrocytes were stored in a conventional storage medium for up to 10 days at 4°C. All standard reagents used were of high or highest purity. Sodium metavanadate and orthovanadate were from Sigma; dextran 4 (M_r about 4000–6000 Da) from Serva, Heidelberg, and bovine serum albumin (BSA) from Paesel-Lorei, Frankfurt a.-M. 1-Oleoyl-2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) aminohexanoyl]-*sn*-glycero-3-phosphocholine (NBD-PC) was purchased from Avanti Polar Lipids, Birmingham, AL, USA. NBD-phosphatidylserine (PS) and NBD-phosphatidylethanolamine (PE) were prepared from NBD-PC by transphosphatidylation using phospholipase D from *Streptomyces* species [20]. *N*-NBD-aminohexanoyl-sphingomyelin was from Molecular Probes, Eugene, OR, USA.

2.2. Methods

2.2.1. General procedure

Erythrocytes were isolated from the storage medium by centrifugation (5 min, $6000 \times g$), and washed twice in isotonic saline. Cells were usually suspended in media containing (concentrations in mM): KCl (90), NaCl [45], NaH_2PO_4/Na_2HPO_4 (12.5), dextran 4 (8.8 g dl^{-1}) (= medium A). Medium B was medium A containing sucrose [44] instead of dextran 4. pH was always adjusted to 7.4.

2.2.2. Exposure to electric field pulses

Suspensions of cells in medium A (Hct 30%, 0°C) were filled into an acrylic glass discharge chamber (4 ml) with two stainless steel electrodes arranged at a distance of 0.5 cm. The suspensions were then immediately subjected to brief, exponentially decaying ($t = 20$ μs) electrical pulses of high field strength (3–9 kV cm^{-1}) by means of a discharge equipment con-

sisting 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. In most experiments, these electroporated cells were then 'resealed' by incubation at 37°C for various periods of time.

2.2.3. Measurement of flip rates

Trace amounts of fluorescent NBD-phospholipids were inserted into the outer membrane leaflet of erythrocytes by addition of 5 μl of a stock solution of the probe (0.1 mM in ethanol) to 1 ml of erythrocyte suspension (5 min, 0°C, Hct 10%). Subsequently, cells were washed once (0°C), resuspended in medium A (Hct 2%) and exposed to electric field pulses. Alternatively, the cells were loaded with the lipid probe after field pulse exposure at 0°C and resealing at 37°C. Rates of reorientation of the probes to the inner leaflet during a subsequent incubation were derived from the time-dependent decrease of extractability of the lipid probe by albumin (usually 0°C, but 22°C in the case of NBD-PE) as described before [20]. Briefly, 0.5 ml samples were mixed (2 min, 0°C) immediately after electroporation as well as after various incubation periods with 0.5 ml of medium A containing bovine serum albumin (3 g dl^{-1}) and incubated for 2 min at 0°C. After centrifugation two further extractions of the cells with 0.7 ml medium A containing 1.5 g dl^{-1} albumin (2 min, 0°C) followed. Cells were then washed once with medium A, packed, and hemolysed by addition of 100 μl water. Phospholipids were extracted by addition of 900 μl isopropanol. Following centrifugation, the fluorescence of the isopropanol phase was measured at 522 nm (excitation at 466 nm) using a Shimadzu spectrofluorometer (RF 5001 PC). The fluorescence of the isopropanol extract after albumin extraction of the cells (= probe localized in inner leaflet) was related to the total isopropanol-extractable fluorescence in the same amount of cells.

2.2.4. Measurement of flop rates

Probes were inserted into the outer membrane leaflet as described above. In the case of NBD-PS the cells were then incubated at 37°C for 60 min. After this period more than 70% of the probe had been translocated to the inner layer by the flippase. Loading of the inner leaflet with NBD-PC and NBD-PE (90 min, 37°C) required the presence of ethanol known to reversibly 'permeabilize' the membrane to lipid probes [20]. The alcohol was removed by subsequent washing of the cells at 0°C. After the loading phases probe left in the outer leaflet was removed by albumin treatment at 0°C. Depending on the experiment, the cells were then electroporated or not. The transbilayer redistribution of the probes and their final stationary distributions were quantified by the albumin extraction procedure using the equilibrative or the 'zero-trans' technique described in Section 3.

2.2.5. Determination of rate coefficients and stationary transbilayer distributions

The fluorescence extractable after albumin treatment (i.e. the probe in inner membrane leaflet, P_i) was related to the total isopropanol-extractable fluorescence, P_{tot}, in the same amount of hemolysed cells. In the case of flip measurements an exponential function, $(P_i/P_{tot})_t = q \cdot [1 - \exp(-k_{in} \cdot t/q)]$ was fitted to the kinetic data of P_i/P_{tot}, where q represents the fraction of probe in the inner leaflet after attainment of stationary conditions and k_{in} is the rate constant for the unidirectional flip. In the case of flop measurements by the equilibrative technique an exponential curve, $(P_i/P_{tot})_t = (1 - q) \cdot \exp[-k_{out} \cdot t/(1 - q)] + q$, was fitted to the kinetic data of the non-extractable fractions (P_i/P_{tot}), where k_{out} is the rate constant for the unidirectional flop. For flop measurements by the 'zero-trans' technique, q was set to zero.

3. Results

3.1. Transbilayer movements of a phosphatidylcholine probe in electroporated cells

3.1.1. Flip measurements

NBD-PC introduced into the outer membrane layer of native human erythrocytes at a concentration of about 0.015 μmol/ml cells, equivalent to 0.35 mol% of the sum of endogenous phospholipids, moves inward ('flips') very slowly at a rate of about 0.02 h^{-1} ($3.3 \cdot 10^{-4}$ min^{-1}) at 37°C. This is about the rate observed earlier [21] for ^{14}C-labelled 16:0-lyso-PC (0.016 h^{-1}).

To study the effect of electroporation on this probe,

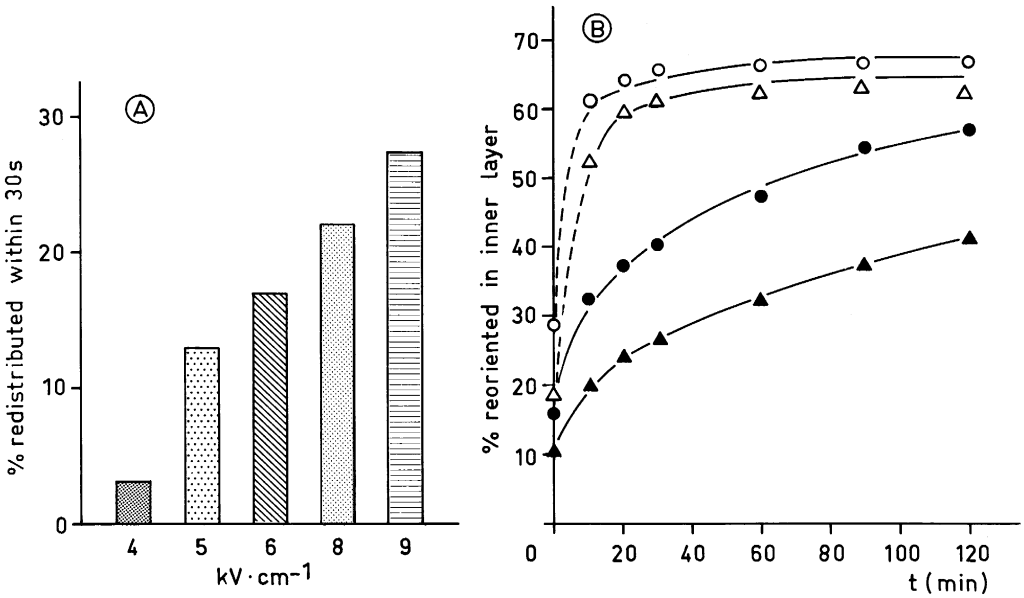

Fig. 1. (A) Fractional amounts of exofacial NBD-PC reoriented to the inner membrane layer of human erythrocytes during and within 30 s after field pulses of different intensities ($\tau = 20$ μs). Cells were loaded with the fluorescent probe at 0°C before the pulse and extracted with bovine serum albumin at 0°C immediately after the pulse. (B) Progress, at 0°C and 37°C, of the inward reorientation of NBD-PC after field pulses, applied at 0°C, of different intensities. Experimental conditions as in A, except that the incubation temperature in two samples was elevated to 37°C immediately after the pulse. Open symbols: incubation at 37°C; closed symbols: incubation at 0°C. Triangles: 5 kV cm^{-1}; circles: 6 kV cm^{-1}.

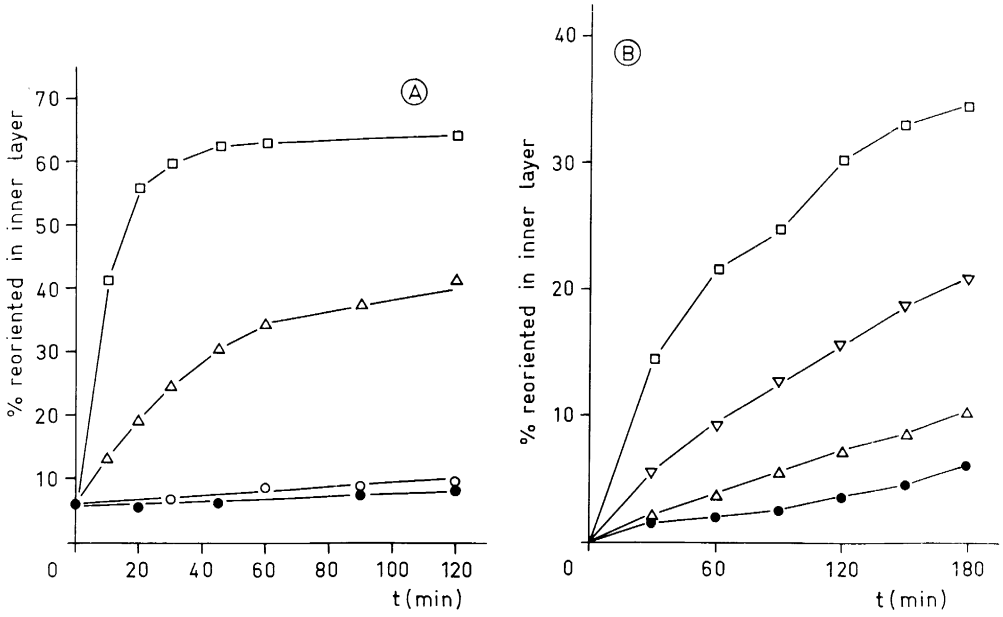

Fig. 2. Time course of the flip, at 37°C, of NBD-PC (A) and [^{14}C]lyso-PC (B) in erythrocytes resealed at 37°C for 60 min after exposure to field pulses of different intensities at 0°C. Cells were loaded with the probe after the resealing period. (●) Controls; (○) 2 kV cm^{-1}; (△) 4 kV cm^{-1}; (▽) 6 kV cm^{-1}, (□) 8 kV cm^{-1}. Data in B obtained using the procedures described in Ref. [21].

cells were loaded with NBD-PC, porated at 0°C and analysed for its transbilayer distribution immediately (< 30 s) after poration and during a subsequent incubation at 0°C. As is evident from Fig. 1A, increasing amounts of exofacially inserted probe become inextractable by albumin within 30 s after field pulses of increasing voltage. In contrast, essentially all of the NBD-PC can be removed from control cells. During a subsequent incubation of the porated cells at 0°C, a time-resolvable flip proceeds at a considerable rate towards a stationary distribution of the probe of about 60–65% inside for high field strengths (Fig. 1B) in contrast to the stationary distribution of about 20–25% inside in native cells [22]. Flip rates calculated for these kinetics in porated cells amount to only about 10–25% of the flip rates during the initial 30 s, approximated from the data in Fig. 1A. They are, however, about 120–140-times higher than flip rates of NBD-PC at 0°C in native cells, calculated from the measured value at 37°C (0.02 h^{-1}) and the activation energy for the flip of NBD-PC in native cells (about 65 kJ/mol between 32°C and 42°C, data not shown). These elevated rates, which are similar to those obtained earlier for [^{14}C]18:1-lyso-PC in porated cells [12], remain essentially time-stable at 0°C.

When cells are brought to 37°C immediately after poration at 0°C, flip rates measured during this period are even higher than those observed at 0°C (Fig. 1B), although during this period a 'resealing' of the leaky state of the membrane takes places (see Ref. [2] and below). A steady state distribution (about 65% inside) is reached within less than 30 min. The half-time is only a few minutes. This rapid inward redistribution of NBD-PC suggests a similar, although not necessarily equally fast inward reorientation of endogenous PC during resealing after electroporation.

After 60 min resealing at 37°C, flip rates of NBD-PC measured at 37°C are still remarkably high (Fig. 2A). They range from $k_{in} = 0.0096$ min^{-1} after a 4 kV cm^{-1} pulse to $k_{in} = 0.0552$ min^{-1} after a 8 kV cm^{-1} pulse. These rates are 30 to 150-times higher than those in the native membrane prior to poration, but about 40-times lower than those estimated for the flip immediately after poration at 0°C. The flip rates of NBD-PC after 60 min resealing are about 5- to 6-times higher than those obtained for the lysophospholipid probe [^{14}C]16:0-lyso-PC under the same experimental conditions (Fig. 2B). During further prolonged incubation at 37°C, flip rates diminish only very little. The rate measured after 24 h resealing amounts to 70% of that after 60 min resealing (data not shown). It has to be concluded from these results, that (a) the barrier function of the membrane for transbilayer movement of phospholipids is only incompletely restored during 'resealing', and (b) the flip rates in these 'resealed' cells vary for different PC analogues.

Enhanced flip rates obtained by poration/resealing at a certain field strength can be further increased by repetitive pulsing at the same voltage. Rates measured after resealing increase almost linearly with the number of pulses (data not shown).

The activation energy of the enhanced flip of NBD-PC in electroporated/resealed cells was found to be about 80 kJ/mol (10–37°C), somewhat higher than the above mentioned value for the native flip, but much higher than the activation energy of leak fluxes of small ions across the electroporated and resealed cell membrane. K^+ leak fluxes in such cells increase only about 2-fold between 0°C and 30°C, corresponding to an activation energy of about 20 kJ/mol (data not shown). This low activation energy is indicative of the presence of aqueous leaks in such membranes (see below).

From Fig. 2A it also becomes evident that the steady-state distribution of NBD-PC is changed in electroporated/resealed cells. Although in native cells the flip rates are too low to allow reliable determinations of steady state distributions, and flop kinetics have to be used for that purpose, the flip kinetics for cells electroporated at different field strengths suggest a progressive shift of the steady-state distribution with increasing field strength. One possible factor contributing to this shift will be worked out in the subsequent, more detailed, analysis of the flip and flop of NBD-PC.

3.1.2. Flip as a composite process

The net inward movement of phosphatidylcholine in erythrocytes was originally supposed to reflect a single passive, non-mediated process. The recent discovery of a floppase activity in human erythrocytes [22–24], mediating an ATP-dependent *outward* translocation of diacyl-glycerophospholipid probes and of newly synthesized PC, has complicated the

situation. We have to regard the observed rate of (inward) net flip of NBD-PC and its steady-state distribution as the result of two oppositely directed processes, downhill inward (passive) diffusion and uphill outward (active) transport via the floppase. This translocase is sensitive to inhibitors such as vanadate [21], NEM [23] and fluoride [22] as well as to depletion of ATP and Mg^{2+} [23]. Floppase inhibition should accelerate the net inward flip of NBD-PC if it is composed of two opposite processes. Indeed, inhibition by the above-mentioned means consistently increases the flip rate coefficients of NBD-PC measured in electroporated/resealed cells by a certain amount (Fig. 3, mean value ± S.D. obtained for various field strengths = 0.0050 ± 0.0028 min^{-1}) under the conditions of our experiments. Moreover, the steady-state distribution of NBD-PC increases from about 35–60% inside in the absence of inhibitors of floppase to about 60–70% inside in their presence, depending on the voltage applied (Fig. 4).

The flip kinetics of NBD-PC measured in the presence of inhibitors of floppase activity thus provide information on the true 'leak' for this phospholipid probe in electroporated/resealed cells as a function of the field strength applied. If these leak *flip* rates follow the principle of simple diffusion, leak *flop* rates, measured after inhibition of floppase, should be of similar magnitude. Moreover, rates of floppase-mediated flop of NBD-PC calculated from

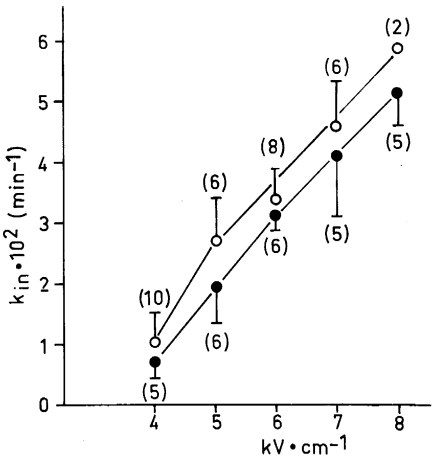

Fig. 3. Flip rates of NBD-PC at 37°C in electroporated/resealed erythrocytes before (●) and after (○) blockage of floppase activity by vanadate (1 mM), NEM (0.8 mM 20 min 37°C), or ATP-depletion [22]. Mean values ± S.D., $n = 5–10$.

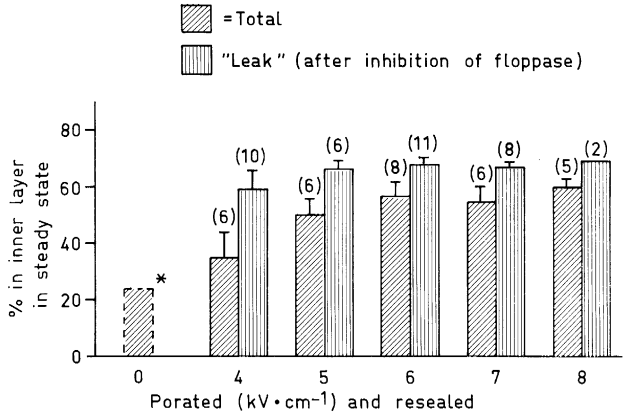

Fig. 4. Stationary distribution of NBD-PC in native and electroporated/resealed cells before (= total) and after (= 'leak') inhibition of floppase activity. Values for porated/resealed cells derived from flip measurements, value for native cells (*) from flop measurements, all at 37°C, as described in the text. Mean values ± S.D. Number of experiments in brackets.

flip and flop rates in the absence and presence of vanadate should at least be comparable. To test this hypothesis we studied the outward flop of NBD-PC in native and electroporated/resealed erythrocytes.

3.1.3. Flop measurements

Flop measurements require preloading of the inner leaflet of the bilayer with a probe within reasonable times. In the case of phosphatidylcholine-derived probes, loading of native cells can be achieved by addition of small amounts of ethanol which reversibly enhances passive flip processes [20,25]. Using this procedure as described in Section 2, NBD-PC was selectively inserted into the cytoplasmic membrane leaflet of native erythrocytes. In electroporated/resealed cells rapid loading could be achieved via the 'leak'. The return of the probe to the outer layer was then followed under two conditions, presented in the following.

3.1.3.1. Equilibrative flop. In this mode the probe moves from the inner to the outer leaflet of the bilayer and approaches a steady-state distribution between the two. In native cells (Fig. 5A) this flop of NBD-PC is strongly inhibited by orthovanadate, indicating that it is floppase-mediated to a major extent. A rate coefficient of 0.0089 ± 0.0022 min^{-1} could be calculated for the floppase-mediated component in

native cells from the difference of the rate coefficients obtained in the absence and the presence of inhibitors of floppase. The residual 'leak flop', i.e. the rate obtained in the presence of floppase inhibitors, amounts to about 0.0016 min^{-1}. The calculated steady-state distribution (inner leaflet: outer leaflet) of the probe in the native cells is also affected considerably by the inhibitors. It changes from 25% inside in the absence of inhibitors (cf. also Fig. 4) to

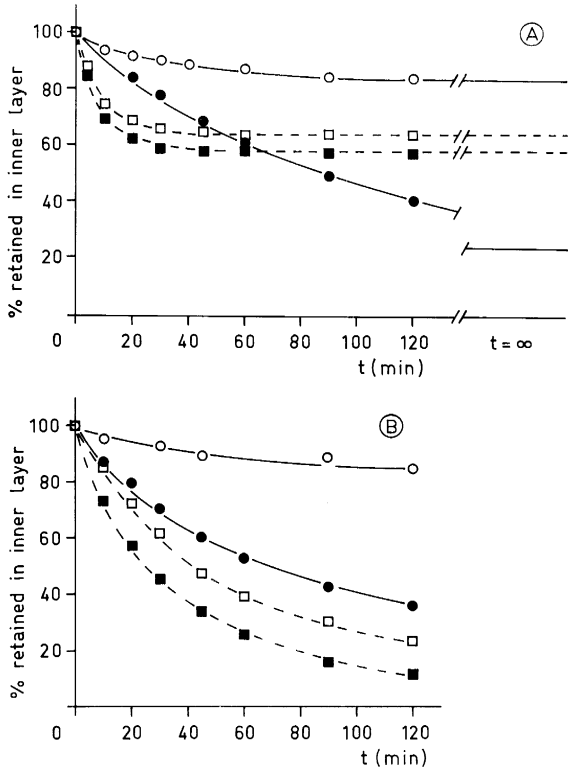

Fig. 5. (A) Time course of outward reorientation (flop) of NBD-PC at 37°C in native (●, ○) and electroporated/resealed (■, □) erythrocytes in the absence (closed symbols) and presence (open symbols) of orthovanadate (1 mM), measured under 'equilibrative' conditions as defined in the text. Field strength 8 kV cm^{-1} (0°C), 60 min resealing at 37°C. Data for fractional retention in the inner leaflet obtained as described in Section 2. The stationary distribution (at $t =$) for the native cells was calculated, assuming exponential flip kinetics, by non-linear fitting of the data (see Section 2). (B) Time course of outward reorientation (flop), at 37°C, of NBD-PC in native (●, ○) and electroporated/resealed (■, □) erythrocytes under 'zero-trans' conditions established by continuous extraction of probe from the outer membrane leaflet with albumin as described in the text. Field strength 6 kV cm^{-1} (0°C), 60 min resealing at 37°C. Closed symbols: no additive; open symbols: orthovanadate 1 mM.

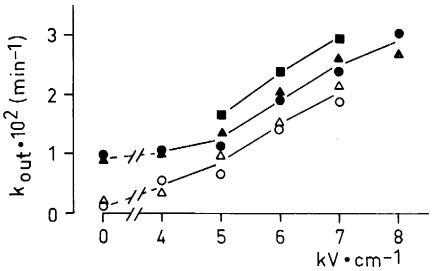

Fig. 6. Flop rates of NBD-PC in electroporated/resealed erythrocytes before (●, ▲) and after (○, △) suppression of floppase activity by vanadate (1 mM), NEM (0.8 mM 20 min 37°C), or ATP-depletion [22]. Rate coefficients derived from measurements under 'equilibrative' (●, ○) and 'zero-trans' (▲, △) conditions as defined in the text. (■) Cells porated and resealed in presence of Mg^{2+} (4 mM). Floppase activity corresponds to the difference between rates measured before and after suppression of floppase activity.

almost the opposite distribution in their presence (Fig. 5A). This supports the concept outlined above, that steady-state distributions of NBD-labelled and possibly native glycerophospholipids are determined by rates of flip and flop occurring via more than one pathway.

After electroporation (at 8 kV cm^{-1}) and resealing, the equilibrative flop kinetics of NBD-PC look quite different (Fig. 5A, broken lines). The rates are considerably enhanced and the steady state distribution is much more symmetric, in line with the results of the flip measurements shown in Fig. 4. From the flop kinetics for electroporated/resealed cells in the presence of inhibitors of floppase one obtains the rate of non-mediated 'leak flop' which, though elevated relative to the leak flop in native cells, is only slightly lower than that of the total flop in the absence of vanadate. The difference between the two kinetics provides the rate of the floppase-mediated transport in porated/resealed cells. From a number of such experiments at different field strengths (Fig. 6, circles), we obtained a mean value for floppase-mediated flop of about 0.0051 ± 0.0003 min^{-1} ($n = 4$), which is somewhat lower than the value for native cells reported above (0.0089 min^{-1}), but agrees with the floppase activity derived from flip measurements on porated/resealed cells (0.0050 min^{-1}, cf. Fig. 3).

3.1.3.2. 'Zero-trans' flop. The steady-state distribution of a lipid probe (cf. Fig. 4), which is usually

obtained by extrapolation from measured data, considerably affects the calculation of flop rates for equilibrative conditions (see Section 2). This complication can be avoided by using a 'zero-trans' type of flop measurement. In such experiments the *trans*-compartment of the flop, i.e., the level of the probe in the outer leaflet of the bilayer, is continuously kept at zero due to the presence of albumin in the suspension medium, which extracts the probe from the outer layer. In experiments of this type (cf. Fig. 5B), the final state is by definition the same in all cases and the curves are directly comparable. First-order rate constants, calculated from the curves, can be used to obtain rate coefficients of floppase-mediated and of leak flop for native and electroporated/resealed cells. Data collected for native and porated/resealed cells are compiled in Fig. 6 (triangles).

In *native* cells the total 'zero-trans' flop proceeded at a rate of 0.0096 ± 0.0006 min^{-1} ($n = 4$), separable into a floppase-mediated ($k = 0.0077$ min^{-1}) and a non-mediated, i.e. inhibitor-insensitive component ($k = 0.0019$ min^{-1}) at 37°C. Both values closely correspond to those for equilibrium flop. The mean floppase-mediated flop rate derived for *porated / resealed* cells from all data in Fig. 6 ($k = 0.0049 \pm 0.0019$ min^{-1}) is somewhat lower than that of native cells, but agrees with the corresponding value derived from equilibrium flop measurements (see above). Rates of non-mediated (i.e. inhibitor-insensitive) leak flop increase with the field strength applied. They are the same under equilibrium and zero-trans conditions, but only half of those of the corresponding leak flips at all field strengths tested (Table 1). This result can be understood on the basis of the passive steady-state distribution of the probe. Since flips and flops of lipid probes are – like fluxes – the products of rate coefficients and concentrations (or compartment sizes), and passive flips must equal passive flops in the steady state (i.e. $k_{in} \cdot C_0 = k_{out} \cdot C_i$), it follows that $k_{in}/k_{out} = C_i/C_0$. The experimental results roughly conform to this prediction (mean $k_{in}/k_{out} \approx 2.5$ according to Table 1, mean $C_i/C_0 \approx 2.0$). It should be noted, that even under the conditions of a mere passive, non-mediated translocation the probe attains an asymmetric distribution.

The increasing rate of passive leak flop of NBD-PC in electroporated/resealed cells (Fig. 6) in combination with the constant floppase-mediated transport also explains, why in the presence of floppase activity the steady-state distribution of NBD-PC is highly asymmetric in native cells but becomes much more symmetric (60% inside in the steady state) following electroporation/resealing (cf. Fig. 4).

Table 1
Rates of non-mediated flip and flop (at 37°C) of NBD-PC and stationary distributions of the probe in electroporated/resealed cells

Field pulse intensity (kV cm^{-1})	Flip $k_{in} \cdot 10^2$ (min^{-1})	Flop * $k_{out} \cdot 10^2$ (min^{-1})	$(k_{in})/(k_{out})$
4	1.03 ± 0.50	0.41 ± 0.17	2.51
5	2.71 ± 0.68	0.73 ± 0.30	3.71
6	3.35 ± 0.49	1.53 ± 0.05	2.18
7	4.50 ± 0.76	2.10 ± 0.40	2.14
8	5.83	2.74	2.12
			2.53 ± 0.67

	(C_i / C_{tot})	(C_0 / C_{tot}) **	C_i / C_0
4	0.53 ± 0.08	0.36	1.6
5	0.67 ± 0.02	0.33	2.1
6	0.68 ± 0.02	0.34	2.0
7	0.67 ± 0.02	0.34	2.0
8	0.70	0.30	2.3
			2.0 ± 0.3

Flippase activity was inhibited by vanadate (1 mM), NEM (0.8 mM, 20 min, 37°C) or ATP-depletion [22]. Data from 3–5 experiments ± S.D.
* Numbers derived from measurements under equilibrative and 'zero-trans' conditions.
** Numbers derived from measurements under equilibrative conditions.

3.1.4. Magnesium and floppase activity in porated / resealed cells

A comparison between the calculated floppase activities in native (0 kV cm^{-1}) and porated/resealed (4–8 kV cm^{-1}) cells reveals a significant decrease, evident after poration/resealing under both, equilibrative and zero-trans conditions (see Fig. 6). Since floppase activity requires ATP, one might speculate that a loss of ATP and/or of Mg^{2+} following poration might be responsible for this decrease. To compensate for such a possible effect, cells were porated and resealed in the presence of external ATP (2 mM) or Mg^{2+} (4 mM). While ATP had no effect on flop rates (data not shown), addition of Mg^{2+} (Fig. 6,

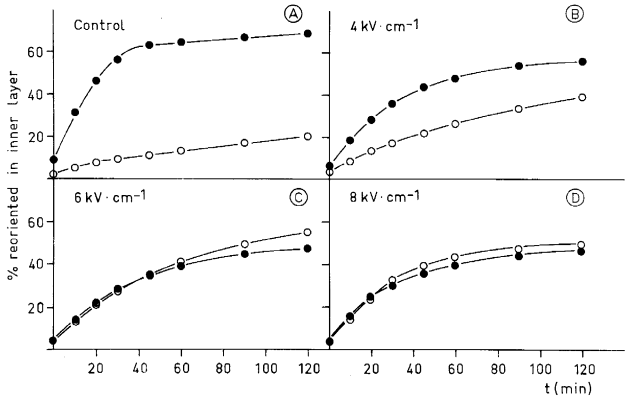

Fig. 7. Disappearance of the vanadate-sensitive (= flippase-mediated) component of the flip of NBD-PS (at 37°C) in electroporated/resealed cells. (●) controls; (○) vanadate (1 mM).

squares) brought floppase activity in porated/resealed cells back to the level observed in native cells, i.e. from a mean of 0.0050 min^{-1} to 0.0089 min^{-1}. A detailed study of the loss of Mg^{2+} responsible for this effect will be the object of a forthcoming study.

3.2. Transbilayer movements of a phosphatidylserine probe in electroporated / resealed cells

3.2.1. Flip measurements

Phosphatidylserines are concentrated in the inner leaflet of the erythrocyte membrane by an active, ATP-dependent process mediated by a flippase, the aminophospholipid translocase [26,27]. Exofacially inserted NBD-PS, although not an optimal substrate of this carrier [28], is transported inward at a rate, at 37°C, of 0.0286 ± 0.0061 min^{-1} ($n = 10$), to be compared, e.g., with a spin-labelled PS analogue, which moves at a rate of 0.21 min^{-1} [29]. Vanadate [27], NEM [27], fluoride [27], and in particular ATP-depletion [15], inhibit this process by more than 90% (Fig. 7A).

In unexpected contrast to our observations for NBD-PC, electroporation and subsequent resealing do not accelerate the inward flip rate of NBD-PS but produce inhibition up to a certain extent (Fig. 7B–D, closed symbols). Even more surprising, the vanadate-sensitive component of the flip of NBD-PS gradually disappears with increasing intensity of the field pulses, while the vanadate-insensitive component (Fig. 7, open symbols) becomes more prominent. Combining these observations, the conclusion seems justified that the mediated flip of NBD-PS is impeded after electroporation/resealing, while the non-mediated component is enhanced just like the flip of NBD-PC (see above). This concept is supported by the data compiled in Fig. 8.

In analogy to the observations concerning floppase activity in porated/resealed cells one might attribute the decrease in flippase activity to a loss of Mg^{2+}. Addition of Mg^{2+} to the suspension medium prior to resealing indeed stimulated the inhibitor-sensitive component of the flip in porated/resealed cells but did not fully restore the native activity. As a possible explanation for the Mg^{2+}-insensitive loss of flippase activity in electroporated cells one might postulate a partial inactivation of the transporter by electric field pulses. This concept could, however, be discarded on the basis of our observation (S. Schwarz, personal communication) that the flippase-mediated flip of another PS-probe, dilauroyl-PS (DLPS), which can be assessed by following red cell shape changes [15], is not abolished in electroporated/resealed cells.

Incorporation of DLPS into the outer leaflet of the erythrocyte membrane did, however, by itself produce inhibition of the flippase-mediated flip of NBD-PS. At a level of about 6 mol% DLPS in the outer

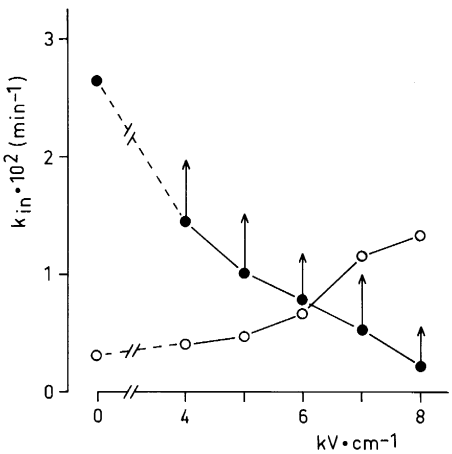

Fig. 8. Rate constants of inward reorientation (flip) of NBD-PS in native and electroporated/resealed erythrocytes. (●) Flippase-mediated component, defined as the difference between total and inhibitor-insensitive flip (inhibition by orthovanadate (1 mM) or NEM (0.8 mM, 20 min, 37°C)). The arrows indicate the increment of the flippase-mediated process when the cells are porated and resealed in the presence of 4 mM Mg^{2+}. (○) Non-mediated component defined as the inhibitor-insensitive flip.

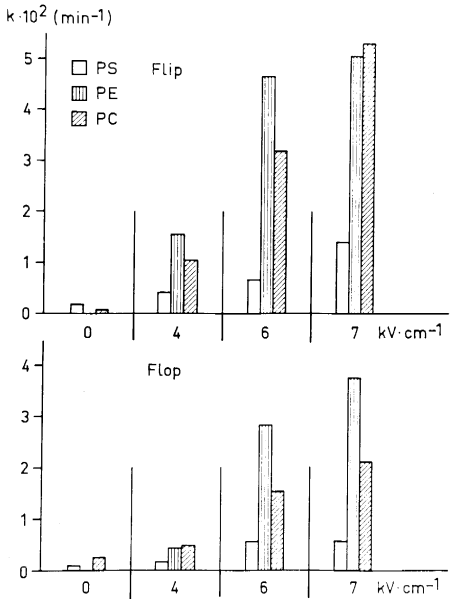

Fig. 9. Rate constants for non-mediated flip and flop of NBD-glycerophospholipids in native and electroporated/resealed erythrocytes at 37°C. The non-mediated flips and flops are defined by the rate coefficients obtained after inhibition of flippase and floppase.

membrane layer the flippase-mediated flip of NBD-PS was inhibited by about 50% (data not shown). Since it is established that in electroporated/resealed cells endogenous PS is partly shifted from the inner to the outer leaflet of the membrane [12,13], one may speculate that this endogenous PS, like exogenous DLPS, inhibits the flippase-mediated flip of NBD-PS, presumably by competition. This concept is further supported by our observation (data not shown), that the flippase-mediated inward reorientation of NBD-PS, when studied, like that of DLPS, by monitoring cell shape changes, is markedly suppressed in electroporated/resealed cells even after compensation for loss of Mg^{2+}. These data demonstrate, in our view, that PS-probes which are transported by the flippase with a lower affinity than endogenous PS may provide misleading information on flippase activity under conditions under which endogenous PS is present in the outer leaflet of the erythrocyte membrane.

A further interesting aspect concerns the enhancement of the leak flip of NBD-PS following electroporation/resealing. As evident from Fig. 8 and Fig. 9 (upper panel), this anionic phospholipid responds to field pulses of increasing intensity to a considerably lesser extent than its zwitterionic analogue NBD-PC.

3.2.2. Flop measurements

In order to characterize the *outward* translocation of NBD-PS in normal and electroporated/resealed cells, we measured its flop rates in native and electroporated/resealed cells under the two conditions outlined in the previous section. Since rate coefficients did not differ between the two modes, numbers were lumped together, while stationary distribution ratios were derived for equilibrative conditions only.

In native cells the probe distributes (Table 2) to a ratio of 80% inside, presumably due to the action of the flippase which counteracts the outward movement of the probe. Outward translocation is almost completely inhibited by inhibitors such as vanadate, fluoride and NEM. This must be regarded as a result of floppase inactivation. The residual inhibitor-insensitive flop characterizes the very small native leak for NBD-PS. It cannot be used for calculating steady state distributions. The floppase activity calculated from the total and the inhibitor-insensitive flop of

Table 2
Flop rates and steady-state distribution (at 37°C) of NBD-PS in native and electroporated/resealed cells.

	$k_{out} \cdot 10^2$ (min^{-1}) *	C_i / C_e **
Native		
No inhibition (= Total)	0.94 ± 0.16 (5)	80:20
Inhibition (= Leak)	0.07 ± 0.04 (9)	—
'Floppase'	0.87	
Elektroporated/resealed (7 kV cm^{-1})		
No inhibition (= Total)	1.10 ± 0.09 (3)	50:50
Inhibition (= Leak)	0.55 ± 0.09 (4)	63:37
'Floppase'	0.55	

7 kV cm^{-1}, 60 min resealing. Inhibitions used: orthovanadate (1 mM); NEM (0.8 mM 20 min, 37°C), or ATP-depletion [22]. Rates of floppase-mediated flop calculated as the difference of flop rates in the absence and presence of inhibitors. Number of experiments in brackets.

* From equilibrative and 'zero-trans' measurements.

** From equilibrative measurements.

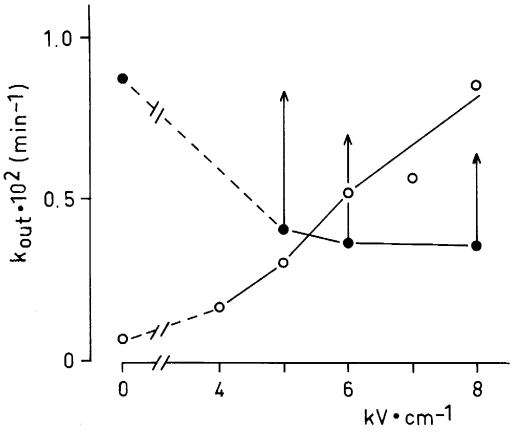

Fig. 10. Rate constants of the outward reorientation (flop) of NBD-PS in native and electroporated/resealed erythrocytes. (●) Mediated component (floppase-dominated), defined as the difference between total and inhibitor-insensitive flop (inhibition by orthovanadate (1 mM), fluoride (6 mM), ATP-depletion [22], or NEM (0.8 mM, 20 min, 37°C)). The arrows indicate the increment of the mediated flop, when the cells are porated and resealed in the presence of 4 mM Mg^{2+}. (○) Non-mediated component, defined as the inhibitor-insensitive flop.

NBD-PS (0.0087 min^{-1}) is very similar to that obtained for NBD-PC (0.0089 min^{-1}).

Following electroporation / resealing at increasing field strengths the total flop of NBD-PS first decreases and then becomes slightly stimulated (not shown). Data for cells porated at 7 kV cm^{-1} are given in Table 2. The steady state distribution drops to 50% inside, in contrast to 80% in native cells. The inhibitor-sensitive component of the flop, which is dominated by floppase activity, is diminished in porated/resealed cells. According to Fig. 10, this is partly due to a loss of Mg^{2+}, but additional effects must also play a role, since addition of Mg^{+} does not fully restore the mediated component. The inhibitor-insensitive leak flop, which like the leak flip, proved to be much slower than that of NBD-PC (Fig. 9, lower panel), increases considerably (Fig. 10).

3.3. Transbilayer movements of phosphatidylethanolamine and sphingomyelin probes in electroporated / resealed cells

Besides PC and PS, the other two major phospholipids of the erythrocyte membrane, PE and SM, are also affected by electroporation/resealing in their translocation rates and steady-state distribution.

Native PE is normally maintained to a major extent in the inner leaflet of the erythrocyte membrane by flippase-mediated active transport [26,27]. The *N*-(NBD)-aminohexanoyl-PE used in our study is not accepted by this transporter [28], while floppase transports the probe at a rate equal to that observed for NBD-PC and NBD-PS (data not shown, see also Ref. [22]).

In order to characterize the influence of electroporation/resealing on NBD-PE we have studied its passive non-mediated flip and flop (Fig. 9). Even at low field strengths, the membrane becomes highly leaky to NBD-PE. Leak flip rates are about 4–8-times higher than those of NBD-PS and also exceed those of NBD-PC. The same is true for the passive flop rates. In electroporated/resealed erythrocytes the non-mediated flips and flops of NBD-PE end at a distribution of about 70% inside, which is comparable to that observed for NBD-PC (data not shown).

NBD-SM has an unmeasurably low transbilayer mobility in native cells. Electroporation, however, mobilizes this phospholipid, too. After a single 7 kV cm^{-1} pulse and 60 min resealing a flip rate of about 0.0006 min^{-1} (37°C) can be measured, which is about 50-times lower than that of NBD-PC under the same conditions. The flip of NBD-SM can be further enhanced by repetitive pulses. 5 sequential pulses of 7 kV cm^{-1} produce a rate of 0.003–0.006 min^{-1} (37°C) which allows to calculate a steady-state transbilayer distribution of the probe of about 50% inside in electroporated/resealed cells. Under these conditions, the flip rates of NBD-PC are of the order of 0.25 min^{-1}. No evidence could be obtained in these studies for an involvement of floppase in the transbilayer movements of NBD-SM.

3.4. Head group-dependent extent of stimulation of flip and flop: specific for electroporation?

As evident from Fig. 9, the enhancement of transbilayer mobility of the NBD-phospholipids in electroporated/resealed cells varies for different headgroups. An increased flip-flop of PC analogues in erythrocytes has previously been demonstrated for various types of membrane perturbation, e.g. by membrane-active and channel-forming agents

Table 3
Extent of enhancement of the passive flip of NBD-phospholipids in the erythrocyte membrane following various types of perturbation

	PC (min^{-1})	PC (normalized)	PE (normalized)	PS (normalized)	SM (normalized)
Electroporation * 6 kV cm^{-1}, 0°, t = 20 μs, resealing 60 min, 37°C	0.0292	1.0	1.44	0.18	0.03
Butanol ** 155 mM	0.0362	1.0	1.35	0.25	0.09
Amphotericin B ** 25 μg/ml medium Hct 10%	0.0798	1.0	0.98	0.11	0.02
Diamide * 5 mM, 45 min, pH 8	0.0235	1.0	1.06	0.36	0.09
t-Butylhydroperoxide * azide (1.5/2.5 mM) 30 min, stop by BHT (0.1 mM)	0.0257	1.0	1.15	0.20	0.06
Ca^{2+}/A23186 ** (0.5 mM/10 μM)	0.0158	1.0	1.11	1.10	0.09
Tetracaine 2.5 mM **	0.0150	1.0	1.85	0.78	0.08

Flippase activity was inhibited by ATP-depletion [2] or by NEM (0.8 mM, 20 min, 37°C), and the cells treated with the perturbing agents either before * or during ** the flip measurements, carried out at 37°C. Flip rates normalized to the enhanced flip rate of NBD-PC, which is given in absolute values in the first column. Mean value from 2–3 experiments. For the passive flip rate of NBD-PC in the native membrane, see Table 4.

[20,30,31], by modification of membrane protein SH groups [32–34] or by calcium loading [35–37]. In order to put our data for electroporated/resealed cells into the framework of these earlier results, we have established the flip rates of all four NBD-phospholipids for various types of membrane perturbation. As is evident from Table 3, in all cases studied except Ca^{2+} loading and tetracaine treatment, the leak flip of NBD-PS is much slower than that of NBD-PC. NBD-PE moves considerably faster than NBD-PC in cells subjected to electroporation, tetracaine or butanol and as fast as NBD-PC under all other conditions. NBD-SM, on the other hand, remains much less mobile than the glycerophospholipids in all cases.

4. Discussion

4.1. Characteristics and possible mechanisms of the field-pulse induced enhancement of transbilayer phospholipid mobility

In this study, we have characterized in more detail than hitherto available the enhancement of the transmembrane mobility of phospholipid probes in erythrocytes after exposure to electric field pulses, using fluorescent NBD-phospholipids. Electroporation goes along with an instantaneous redistribution of the phospholipid probe, NBD-PC, due to a marked increase of its transbilayer mobility. This increased mobility rediminishes, however, to a lower but stably elevated level within a few minutes, even at 4°C. A similar biphasic change is already known [38,39] for the field-pulse-induced leakiness of the erythrocyte to polar permeants which are assumed to penetrate the modified membrane via aqueous pores [2,38].

Warming of electroporated erythrocytes from 4°C to 37°C induces resealing of the leaky membrane [2,3,41,42]. Contrary to suggestions based on earlier observations [41,43], however, this resealing is not complete except after very mild poration. Even after single porations at moderate field strengths (4–8 kV cm^{-1}, $\tau = 8–80$ μs) cells retain some permanent leakiness to small hydrophilic ions (K^+) and to larger hydrophobic ions (methyltriphenylphosphonium), while the residual leakiness to larger hydrophilic probes such as mono- and disaccharides drops below the level of detection in many cases (B. Deuticke, data not shown). Statements concerning a 'resealing' of an electroporated biomembrane are thus only useful when given with regard to specified probes and for defined experimental conditions.

According to our present data, the enhanced transbilayer mobility of the phospholipid probes does never return to normal even during prolonged (24–27 h) resealing of field-pulse-treated cells. It is conceivable that the early 'large defects' in the bilayer observed by Chang and Reese [40] in a morphological study act as the flip sites responsible for the immediate, very rapid reorientation of phospholipid documented in Fig. 1A. Since these defects have been shown, however, to be rather short-lived, one has to postulate a second phase of persistently increased transbilayer mobility which does not disappear at 0°C but can at least be partly abolished by elevated temperatures. Data obtained for the permeability of a hydrophobic cation (methyltriphenylphosphonium) under the same conditions indicate that the 'resealing' processes may reach different stationary levels depending on the temperature of resealing (B. Deuticke, data not shown). Resealing therefore seems to be governed not only by kinetic but also by thermodynamic phenomena.

The molecular basis of the persistent component of field-pulse-induced membrane damage is presently not clear. While the transient large leaks in electroporated cells are usually ascribed to short-lived alterations of the lipid domain, the stable 'leaks' might be related to alterations of membrane proteins. Specified concepts have been proposed to explain such alterations for the case of leaks for hydrophilic permeants. Current flow and local heating within preexisting specific channels or pores, i.e., integral membrane proteins were claimed to induce their conversion into leak pathways for such probes [3,44]. Since the number of such unspecific leaks necessary to account for the observed leak permeabilities is certainly very small (at best a few hundred per cell, but probably much less) [2], the corresponding loss of specific transport sites would remain below the level of detection.

Our present results suggest that modified proteins may also serve as flip sites. This is not quite unexpected in view of the observation that pore-forming peptides [45] as well as peptide-analogous membrane-active channel formers [30,31] enhance flip rates in artificial and erythrocyte membranes. A 'mismatch' between modified transmembrane domains of intrinsic proteins and the surrounding lipid could serve as a pathway facilitating the passage of the phospholipid headgroup through the hydrophobic core by lowering the Born energy barrier. On the other hand, modified peripheral membrane proteins might lower the energy barrier by perturbing the lipid bilayer. Support for this concept comes from the stimulation of transbilayer mobility of phospholipids by the SH oxidizing agent, diamide, which modifies and crosslinks membrane skeletal protein of the erythrocyte with some preference [32,46].

Alternatively, the irreversible leak in electroporated/resealed erythrocytes might have its origin in a field-pulse-induced stable reorganization of membrane phospholipids. There is evidence now that losses of aminophospholipid asymmetry occurring, e.g., in sickle cells but also in other types of damaged cells [27,46], induce the formation of non-bilayer structures as indicated by binding of phospholipid antibodies [47,48] and by fusogenicity [49]. Non-bilayer structures in artificial bilayers are known to induce a marked leak permeability and enhancement of phospholipid flip-flop [50,51]. Electroporated/resealed erythrocytes exhibit many of these functional indicators of non-bilayer structures in combination with the established [12,13] loss of phospholipid asymmetry.

4.2. Specificity of enhanced phospholipid transbilayer mobility

In the present study we have also demonstrated characteristic differences in transbilayer mobility of probes with different headgroups in electroporated/resealed erythrocytes. NBD-SM remains very immobile even in cells subjected to strong field pulses. The glycerophospholipids exhibit increasing mobilities in the order PS $\ll$ PC $<$ PE. This sequence concerns the leak properties derived from flip and flop rates measured after inhibition of all mediated components (Fig. 9).

These findings raise three questions:

(a) Is the mobility sequence in porated/resealed cells equal to that in the native unperturbed membrane and in membranes perturbed by other modifications?

(b) Which properties of the probes are responsible for the sequence?

(c) Are the data obtained for the probes also valid for the corresponding endogenous phospholipids?

Table 4
Passive flip of NBD-phospholipids in the native, unperturbed membrane

NBD-Phospholipids	$k_{in} \cdot 10^2$ (min^{-1})	
PC	0.062 ± 0.011	(1.00)
PE	0.094 ± 0.008	(1.46)
PS	0.048 ± 0.001	(0.77)
SM	< 0.002	(< 0.003)

Flippase activity was inhibited by ATP-depletion [22] or by NEM (0.8 mM, 20 min, 37°C). Flip rate constants (k_{in}) represent mean values $\pm$ S.D. of 3–7 experiments. Values normalized to PC given in brackets.

Question (a) cannot be answered conclusively at present. The set of preliminary data compiled in Table 4 suggests at least, that the sequence of passive flip rates in the native, unperturbed membrane differs only quantitatively from that in the electroporated/resealed membrane (cf. Table 3). This observation might support the view that flip and flop in the porated/resealed membrane occur via a pathway not essentially different from that in the native membrane.

One might then ask, whether the mobility sequence of NBD-phospholipids in electroporated/resealed cells is specific for that condition or a general property of perturbed erythrocyte membranes. The data compiled in Table 3 do not provide an unambiguous answer. On the one hand, most studied types of perturbation – with the notable exception of that by Ca^{2+} and tetracaine – share the low relative mobility of the anionic NBD-PS and the much lower mobility of NBD-SM. On the other hand, only electroporation/resealing, butanol, and tetracaine go along with a mobility of NBD-PE significantly higher than that of NBD-PC. This heterogeneity may indicate that the putative 'flip sites' in perturbed membranes vary in a yet unexplainable dependence with the underlying alteration. The exceptional behaviour of the Ca^{2+}-exposed membrane, in which all glycerophospholipids have the same passive mobility, may support the concept [52,53] that 'Ca^{2+}-scrambling' of phospholipids in biomembranes [16,54,55] arises from the activation of a specific protein-mediated process [16,52–54].

Concerning question (b), the maintenance of a low relative transbilayer mobility of NBD-SM even after pulse treatments and other perturbations (see Table 3) which raise the mobility of the glycerophospholipids to very high values, indicates that properties of the probe per se, irrespective of its environment, are involved. The major structural peculiarity of sphingomyelin concerns its intermediate region between the zwitterionic headgroup and the hydrophobic tail. The amide bond and/or the lack of an acyl ester oxygen may fix NBD-SM in its native orientation and prevent an easy transbilayer reorientation. Particular H-bonds between sphingomyelins and their next neighbours could play a role in this context. The differences in mobility – in electroporated/resealed cells – between the various NBD glycerophospholipids, on the other hand, most probably arise from the headgroup structure. Not unexpectedly, the negative charge of PS goes along with a low leak permeability. The anionic headgroup has to overcome a much higher Born energy barrier in the core of the lipid bilayer than that of the analogous zwitterions PC and PE.

The above considerations are based on data for NBD-phospholipids. Concerning question (c), it remains to be seen whether they are also applicable to endogenous phospholipids and thus allow predictions concerning the extent of mobilization and subsequent 'scrambling' of endogenous phospholipids in electroporated cells. While the absolute mobilities of endogenous phospholipids are most likely lower than those of the NBD probes, the *ratios* of the enhanced leak mobilities following perturbation might well be the same.

4.3. Transbilayer distribution of phospholipids as a dynamic non-equilibrium state

Flip and flop measurements in electroporated/resealed cells can also be used to study processes which contribute to the steady-state transbilayer distribution of phospholipids or their probes. Such studies should be helpful to test predictive mathematical models (e.g., Ref. [56]) of the mechanistic basis of phospholipid asymmetry in biomembranes.

In the case of NBD phospholipids, one can investigate the contribution (a) of leak permeabilities which can be set in their extent via the strength or the duration of field pulses, (b) of the influence of the aminophospholipid flippase, which can be switched off by energy deprivation or inhibitors, and (c) of the

NBD-glycerophospholipid floppase, for which the same is true. Since the flippase accepts NBD-PS but not NBD-PC or -PE, while floppase accepts all three glycerophospholipids, one can select among various test conditions.

Using NBD-PC, we have studied the case of a steady-state distribution governed by an outward-directed active and a bidirectional passive transport. In the native cell the stationary distribution of NBD-PC (20% inside) is obviously dominated by floppase activity since floppase inhibition induces a marked shift of the distribution (Fig. 5A). The very slow leak flop in the native cell, however, prevents a reliable calculation of the true final distribution from first-order-kinetics. Enhancement of leak mobility by poration/resealing provides a direct measure of a true passive distribution in such cells. This turns out to be 65% inside, which is shifted to about 60% inside when floppase is active (Fig. 5A).

It should be noted that this passive distribution of the probe is asymmetric in favour of the inner leaflet. There are at least three possible explanations for a passively established 'asymmetry' of a phospholipid probe: First, it might only be an apparent asymmetry due to differences in the 'spaces' available to the lipid probe in the two leaflets of the bilayer. Second, different specific or colligative properties of the endogenous lipids in the two leaflets might induce different affinities or standard chemical potentials for the probe in the two leaflets. The passive transbilayer distribution of endogenous phospholipids following electroporation/resealing is not well known, although it is clear from the results of Dressler et al. [12] and Song et al. [13], that it is not identical with the native situation. Third, an interaction of lipid probes with cytoskeletal or integral proteins might induce a slight preference for the inner leaflet. Such effects have been claimed [57] since the first description of phospholipid asymmetry, but were later discarded by most investigators.

4.4. NBD-phospholipids: suitable probes for demonstrating scrambling and recovery of asymmetry for endogenous phospholipids?

As shown in a forthcoming paper (see also Ref. [19]), electroporation induces a rapid transformation of discocytic erythrocytes into echinocytes. Resealing at 37°C brings about shape normalization and stomatocyte formation. Comparable observations in calcium-loaded cells [15,16] have been attributed to scrambling and subsequent partial normalization of phospholipid asymmetry. The underlying movements of phospholipids, in particular of PS, could be demonstrated using short chain ^{14}C- and spin-labelled diacylphospholipid probes. In view of the commercial availability and the easy handling of NBD-phospholipids they would seem to be the obvious probes for studying phospholipid scrambling in electroporated cells. While this is clearly possible for NBD-PC, NBD-PS proves to be inappropriate for that purpose for two reasons. (1) The difference between the steady-state distribution of NBD-PS in native cells (about 70–80% inside), set by high flippase activity and low leak permeability, and that in porated/resealed cells (about 50–60% inside), set by high leak permeability but low flippase activity, is too small for reliable kinetic studies of 'scrambling'. (2) Aminophospholipid translocase loses transport capacity for NBD-PS in electroporated cells. Thus, a recovery of PS asymmetry cannot be studied with this probe. In view of these shortcomings, other strategies will be needed to quantify the movements of PS in electroporated/resealed cells.

4.5. Lessons for future work

Summarizing, the data obtained on electroporated/resealed cells provide direct experimental insights into the interplay of passive and active processes in the establishment of the steady state phospholipid distribution in the erythrocyte membrane. Due to the different mobilities and translocation rates of probes and native phospholipids, the quantitative results have mainly model character. The qualitative aspects, however, can probably be generalized. Electroporation as a purely physical means to modify passive transbilayer mobilities may presently be the choice procedure for the investigation of factors governing phospholipid distribution. Analysis should be refinable by the development of more physiological fluorescent phospholipid probes.

The irreversible enhancement of phospholipid mobility in the porated/resealed membrane may contribute to the 'long-lived fusogenic state' observed in field pulse-treated erythrocytes [58,59]. Therefore,

complementary studies relating fusion rates and yields to phospholipid mobility would seem worthwhile.

Membrane perturbations by different procedures seem to alter transbilayer mobilities of phospholipids relative to each other to a different extent, indicating differences in the underlying processes. In view of these differences, transbilayer redistributions of endogenous phospholipids in membrane-perturbed cells may be expected to vary in dependence on the experimental conditions. This will in turn give rise to variations in the type and extent of secondary alterations, e.g. of cell shape. Forthcoming papers will present material to further illustrate this view.

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