Nonmediated Flip-Flop of Anionic Phospholipids and Long-Chain Amphiphiles in the Erythrocyte Membrane Depends on Membrane Potential

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ABSTRACT: The nonmediated inward translocation (flip) of the anionic fluorescent *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)- (NBD-)labeled phospholipid phosphatidylmethanol (PM) from the outer to the inner membrane leaflet of human erythrocytes and vice versa depends on membrane potential. Interestingly, inside-positive potentials due to chloride gradients and the native chloride conductance of the cells resulted in an increase of the flip rates. This flip enhancement could be suppressed by addition of gramicidin D, which increases cation conductance, or 4,4′-diisothiocyanatostilbene-2,2′-disufonate (DIDS), which inhibits anion conductance. Conversely, inside negative potentials established by an outward-directed K^+ gradient in the presence of gramicidin on DIDS-treated cells resulted in a decrease of flip rate. Flip rate exhibited an exponential dependence on membrane potential. The opposite effects of the positive and negative potentials were obtained for the outward translocation (flop) from the inner to the outer membrane leaflet. Similar potential dependencies were found for the nonmediated flip of anionic NBD-labeled phosphatidic acid (PA) and 2-(*N*-decyl)aminonaphthalene-6-sulfonic acid (2,6-DENSA) following blockage of the band-3-mediated component of flip. The membrane potential also influences the stationary distribution of the anionic lipids between the inner and outer leaflets. The distribution is shifted to the inner leaflet by increasingly positive potentials and to the outer leaflet by increasingly negative potentials. It is concluded that nonmediated flip-flop of the anionic phospholipids and the long-chain sulfonate represents electrogenic translocation of the unprotonated charged lipids across the hydrophobic barrier.

The dependence on membrane potential of the diffusion of hydrophobic ions across membranes has been studied in some detail (*1*−*3*). In these studies both the potential dependence of partitioning of ionic amphiphiles between the aqueous medium and the membrane and that of their diffusion across the hydrophobic membrane barrier have to be considered (*4*−*7*). Little information concerning the dependence on membrane potential of the mere transbilayer translocation (flip-flop) of ionic lipid probes (*8*, *9*) or phospholipids is available as yet. In the case of the flip-flop of these lipid probes with high membrane/water partition coefficients, effects of membrane potential only concern the transbilayer movement of the lipid probe without the contribution of its partitioning into the membrane. The steep pH dependence in lipid vesicles of the flip-flop of anionic phospholipids like phosphatidic acid and phosphatidylglycerol was taken as evidence for the electroneutral movement of the protonated species across the barrier (*10*, *11*). Recently, the potential-dependent translocation of a cationic lipid probe from the outer to the inner leaflet of lipid bilayers was demonstrated qualitatively by an indirect method (*8*).

For biological membranes no experimental information on the dependence of flip-flop of anionic lipids on membrane potential is available as yet. From the small pH dependence of nonmediated (meaning not catalyzed by reversible binding to a protein) flip-flop of anionic phospholipids in erythrocytes (*12*), this process is unlikely to be a nonionic diffusionlike process. In excitable membranes with rapid flip-flop, the distribution of charged phospholipids between the two leaflets has been speculated to follow the electric gradients across the membrane (*13*).

In the present study we investigated the effect of changes of membrane potential on the nonmediated flip-flop of the NBD-labeled anionic phospholipids, phosphatidylmethanol (PM)[1] and phosphatidic acid (PA) and the long-chain sulfonate 2-(*N*-decyl)aminonaphthalene-6-sulfonic acid (2,6-DENSA), using the erythrocyte membrane as a model system. Since these lipids have high membrane−water partition coefficients greater than 10^5 (*12*, *14*, *15*), membrane potential-dependent changes of partitioning of the probes into the membrane can be neglected and the mere effect of membrane potential on the translocation across the hydrophobic membrane domain can be studied. From the observed potential dependencies, it is concluded that nonmediated flip-flop of these lipid probes is an electrogenic process.

MATERIALS AND METHODS

Materials. Human erythrocyte concentrates were obtained from the local blood bank and used within 8 days. Eryth-

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[1] Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonate; 2,6-DENSA, 2-(*N*-decyl)aminonaphthalene-6-sulfonic acid; 5,2-DENSA, 5-(*N*-decyl)-aminonaphthalene-2-sulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; NBD-PC, 1-oleoyl-2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminohexanoyl]-*sn*-glycero-3-phosphocholine; NBD-PA, 1-oleoyl-2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminohexanoyl]-*sn*-glycero-3-phosphoric acid; NBD-PM, 1-oleoyl-2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminohexanoyl]-*sn*-glycero-3-phosphomethanol; TAPS, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid.

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rocytes were isolated by centrifugation and washed three times with isotonic saline.

1-Oleoyl-2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminohexanoyl]-sn-glycero-3-phosphocholine (NBD-PC) was obtained from Avanti Polar Lipids (Birmingham, AL), 2-(*N*-decyl)aminonaphthalene-6-sulfonic acid sodium salt (2,6-DENSA) was from Molecular Probes (Eugene, OR), 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) was from Calbiochem (Bad Soden), gramicidin D was from Sigma (Deisenhofen), and *N*-ethylmaleimide (NEM) was from Fluka (Neu-Ulm). The anionic phospholipid probes NBD-phosphatidic acid (PA) and NBD-phosphatidylmethanol (PM) were prepared from NBD-PC using phospholipase D from *Streptomyces* species (Sigma, Deisenhofen) as described before (*12*).

Pretreatment of Erythrocytes. Erythrocytes were suspended in medium A (pH 7.4) containing (in millimoles per liter) KCl (90), NaCl (45), Na_2HPO_4/NaH_2PO_4 (12.5), and sucrose (44). In experiments with NBD-labeled PM and PA, cells were pretreated with NEM (0.8 $mmol{\cdot}L^{-1}$, 20 min, pH 7.4, 37 °C) to block the active component (to about 90%; *12*, *16*) of outward phospholipid translocation, and in the case of NBD-PA and 2,6-DENSA, cells were pretreated with DIDS (20 $\mu mol{\cdot}L^{-1}$, 30 min, 37 °C) to block the band-3-mediated component (to 95%; *17*) of inward and outward translocation (*12*, *14*, *15*). These treatments were followed by three washes with medium A.

Establishment of Membrane Potentials. Inside positive membrane potentials were established by a decrease of the extracellular KCl concentration and maintenance of isotonicity by addition of potassium glutamate. Since the chloride conductance of the erythrocyte membrane is much higher than that for cations (*18*−*20*), membrane potentials arising under these conditions can be calculated from the ratio of the intra- and extracellular chloride concentrations using the Nernst equation (*21*−*24*). Chloride concentrations were determined using a Chlor-o-counter (Marius, The Netherlands). Intracellular chloride was released from the cells by hemolysis and protein precipitation with $HClO_4$. The amount of chloride in the samples was then related to the volume of cell water calculated from the measured dry weight per volume of packed cells. Considering the fast exchange of chloride for bicarbonate (or OH^-) by the anion exchanger, the resulting redistribution of OH^- will end up in the stationary distribution of H^+ opposite to that of chloride. Indeed, membrane potentials calculated from the chloride distribution and those calculated from the difference between the intra- and extracellular pH were quite similar (data not shown), as reported before (*22*, *23*).

In the case of inhibition of anion conductance by DIDS pretreatment of cells, membrane potentials could not be derived from the equilibrium potential for chloride (*24*). The slopes of the lines relating membrane potential measured with CCCP (see below) to extracellular chloride concentration at 41 and 25 °C were respectively, 41 and 24 mV/10-fold change of the chloride concentration. These values are well below the theoretical value of about 60 mV obtained from the Nernst equation, and therefore potentials had to be calculated from the difference between the pH of cell interior and medium measured in the presence of 20 $\mu mol{\cdot}L^{-1}$ carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) to enhance pH equilibration (*25*−*30*). In some experiments, KCl was exchanged for K-HEPES.

Inside negative membrane potentials were established by the simultaneous increase of the membrane conductance for cations by addition of 0.5 $\mu mol{\cdot}L^{-1}$ gramicidin from ethanol (*31*, *32*), blockage of anion conductance by pretreatment of cells with DIDS, and decrease of the extracellular K^+ (or Na^+) concentration by its isotonic exchange for choline (both as chloride salts). Membrane potentials were calculated from the pH difference between cell interior and medium in the presence of 20 $\mu mol{\cdot}L^{-1}$ CCCP (*24*−*32*). The slope of the line relating the measured potential to the extracellular K^+ (Na^+) concentration was 55 mV/10-fold change of the cation concentration, which is close to the theoretical value of 59 mV for 25 °C obtained from the Nernst equation.

Titration of Cells to Various pH Values. Cell suspensions (5% hematocrit) were titrated to pH values between 5.5 and 8.5 in media containing 150 mmol l^{-1} KCl and 10 $mmol{\cdot}L^{-1}$ MES, HEPES, or TAPS buffer. For titration of cells to pH values above and below 7.4 without changes of membrane potential, the extracellular chloride concentration was respectively decreased and increased according to procedures described in the literature (*33*, *34*). To set membrane potentials to zero (measured using the CCCP method), the extracellular chloride concentrations ranged from 180 $mmol{\cdot}L^{-1}$ for pH 6.8 and 55 $mmol{\cdot}L^{-1}$ (with 35 $mmol{\cdot}L^{-1}$ sodium/potassium tartrate) for pH 8.4.

Measurement of Inward Translocation (Flip) of Lipid Probes. Trace amounts of fluorescent NBD-phospholipid and 2,6-DENSA (ratio of probe to membrane phospholipid, respectively, 1:300 and 1:100) from a stock solution in, respectively, ethanol and dimethyl sulfoxide (5 μL/mL of suspension, which does not affect flip) were inserted into the outer membrane leaflet of erythrocytes (respectively 7 and 15 min, 0 °C), and the cells washed once with the flip medium. Subsequently, cells were resuspended in the flip medium (Hct 10%) and the time-dependent flip of NBD-PM (25 °C), NBD-PA (41 °C), and 2,6-DENSA (41 °C) were measured by the increase of fluorescence (excitation respectively 466 and 350 nm, emission respectively 522 and 410 nm) in the inner membrane leaflet (P_i) using the albumin extraction method described before (*12*). An exponential function, $q[1 - \exp(-k_1 t/q)]$, was fitted to the kinetic data of the fractions of total fluorescence (P_{tot}) in the inner leaflet (P_i/P_{tot}), where q represents the fraction of probe in the inner leaflet under stationary conditions and k_1 is the rate constant for the unidirectional flip. Thus, the ratio of the probe in the inner (P_i) and outer (P_o) leaflet is $q/(1 - q)$.

Measurement of Outward Translocation (Flop) of Anionic Lipid Probes. Following a flip period (30−90 min) of the anionic lipid probe, the probe was removed from the outer membrane leaflet by two repetitive albumin (1.5 $g{\cdot}dL^{-1}$) extractions. After washing of the cells with the flip medium, the flop of the lipid probe was followed by measuring the time-dependent decrease of albumin-inextractable fluorescence as described before (*12*). An exponential curve, $(1 - q)\exp[-k_{-1}t/(1 - q)] + q$, was fitted to the kinetic data of the nonextractable fractions (P_i/P_{tot}), where k_{-1} is the rate constant for the unidirectional flop.

RESULTS

In the present study, effects of changes of membrane potential of human erythrocytes on the nonmediated translocation of the anionic phospholipids NBD-labeled PM and

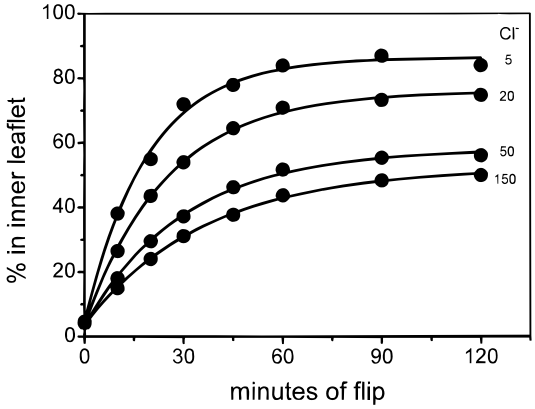

FIGURE 1: Time-dependent translocation of NBD-PM from the outer to the inner membrane leaflet (flip) as a function of the extracellular chloride concentration. NBD-PM was inserted into the outer membrane leaflet of erythrocytes and flip measured (25 °C) in media containing 150, 50, 20 and 5 mmol·L^{-1} KCl supplemented to isotonicity with potassium glutamate. Glutamate is an anion nontransportable via the anion exchanger.

PA and the long-chain amphiphilic sulfonate 2,6-DENSA from the outer to the inner membrane leaflet (flip) and vice versa (flop) were investigated. In medium containing 150 mmol·L^{-1} chloride that produces a slightly negative membrane potential (about −9 mV; *21*), the flip of NBD-PM with a rate constant of 0.014 ± 0.002 min^{-1} ($n = 9$) and a corresponding half-time of 23 min (25 °C) is rather fast. A gradual decrease of the extracellular chloride concentration to 5 mmol·L^{-1} by isotonic exchange of chloride for the impermeable anion glutamate, which goes along with a change of the slightly negative membrane potential to increasingly positive potentials (*22*, *23*) of up to about +60 mV (determined as described under Materials and Methods), results in stimulation of the flip of anionic NBD-labeled PM (Figure 1).

To confirm that the flip enhancement induced by a decrease of the extracellular chloride concentration is indeed due to the established positive membrane potential, the membrane potential was short-circuited by an increase of the cation conductance by addition of the ionophore gramicidin D or a decrease of the anion conductance by the addition of DIDS to the flip medium and their effects on flip were investigated. Both gramicidin and DIDS suppress the flip stimulation by a decrease of extracellular chloride to 5 mmol·L^{-1} (Figure 2) and decrease the positive membrane potential from about +60 mV to about, respectively, +30 and 0 mV (25 °C). This result supports a causal role of the positive membrane potential in flip stimulation.

In further experiments, the effect of increasingly negative membrane potentials (i.e., hyperpolarization of the membrane) on the flip of NBD-PM was investigated. To establish negative membrane potentials of up to about −100 mV (*32*), the membrane conductance for anions was suppressed by a pretreatment of cells with DIDS, the membrane conductance for small monovalent cations was increased by the addition of gramicidin, and extracellular K$^+$ was exchanged for choline during flip measurement (see Materials and Methods). As expected, the flip rate of NBD-PM diminished upon a gradual decrease of the extracellular K$^+$ concentration (Figure 3).

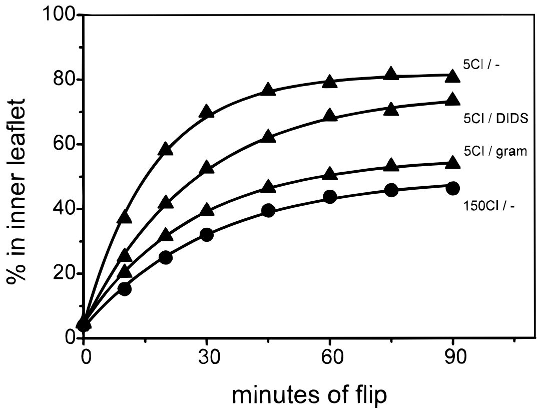

FIGURE 2: Addition of gramicidin (0.5 μmol·L^{-1}), which increases cation conductivity, or DIDS, which decreases anion conductance, suppresses stimulation of NBD-PM flip (25 °C) by a decrease of extracellular chloride concentration from 150 (●) to 5 (▲) mmol·L^{-1}.

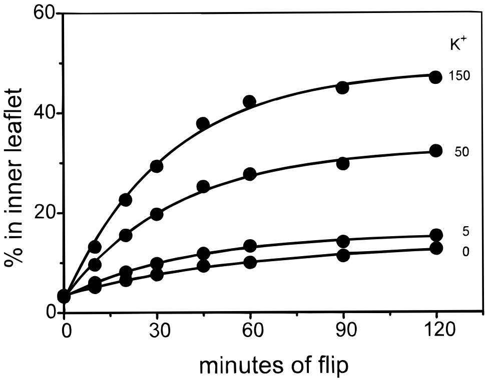

FIGURE 3: Gradual decrease of the extracellular K$^+$ concentration from 150 to 50, 5, and 0 mmol·L^{-1} by isotonic exchange of KCl for choline chloride in the presence of gramicidin (0.5 μmol·L^{-1}) which goes along with increasingly negative potentials decreases flip rate of NBD-PM (25 °C).

Valinomycin could not be used to increase K$^+$ conductance because of its potential-independent stimulation of the flip rate. At 0.5 and 2 μmol·L^{-1} valinomycin and 90 mmol·L^{-1} K$^+$, the flip rates of NBD-labeled PA and PM are enhanced respectively 2- and 4-fold, which is comparable to the effect of valinomycin on the flip rate of lysophosphatidylmethanol (*35*). Although this stimulation is smaller at lower concentrations (*35*), it complicates the evaluation of the mere membrane potential dependence. This flip stimulation is probably due to the binding of the anionic phospholipid to the cationic valinomycin−K$^+$ complex and the enhanced transbilayer movement of the electroneutral complex as described previously for the transport of lipophilic and hydrophobic ions across the membrane (*36*−*38*). Gramicidin at 0.5 μmol·L^{-1} when added from ethanol does not have such a side effect (*39*).

From the flip kinetics of cells with different membrane potentials, rate constants were calculated (see Materials and Methods) and plotted against the corresponding membrane potential determined as described under Materials and Methods. As shown in Figure 4, the flip rate exhibits an exponential dependence on membrane potential. Similar results were obtained for the nonmediated (DIDS-insensitive) flip of anionic NBD-PA and 2,6-DENSA at 41 °C (Figure 4, inset).

Variation of membrane potential not only influences flip rates but also alters the stationary transbilayer distribution of anionic NBD-PM (Figures 1 and 3). The ratio of NBD-

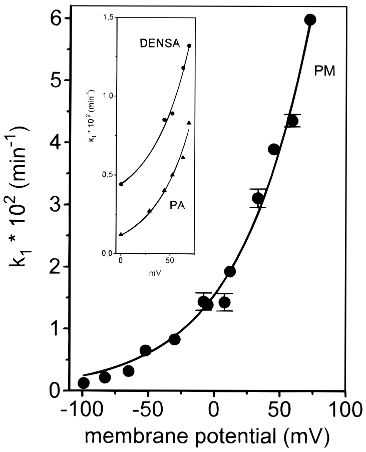

FIGURE 4: Exponential dependence of the flip rates of NBD-PM (●) at 25 °C and of NBD-PA (inset, ▲) and 2,6-DENSA (inset, ●) at 41 °C on membrane potential. k_1 is the flip rate constant.

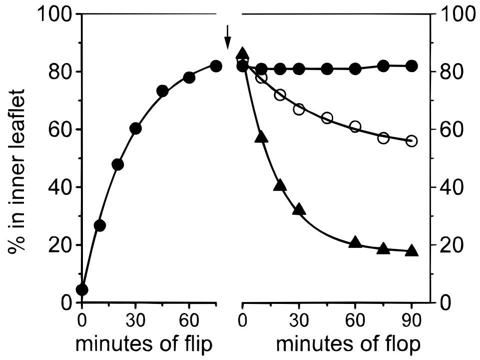

FIGURE 5: Accumulation of NBD-PM in the inner membrane leaflet as a result of a positive membrane potential (●) established by suspension of cells in 150 mmol·L^{-1} glutamate is reversed (see arrow) upon membrane depolarization by resuspension of cells in 150 mmol·L^{-1} KCl (○) or establishment of a negative potential by resuspension of cells in 150 mmol·L^{-1} choline in the presence of 0.5 μmol·L^{-1} gramicidin (▲).

PM between the inner and outer leaflet (P_i/P_e) increases from 0.18 ($n = 2$) in the presence of a highly negative potential (−90 mV) to 1.1 ($n = 6$) in the essential absence of a membrane potential (150 mmol·L^{-1} chloride) to 5.7 ($n = 4$) in the presence of a highly positive potential (+70 mV). The highly asymmetric stationary distribution of NBD-PM in favor of the inner membrane leaflet achieved by a highly positive membrane potential in 150 mmol·L^{-1} potassium glutamate is fully reversed by washing the cells in the cold and resuspending them in media that depolarize (150 mmol·L^{-1} KCl) or establish a negative (150 mmol·L^{-1} choline chloride in the presence of gramicidin) membrane potential (Figure 5).

Effects of membrane potential opposite to those on flip rates were obtained for rates of flop, i.e., the outward translocation from the inner to the outer membrane leaflet. Upon an increase of the inside negative (outside positive) membrane potential, an exponential increase of flop rates of anionic NBD-PM was observed (Figure 6). The negative potential also alters the stationary distribution of NBD-PM between the inner and outer membrane leaflet. The ratio of NBD-PM between inner and outer leaflet (P_i/P_e) increases from 0.2 in the presence of highly negative membrane potential (about −70 mV) to 1.0 in the absence of a membrane potential. The stationary distributions of NBD-PM obtained by a flop process are thus comparable to those found after a flip process under the same conditions (Figure 3).

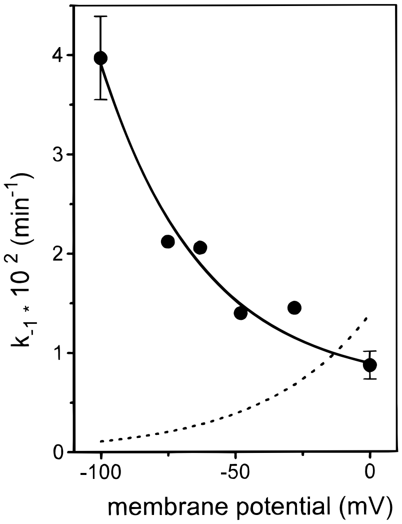

FIGURE 6: Exponential increase of flop rate of NBD-PM (25 °C) by increasingly negative membrane potentials established by a gradual decrease of the extracellular K$^+$ concentration in the presence of 0.5 μmol·L^{-1} gramicidin. The dotted line represents the corresponding relationship for the flip process. k_{-1} is the flop rate constant.

DISCUSSION

This study presents for the first time detailed information on the influence of membrane potential on nonmediated transbilayer reorientation (flip-flop) and stationary distribution between inner and outer membrane leaflet of anionic phospholipid probes and a long-chain sulfonate in a biological membrane. Potential changes did not influence the flip of zwitterionic phosholipid NBD-PC (data not shown), excluding unselective effects of the conditions required to establish different membrane potentials. Only recently, the potential-dependent translocation of a cationic lipid probe from the outer to the inner leaflet of artificial lipid bilayers was demonstrated qualitatively (*8*).

In the case of the inward translocation of the anionic phospholipid probes, NBD-PM and NBD-PA, and the anionic long-chain amphiphile, 2,6-DENSA, from the outer to the inner membrane leaflet (flip) in erythrocytes, two pathways have to be considered (*12*, *14*, *15*), namely, nonmediated flip and band-3-mediated flip, and in the case of the outward translocation from the inner to the outer membrane leaflet (flop), three pathways have to be considered, namely, nonmediated flop, band-3-mediated flop, and active flop mediated by an ATP-dependent floppase (*12*). For 2,6-DENSA the active flop is absent and for NBD-PM the band-3-mediated components of flip and flop are essentially absent (*12*). In order to study the isolated effect of membrane potential on nonmediated flip, the active flop of NBD-labeled PA and PM was blocked by a pretreatment of cells with the SH reagent *N*-ethylmaleimide and band-3-mediated flip and flop of DENSA and NBD-PA were blocked by a DIDS pretreatment (see Materials and Methods). By this method the remaining flip and flop processes are essentially nonmediated and probably take place in the membrane lipid domain (*12*).

Membrane potential was varied and measured using well-established procedures (*21*−*34*). Values for membrane

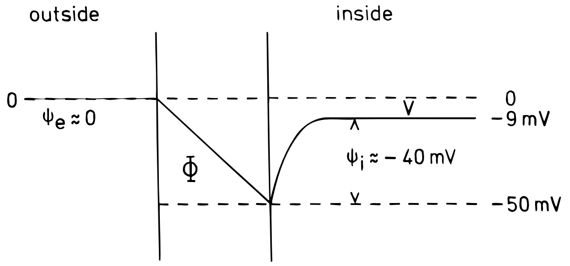

FIGURE 7: Schematic drawing showing the membrane diffusion potential *V*, the transbilayer potential Φ, and the surface potentials of, respectively, outer and inner membrane surface Ψ_e and Ψ_i.

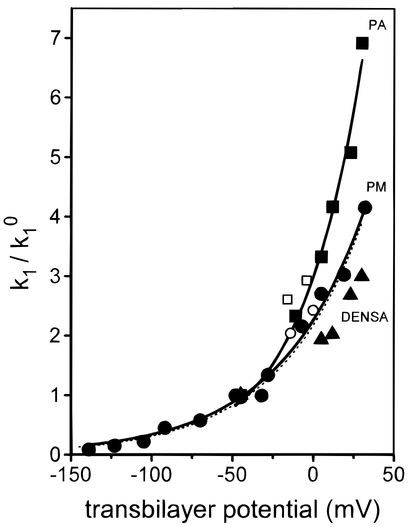

FIGURE 8: Exponential dependence of the flip rate of NBD-PM (circles), NBD-PA (squares), and 2,6-DENSA (triangles) established by exchange of extracellular chloride for glutamate (closed symbols) or HEPES (open symbols) on the transbilayer potential. The dotted line represents the theoretical relationship for an anionic probe with valence $z = 1$ on the basis of a rate analysis using the Eyring approach (see text). The flip rate constants in the presence of a transbilayer potential, k_1, were normalized to k_1^0, i.e., the rate constant at a membrane potential of about 0 mV or a transbilayer potential of −40 mV.

potential determined under our experimental conditions as described under Materials and Methods were comparable to those reported for corresponding conditions (*22*, *23*, *31*−*34*).

In the case of the anionic lipid probes with high membrane/water partition coefficients (about 10^5) (*14*, *15*), the membrane potential dependence only concerns the transmembrane movement of the anionic probes from the outer to inner membrane leaflet and vice versa without contribution of a potential-dependent partition of the probes between membrane and medium. Since the anionic lipid probes are oriented in parallel to the membrane phospholipids, the electric driving force for translocation is not the transmembrane potential, *V*, but the intramembrane or transbilayer potential (*40*), Φ, which is the potential difference between the surfaces of the two lipid leaflets (Figure 7). The transbilayer potential can be obtained by the sum of the membrane potential, *V*, and the surface potential of the inner membrane leaflet (Ψ_i) minus the surface potential of the outer leaflet (Ψ_0). For the erythrocyte membrane, the surface potential of the inner membrane leaflet is about −40 mV due to the presence of negatively charged phosphatidylserine (*41*) and that of the outer membrane surface is about 0 mV (Figure 7). The insertion of small amounts of anionic phospholipid probe (1 per 300 membrane phospholipids) into the outer membrane leaflet only slightly affects the outer surface potential, namely, by about −1 mV as calculated from the surface charge (*42*). In isotonic saline the membrane potential is about −9 mV (*21*) and thus the transbilayer potential is almost −50 mV. Accordingly, membrane potentials of +70 and −90 mV correspond to transbilayer potentials of respectively +30 and −130 mV. The orientation of PS to the inner membrane surface as probed by NBD-labeled PS inserted into the inner membrane leaflet is maintained under the experimental conditions required to vary the membrane potential (data not shown). Thus, the surface potential is constant during flip measurements.

In order to compare the rate constants in the presence of varying membrane potentials, absolute numbers of flip rate constants of NBD-PM, NBD-PA and 2,6-DENSA were normalized to the flip rate constant in the absence of a membrane potential and plotted against the transbilayer potential (Figure 8). As evident, the flip of the anionic probes depends exponentially on the transbilayer potential. These dependencies may be compared with the potential dependence theoretically expected on the basis of a rate analysis using the simple Eyring approach for a single membrane barrier to translocation of the anionic lipid probe (*3*). According to this theory, $k_\Phi = k_0 \exp(z\, n\Phi F/RT)$, where k_0 and k_Φ are the rate constants for the translocation of an anionic lipid with valence *z*, in the absence and presence of a transbilayer potential (Φ), *n* is the fraction the membrane potential will fall from the membrane surface to the translocation barrier, and *F*, *R*, and *T* have their usual meanings. The sulfonate 2,6-DENSA will be fully ionized above pH 2−3 ($z = 1$) and the phosphate diester NBD-PM above pH 3−4 ($z = 1$), while the phosphate monoester NBD-PA will have one negative charge around pH 4 ($z = 1$) and two negative charges ($z = 2$) above 8 (*43*). Taking valence $z = 1$ and assuming a central symmetric barrier ($n = 0.5$), the dependence predicted by this theory agrees well with the experimental data for the flip of NBD-PM (Figure 8, dotted line). For 2,6-DENSA the potential dependence is somewhat less steep; for NBD-PA it is considerably steeper. The deviation from the theoretical curve could be due to an asymmetric barrier, i.e., *n* could be smaller than 0.5 for 2,6-DENSA and greater than 0.5 for NBD-PA. This would implicate that the localization of the barrier in the bilayer is different for the three probes. In the case of NBD-PA, the steeper dependence of flip could be due to the partial divalent nature of NBD-PA at pH 7.4 (*43*). For divalent NBD-PA, $z = 2$ in the exponential term and the potential dependence would be steeper than that for monovalent phospholipid.

In a more quantitative analysis of the potential dependence of flip, it should be considered that establishment of membrane potentials alters the Donnan equilibrium distribution for protons and thus inevitably goes along with changes of the extracellular or intracellular pH depending on the buffer capacity of the medium. Upon a decrease of extracellular chloride by its isotonic exchange for glutamate to establish positive membrane potentials of up to +70 mV, the extracellular pH gradually decreases from 7.4 to 6.2. Titration of cells from extracellular pH 7.4 to 6.2 at constant high extracellular chloride concentration increases the flip rate of NBD-labeled PM and PA respectively 1.3, and 2.3-fold (data not shown). However, such a decrease of pH is

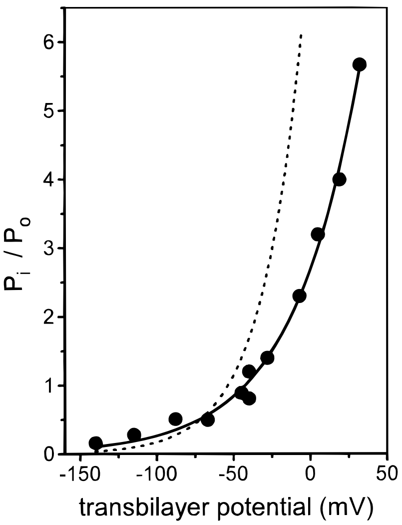

FIGURE 9: Ratio of NBD-PM in the inner (P_i) to that in the outer (P_o) membrane leaflet under stationary conditions highly depends on the transbilayer potential. The dotted line represents the theoretical relationship (see text). The data represent mean values of 2−4 experiments.

well known to change the Donnan equilibrium distribution of chloride and protons (*44*, *45*), i.e., the ratio of chloride concentrations in medium and cells. This makes the membrane potential 10−12 mV more positive and would enhance the flip rate of NBD-PM according to Eyring theory almost 1.3-fold. After correction of pH dependence for a contribution of such a pH-dependent change of membrane potential, the pH dependence of NBD-PM flip would become negligible. To get support for this concept, the flip was measured at different pH but at constant membrane potential. To this end, the ratio of concentrations of extracellular and intracellular chloride was set to 1 and thereby membrane potential to 0 (see Materials and Methods). This was achieved by respectively increasing and decreasing the extracellular chloride concentration during titration of cells to a lower and higher extracellular pH (*33*, *34*). In the essential absence of membrane potentials, the pH dependence of flip of NBD-PM was essentially absent (data not shown) and correction of flip acceleration by established positive membrane potentials for pH-dependent changes of flip is not required. For the flip of NBD-PA, the pH dependence at constant membrane potential becomes only somewhat less than that at constant extracellular chloride. Nevertheless, correction for this pH dependence of the up to 7-fold flip acceleration (Figure 8) by established positive membrane potentials, i.e., a transbilayer potential of up to +30 mV, would still result in a potential-dependent acceleration (3.5-fold) that would be close to the theoretical curve in Figure 8, assuming monovalent NBD-PA. However, the situation is complicated by the pH-dependent protonation of divalent PA in the outer membrane leaflet upon establishment of positive membrane potentials (see above). Such a complication can be avoided by establishment of chloride diffusion potentials in medium containing HEPES to maintain the extracellular pH at 7.4 during flip. For NBD-labeled PA and PM, the dependences of flip on the transbilayer potential at constant extracellular pH but measured variable intracellular pH (open symbols in Figure 8), were comparable to those at variable extracellular pH. The results support the major role of the positive membrane potential in flip stimulation by a decrease of extracelular chloride. Therefore, it can be concluded that the flip-flop of the anionic probes is an electrogenic process representing the translocation of the unprotonated charged lipid probes across the membrane barrier.

Previously, the flip of PA in lipid vesicles was proposed to involve the translocation of the protonated uncharged species since the flip rate increased 10-fold upon a decrease of pH by 1 unit in the range below 5.5 where the flip became rapid enough to measure (*10*, *11*). The flip rates of NBD-labeled PM and PA in erythrocytes only increased respectively 3- and 10-fold by a decrease of pH from 8.5 to 5.5 and are enhanced only 1.8- and 2.3-fold by a decrease of pH from 6.5 to 5.5 (data not shown). After consideration of pH-dependent changes of membrane potential, pH dependence of NBD-PA flip becomes less and that of NBD-PM is essentially absent. These small pH dependences exclude significant contributions to the flip of an electroneutral nonionic diffusionlike process, which would be expected to change 10-fold by a change of 1 pH unit. The mechanism of flip-flop of NBD-labeled PM and PA in the erythrocyte membrane and that of anionic phospholipids in pure lipid bilayers (*10*, *11*), at least around pH 5.5, are therefore different. The electrogenic nonmediated flip-flop of anionic phospholipids in the erythrocyte membrane is proposed to take place at structural defects in the lipid bilayer or at the hydrophobic protein−lipid interface (*46*, *47*).

Changes of membrane potential not only affect the rate of flip-flop but also—not unexpectedly—influence the stationary distribution of the anionic lipid probes (Figure 9). According to theory, the transbilayer potential constitutes a driving force for charged lipids that moves them from one membrane surface to the other. The dependence of their stationary distribution between inner (P_i) and outer (P_o) membrane leaflet on the transbilayer potential will have to satisfy the Boltzmann relation: $(P_i/P_o)_\Phi = \exp(-z\Phi F/RT)$ (*40*). The distribution of NBD-PM strongly responds to changes of the transbilayer potential, although considerably less than theoretically expected. For a change of potential by 100 mV, the distribution changed only 10-fold instead of the 50-fold expected theoretically at 25 °C. Previously, comparable smaller effects of potential changes have been reported for the anionic probe 5,2-DENSA (*48*).

At a transbilayer potential of 0 mV, equivalent to a membrane potential of + 40 mV, the distribution of NBD-PM in the erythrocyte membrane is already asymmetric in favor of the inner membrane leaflet (P_i/P_o about 3; Figure 9). This has also been found for the distribution of 5,2-DENSA (P_i/P_o about 2.6; *48*). Thus, other factors such as a higher affinity of the anionic probe for the inner than for the outer membrane leaflet, different distribution volumes in both leaflets, or shielding of the anionic charge of the probe by membrane skeletal proteins at the inner membrane surface may contribute to the asymmetric distribution of these anionic probes between the inner and outer membrane leaflet in the absence of a transbilayer potential.

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